CHANGES IN ZEBRAFISH (*Danio rerio*) LIVER PROTEIN EXPRESSION DUE TO STARVATION. A PROTEOMICS APPROACH FOR ANALYSIS OF CRUDE MITOCHONDRIAL EXTRACT.

By

ENYU YEE LING

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PERUBAHAN PENGEKSPRESAN PROTEIN DALAM HATI IKAN ZEBRAFISH (Danio rerio) AKIBAT KEBULURAN. SATU PENDEKATAN PROTEOMIK UNTUK ANALISASI PENGEKSTRAKAN BASAL MITOKONDRIA.

ABSTRAK

Pemahaman terhadap perubahan metabolik mekanisma semasa kebuluran dalam organisma telah meningkatkan minat para penyelidik sejak kebelakangan ini. Perhubungan antara mitokondria dan fungsi yang berkaitan dengan kebuluran seperti penghasilan tenaga, metabolisma asid lemak, pemanjangan hayat hidup dan proses penuaan telah menjadikan ia sebagai organ untuk dikaji dalam kajian ini. Berpegang kepada konsep ini, kajian ini dijalankan bertujuan untuk melihat perubahan protein dalam basal ekstrak mitokondria dari hati ikan zebrafish selepas satu tempoh kebuluran. Gel elektroporesis sodium dodesil sulfat poliakrilamida dua dimensi (2D SDS-PAGE) dan analisis gel menunjukkan 99 tompok mengalami perubahan corak ekspresi semasa 15 hari tempoh tanpa makanan. Sejumlah 35 tompok yang mememuhi tiga ciri-ciri utama telah dipilih untuk spektrometri massa; tompok itu mesti dapat dikesan dalam semua gel; sentiasa menunjuk corak penekspresan yang sama; bacaan intensiti tompok tersebut harus menunjuk perbezaan yang signifikasi terhadap golongan yang dibandingkan dalam semua set replikasi. Sembilan belas tompok daripada 35 yang terpilih untuk análisis spektrometri massa telah memberi pengenalan yang positif. Hasil kajian ini telah menunjukkan penigkatkan ekspresi beberapa protein yang terlibat dalam proses ‘chaperoning’ dan reaksi anti-oksidatif seperti protein heat shock, peroksiredoksin dan paraoksonase. Protein yang terlibat dalam proses katabolik pula menunjukkan corak ekspresi menurun semasa kebuluran. Semua reaksi tersebut sebagai kesan untuk mengurangkan aktiviti metabolik,

melambatkan proses penuaan dan memanjangkan hayat. Peningkatan kawalaturan beberapa protein yang terlibat dalam metabolisma asid lemak dan asid amino menunjukkan kebanyakan bekalan tenaga bukan berasal daripada sumber karbohidrat semasa kebuluran. Corak ekspresi protein-protein ini secara keseluruhannya menunjukkan penurunan glikolisis dan peningkatan glukoneogenesis.

CHANGES IN ZEBRAFISH (*Danio rerio)* LIVER PROTEIN EXPRESSION DUE TO STARVATION. A PROTEOMICS APPROACH FOR ANALYSIS OF CRUDE MITOCHONDRIAL EXTRACT.

ABSTRACT

Understanding the metabolic mechanism changes of organisms during starvation has raised the interest of scientists over the past years. The association of mitochondrion with related functions such as energy production, fatty acids metabolism, life span extension and ageing process has made it a target organ in this study. With this in mind, this study was designed to look into the changes of the crude mitochondrial extract after a period of starvation in adult zebrafish liver. Second dimension sodium dodecyl sulphate polyacrylamide gel electrophoresis (2D SDS-PAGE) and gel analysis revealed 99 spots undergoing changes in expression pattern during 15 days of food deprivation. A total of 35 spots which fulfilled three important criteria were selected for mass spectrometry; A) can be detect in all gels, B) shows a consistent expression pattern, and C) intensity of spots must be significantly different from the comparison sample in all replicates. Nineteen spots were positively identified from 35 spots sent for analysis. Interestingly, results showed that several proteins involved in chaperoning and anti-oxidative reactions such as heat shock proteins, peroxiredoxin and paraoxonase were upregulated while a protein involved in the catabolic pathway was downregulated during starvation. These responses lead to lower metabolic activities, slowdown of ageing process and extension of life-span. Several proteins involved in fatty acid and amino acid metabolism were upregulated indicating a majority of energy supplies during prolong starvation were not from a carbohydrate source. As expected, results showed an overall reduction in glycolysis and an increase in gluconeogenesis during starvation.

CHAPTER 1

INTRODUCTION

1.1 General

For the past decades, scientists have been trying to understand the metabolism of organisms during starvation. Starvation is defined as a state where extreme hunger occurrs due to lack of energy sources for a prolonged period. Generally, starvation happens naturally to animals due to unfavourable foraging seasons, weather fluctuation and parental behaviour established by some of the animal species (Pérez-Jiménez *et al*., 2007). In the wild, fish larvae have typically high mortality due to poor tolerance of starvation among other factors. Thus, many investigations in aquaculture were focused on larval starvation (Sheng *et al*., 2007). In humans, self-starvation often happens to anorexia nervosa patients caused by various factors such as 'fat phobia' and depression, to name a few. Understanding these starvation mechanisms would allow the optimisation and improvement of developing a better solution for anorexia nervosa treatment (Lee, 1995; Södersten *et al*., 2006).

An organism suffering from starvation will adapt and survive wholly or partially on its endogenous energy stores. The responses towards starvation is both behavioural and biochemical. During starvation, an organism's response to environmental stimuli is less sensitive compared to normal conditions, thus unnecessary movement is avoided (Campbell, 2007). In most species, liver glycogen serves as the first substrate to obtain energy when glucose level is low during starvation. When starvation persists, protein will be utilised to obtain energy before the final energy substrate, lipid is used (Metón *et al*., 2003). During starvation, some

of the biochemical pathways are eliminated while some minor pathways become extremely important such as ketogenesis, fatty acid metabolism and stress regulation pathways.

Mitochondrion is commonly known as the main power station producing ATP needed for normal cellular function and metabolic homeostasis by oxidative phosphorylation. Besides that, mitochondrion also serves as a biosensor for oxidative stress and an effector for cell death through apoptosis. Studies done on both humans and animals showed that the developing insulin resistance during starvation may lead to impaired mitochondrial function. Mitochondrial dysfunction increases oxidative stress and disorder of this organelle has been implicated in pathogenesis of a number of diseases mostly cardiovascular diseases (Awad *et al*., 2009).

Danio rerio, commonly known as zebrafish has quickly gained its popularity among scientists in recent decades. Zebrafish belongs to the family of cyprinids (Cyprinidae). This tropical freshwater fish originated from the rivers of South Asia, North India, North Pakistan, Bhutan and Nepal (Dahm and Geisler, 2006). These tiny fishes are not only easy to maintain, they also breed readily and are able to produce a large number of eggs in one breeding cycle. Furthermore, an almost complete genome project and continual discovering of new techniques have strengthened the versatility of zebrafish as a model organism in modern biological research (Brand *et al*., 2002; Gilmour *et al*., 2002).

The proteomics approach is a powerful tool which is widely used to study proteome, protein components obtained from a cell or tissue. It identifies and quantifies each of the proteins that constitute the proteome. With the help of these emerging technologies, biologists have been given the great opportunity to gain

greater insight into the cellular and molecular mechanisms involved in key physiology processes (Corthals *et al.*, 2000).

In order to understand the physiological changes of liver mitochondria during starvation, it is important to establish a protocol in isolating the mitochondrial protein from the total liver extract, followed by a good protein separation method and gel image analysis. The identity of selected protein spots will then be determined through mass spectrometry.

1.2.1 Objectives

The objectives of this study are listed as below:

i. To produce a reproducible second dimensional sodium dodecyl sulphate polyacrylamide gel (2D SDS-PAGE) image of the extracted crude mitochondrial proteins by going through a series of gel optimisation.

ii. To compare the expression patterns of crude mitochondrial protein in control and starved zebrafish liver.

CHAPTER 2

LITERATURE REVIEW

2.1 Starvation

Food or nutrient is the main energy source for all living things. Starvation is defined as a state of extreme hunger resulting from the lack of essential nutrients over a prolonged period. All animals in their natural environment will alternate periods of feeding and fasting in response to several factors such as temperature, change of seasons, spawning migration and parental behaviour, to name just a few (Pérez-Jiménez *et al.*, 2007). Cultured fish also experience the same factors mentioned above in addition to those imposed by routine aquaculture procedures. In order to survive this food restriction, animal mobilises reserved energy to sustain its daily activities. This metabolic adjustment process is not only species dependent on the species but also the age, nutrition status and behaviour of the species (Navarro and Gutiérrez, 1995).

A very early research by Greene (1921) on the migration journey of the king salmon (*Oncorhynchus tschawytscha*) has revealed that its journey of about 60 to 100 days to the spawning beds was made without food. The king salmon were fed in the California coastal waters to maturity and allowed to migrate through the San Francisco Bay and up the Sacramento River to the spawning beds. The king salmon stored enormous amount of fat which disappeared during the migration and were probably the main source of energy. Though the kinetic energy during the migration is derived from the oxidation of the stored fats, carbohydrates may have also played a significant role. The study also showed that muscle glucose, presumably glycogen, was present in low amounts during the feeding period and disappeared entirely

during migration. Besides this, they also discovered that the composition of glycogen in king salmon ovaries were constant (Greene, 1921).

The red crab (*Gecarcoidea natalis*) is an example of a species which experiences food depletion during the breeding season. With the arrival of monsoonal rains and after months of inactivity during the dry seasons, the red crabs embark on their annual breeding migration to the coast. Crustaceans are known to have substantial lipid and glycogen reserves in the midgut gland and muscle tissues, which are utilised during starvation (Chang and O'Connor, 1983; Nishida *et al.*, 1995). Migration is the most intense period of the year for *G. natalis*. The red crabs have reduced foraging opportunities due to the unfavourable environment during the dry season, thus the red crabs have to spend up to two months in their burrows before the migration (Green, 1993). The migratory activities are energetically demanding, so they are required to utilise the stored metabolic fuels. Surprisingly, crabs engaged in migration did not use the stored energy sources. In contrast, crabs returning from the breeding activities had significantly depleted glycogen stores (Adamczewska and Morris, 2001).

Besides migratory activities for certain species during the breeding seasons, some species are forced to live without food during the change of seasons, especially winter. These species have evolved better to overcome food scarcity. They utilise passive wintering strategies to overcome the seasonal variation in food availability. Examples of these species are bears *Ursus spp* (Nelson *et al.*, 1973), the raccoon dog *Nyctereutes procyonoides* (Mustonen *et al.*, 2004) and the American badger *Taxidae taxus* (Harlow and Seal, 1981). Mustonen *et al*. (2005) carried out a study on the mink population which is believed to experience periods of food scarcity during the cold and harsh winter. Food deprivation led to degradation of the liver glycogen

stores of minks after five days of fasting. A more drastic decline of liver glycogen stores had been reported by Børsting and Gade (2000) in minks after two days of food deprivation. In addition, they concluded that the minks have relatively poor adaptation to the cold and harsh winter. A similar trend of liver glycogen stores depletion was found in the domestic dog *Canis familiaris,* whereby depletion was the highest during the first two days of fasting (de Bruiine and de Koster, 1983).

Food deprivation or starvation has been applied in certain experiments such as studies on energy expenditure, general changes in composition of muscle tissue and liver glycogen stores utilisation. A good example will be the study of hypothalamic neuropeptide Y (NPY) mRNA by Silverstein and Plisetskaya (2000). Food deprivation is a condition under which hypothalamic NPY mRNA and peptide levels rise. NPY is known to influence a number of physiological parameters, the most avidly researched being food intake and energy balance (Morris and Crews, 1990). It also acts to reduce the autonomic nervous activity and energy expenditure as this might be the reason for an increase in the NPY peptide and mRNA levels when food is restricted (Billington *et al.*, 1991; Marks *et al.*, 1992; Schwartz and Seeley, 1997). Furthermore, to better understand the biology of certain organs during working and fasting, food deprivation has been applied to the studies, especially for the determination of creatine and amino-acids (Greene, 1919).

2.2 Effects of starvation on the organism

Starvation is defined as a condition whereby the exogenous energy or protein intake is inadequate or absent, be it partial or complete. The body of an individual suffering from starvation will adapt and survive either wholly or partially on its

endogenous energy stores. Energy is derived from oxidation of macronutrients such as carbohydrates, fats and proteins. Glucose serves as an immediate source of energy which may be used to maintain glycaemia during the early stage of starvation. Almost parallel with liver glycogen depletion, lipid reserves are used to obtain energy. When both reserves are practically depleted, protein is utilised. Generally, liver glycogen serves as the first substrate to obtain energy for most species (Echevarría *et al*., 1997; Metón *et al*., 2003). However, there are some contradicting explanations found for some species, whereby they try to preserve liver glycogen stores by degrading protein for gluconeogenesis and using the lipids or proteins as energy substrates (Sheridan and Mommsen, 1991; Gillis and Ballantyne, 1996). When starvation persists for a long period of time, lipid is used as the second energy substrate to obtain energy. Fat represents long-term energy storage. Although protein can be used as an energy source, it normally supplies only 10-15% of the body's energy needs. However, the net loss of proteins is accompanied by deterioration of body functions as protein forms the structure of the body (Campbell, 2007).

Responses to starvation are both behavioural and biochemical. During starvation, responses of the organism to environmental stimuli are abated and unnecessary movement is stopped. Some of the biochemical pathways are eliminated while some minor pathways such as gluconeogenesis and ketogenesis become extremely important (Campbell, 2007).

2.2.1 Phases of starvation

The temporal responses to starvation can be categorised into three phases: glycogenolytic, gluconeogenic and ketogenic.

2.2.1.1 Glycogenolysis

This happens during the very early stage of starvation. When food intake stops, the blood glucose level drops while insulin and glucagon rise. The stimulus and response are secondary to the change of glucose concentration. Liver glycogen stores are mobilised through glucagon and the activation of the liver phosphorylase enzymes. The hepatic glycogen is broken down to glucose-6-phosphate, which is then converted to glucose and released into the blood stream. The resulting pyruvate, the end product of glycolysis process, is converted to lactate, which passes through the circulatory system and later into the liver. Lactate that act as a gluconeogenic precursor is converted to glucose for export into the general circulation. The cycle of glucose to lactate happens in peripheral tissues, while the conversion of lactate to glucose takes place in the liver. These cycles are collectively known as the Cori cycle. The glycogen stores in liver and muscle lasts for about 24-48 hours.

2.2.1.2 Gluconeogenesis

This process is very important in synthesising glucose from non-carbohydrate sources such as amino acids, glycerol and also lactate to continue supplying energy to meet the body's energy requirement. The metabolic pathways for gluconeogenesis in fish are quite similar to those found in other animal species. This metabolism is initiated by the release of amino acids from protein breakdown. In the early stages of starvation, gluconeogenic amino acids might come from rapidly turning-over tissue pool such as the lining of the gastrointestinal tract. However, the major protein storage for long term food deprivation is the skeletal muscle. The release of amino acids is stimulated by the decline in insulin concentration and the concomitant rise in glucagon (Nascimento *et al.*, 2008). Amino acids were then converted to glucose by

the liver and to a lesser extent by the kidney. About half of the amino acids are released from peripheral tissues, such as alanine and glutamine. Alanine is the corresponding amino acid to pyruvate, the principal product of glycolysis. Meanwhile, glutamine is the corresponding amino acid to α-ketoglutarate, a constituent of the Krebs cycle. If no further adaptations to starvation take place, this will cause a very rapid depletion of lean body mass.

Another principal of gluconeogenic precursor, in addition to lactate and amino acids, is glycerol. This substrate is a three carbon alcohol and a major constituent of triglyceride; the form in which most of the fat in the body is stored. As adaptation to starvation takes place, triglyceride is broken down to its constituents of glycerol and free fatty acids, and the glycerol moiety is transported to the liver where it is converted to glucose. The energy for the synthesis of glucose in the liver, from these precursors, is thus derived from the body's major energy store.

Gluconeogenesis increases to a maximum at about 48 hours, and then decreases slowly during the following 1-2 weeks as the keto-adaptation takes place. The requirement for gluconeogenesis declines which allows significant sparing of muscle protein as event of survival benefit.

2.2.1.3 Ketogenesis

Ketogenesis is a process which ketone bodies are produced as a result of fatty acids breakdown. This metabolism is the final adaptive process that allows the body to function for a prolonged period without any food intake. At this stage, many tissues are adapted to use fatty acid as their energy source. Those fatty acids that arrive at the liver will esterified to triglyceride; a process stimulated by insulin. In the presence of low insulin concentration, the fatty acids are converted to ketone bodies

(acetoacetate and β-hydroxybutyrate). These substances are oxidised to generate energy instead of glucose. For example, in the brain, about two-thirds of the metabolic requirement is derived from ketone oxidation, thus sparing up to 80 g of glucose per day.

2.2.2 Hormonal changes

The metabolic changes mentioned above result from the hormonal changes in the body during food deprivation. The rise in glucagon promotes gluconeogenesis. Triiodothyronine (T_3) decreases and reverse T_3 increases (Deng *et al.,* 2004). These changes lead to a fall in metabolic rate, and also the fall in T_3 may also have a protein-sparing effect (Guderley *et al.*, 2003). Sympathetic activity is reduced during starvation and long-term starvation difficulties may be experienced, for example, control of blood pressure and posturalhypotension. There may also be difficulties with thermoregulation because one of the body's defences against cold stress is sympathetic activation.

2.2.3 Metabolic rate

Metabolic rate decreases proportionally with weight loss during starvation, whereby energy expenditure decreases by about 40%. There are several reasons: the decrease in sympathetic nervous activity, the decline in T_3 and a relatively greater loss of metabolically active tissues, such as the lining of the gastrointestinal tract and the liver (Figueiredo-Garutti *et al.*, 2002). Although active in gluconeogenic and ketogenic activity, the liver is no longer involved in the daily processes of digestion and assimilation.

2.2.4 Energy value of weight loss

For individuals on a low-energy diet, weight loss is initially rather rapid due to loss of lean tissue with its high water content and then slows until ongoing weight loss is extremely difficult to achieve. As the low-energy diet continues and the body adapts to using mostly fat, the energy density increases as weight loss increases until virtually only adipose tissue is being used in an established fully adapted fast. During the early stage of food deprivation, the energy density is about 1000-2000 kcal/kg, eventually increasing to 7000-8000 kcal/kg when starvation is prolonged.

2.2.5 Duration of starvation

Death normally occurs after 60-70 days of starvation. Survival continues as long as the fat stores last. When the fat stores run out, protein is oxidised for fuel and there is a 'premortal' rise in urinary nitrogen excretion. There are cases of very obese individuals undergoing therapeutic fast, supplemented with vitamins and minerals, for more than 1 year.

2.3 Mitochondria

Mitochondria are eukaryotic organelles that originated from bacteria some 2 billion years ago. It transited to the modern mitochondrion and has been accompanied by great changes in its protein content, or the so-called proteome. These changes include the complete loss of some bacterial pathways. Only a minor group of modern mitochondrial proteome can be traced back to the bacterial endosymbiont. These modern mitochondria have transformed into a highly specialised organelle that play a key role in the metabolism of most eukaryotic cells (Gabaldón and Huynen, 2004)

Mitochondria are recognised as having fundamental roles in several cellular processes especially energy metabolism via the Krebs cycle, the oxidative phosphorylation system and β-oxidation of free fatty acids. At the same time, mitochondria also play an important role for the heme biosynthesis, ketone body formation and urea degradation (Bailey *et al.*, 2005). Mitochondria of all organisms except mammalian have almost all the proteins encoded by the nuclear genome and imported into the mitochondria through a bilayer transmembrane system (Westermann and Neupert, 2003). Mammalian mitochondria are unique in that they contain several copies of their own genome (Bailey *et al.*, 2005).

2.3.1 Research on mitochondria proteome

Nowadays, it has become technically feasible to obtain comprehensive data for the protein content of mitochondria, thanks to the rapid development of technology. The analysis of the mitochondria proteome can be categorised into three steps. Firstly, the mitochondria analysis starts with isolation and purification of the mitochondria organelle from the whole cell lysate by centrifugation on a density gradient. This is followed by a separation of the protein content into individual protein for further identification. The most commonly use techniques are 1D or 2D electrophoresis combined with techniques such as liquid chromatography and isoelectric focusing (IEF). A variety of mass spectrometry techniques is available for protein identification, such as matrix-assisted laser-desorption ionisation mass spectrometry (MALDI-MS), electrospray ionisation MS and tandem MS. Successive proteomics stuies have progressively increased the molecular coverage. Consequently, we now have a few complete sets of mitochondria genome sequences

from human, yeast and some model organisms such as mouse, rice, *Arabidopsis* and zebrafish. (Gabaldón and Huynen, 2004).

By using the proteomics approach, about 90% of yeast mitochondria proteome were successfully identified from highly purified yeast mitochondria (Sickmann *et al.*, 2003). Furthermore, a large scale proteomics survey was carried out on mitochondria purified from mouse brain, liver, kidney and heart resulting in an identification of about 591 distinct mitochondria proteins (Mootha *et al.*, 2003). A database of liver mitochondrial inner membrane proteins played an important role in understanding the mitochondria metabolism (Da Cruz, *et al.*, 2003). These studies emphasised the importance of different mitochondria protein composition with regards to crucial functions played by the mitochondria in the cell (Douette and Sluse, 2006).

Although comprehensive biochemical and genetic approaches combined with impressive computational predictions provide a good vision of the mitochondria proteome, important challenges remain. More input is needed from comparative proteomics such as the analysis of protein profiles from different organs. Protein profiling is important in correlating different structures with different sets of proteins. Apart from that, mitochondria proteomes need to be studied under various conditions in order to gather more information about roles of the mitochondria in certain physiological mechanisms (Westermann and Neupert, 2003).

2.4 Zebrafish as a model organism

In recent years, the zebrafish has become one of the most prominent vertebrate model organism used to study genetics underlying development, human diseases, organ toxicity and also various physiological mechanisms. The growing

interest in zebrafish research was paralleled by an increase in tools and methods available to study zebrafish. The zebrafish (*Danio rerio*, formerly known as *Brachydanio rerio*) is a tropical freshwater fish with natural habitats in rivers of South Asia, chiefly northern India, northern Pakistan, Bhutan and Nepal. They belong to the family of the cyprinids (Cyprinidae) in the class of ray-finned fishes (Actinopterygii) and within this class to the bony fishes (Teleost) (Dahm and Geisler, 2006).

In the past decade, zebrafish has become the favourite vertebrate model organism, particularly for genetic control of embryonic development studies. This is due to the fact that zebrafish combine a number of key embryological and experimental advantages. They are easy to maintain and breed (Brand, *et al*., 2002; Lawrence, 2007). They develop rapidly whereby embryogenesis takes only 24 hours and organogenesis is largely complete after day 5 of development, have enabling the observation of defined aspects of development as well as the completion of experiments within a few hours to days (Dahm, 2002). Furthermore, the translucency of its embryos and early larval development stages allow easy visualisation of the internal processes, such as the formation and function of internal organs within the living animal. They also can be facilitated, for example tracking the expression of fluorescent tagged transgenes and monitoring reporter gene activity (Gilmour *et al*., 2002). Being transparent during the embryonic stage also makes zebrafish an ideal experimental model for monitoring the fate of labeled transplanted cells without causing any harm to the animals (Guyon *et al.*, 2007**)**.

Zebrafish also have large numbers of offspring compared to other vertebrate model organisms. Their continuous production of eggs distinguishes them from most aquaculture fish. When bred under laboratory conditions, zebrafish are able to spawn

throughout the year (Brand *et al.*, 2002). Zebrafish with an average life expectancy of 2 to 4 years, reach sexual maturity after approximately 3 to 4 months, and with high reproductive capacity, a single female zebrafish may produce up to 300 eggs once every 5 to 7 days (Guyon *et al.*, 2007**)**. The constant supply of large numbers of offspring from zebrafish are ideally suited to large-scale genetic approaches aimed at identification of novel genes and uncovering their functions in a vertebrate (Pelegri, 2002). Embryos and early larvae measure only approximately one milimeter in length and are small enough to fit into a 96-well microtiter plate. Therapeutic agents can be directly included in the water in which the embryos develop allowing automated analyses. This latter approach is being applied to toxicology, with mutants that are models for diseases leading to drug discovery and development (Dahm and Geisler, 2006). Mutation exists in most of the organ systems. These mutations potentially serve as models for a multitude of human diseases (Barut and Zon, 2000**).**

In studies of human diseases, for example the muscle disease, pyruvate dehydrogenase deficiency, zebrafish has been recognised as a suitable model (Barut and Zon, 2000; Taylor *et al.*, 2004; Guyon *et al.*, 2007). The technical challenges of working with a small number of human patients have made it difficult to include all the necessary controls and systematically evaluate therapeutic agents. Therefore, the best model for these studies should show similar phenotypic characteristics to those seen in human patients and provide reliable methods for assessing treatment (Taylor *et al.*, 2004**)**. Besides its rapid ex-utero development, small size and short generation (3 to 4 months), there are strong similarities between zebrafish and human genomes (see also *http://www.sanger.ac.uk/Projects/D_rerio/ and http://genome.ucsc.edu/cgi-bin/hgGateway*).

Although reliable data for studies related to human diseases and extrapolating toxicant effects to human are obtained through laboratory rodent studies, these are expensive, time consuming and also often restricted by law. While zebrafish can be an ideal model for vertebrate development, there are some disadvantages to the system, as zebrafish are evolutionarily more distant from humans compared to mammals. As for genes, receptors and molecular processes are highly conserved across animal phyla, therefore, studies with lower class animal species could be representative for higher classes or more-complex animal (Hill *et al.*, 2005)**.** After all, findings from fish experiments should be replicated in mammals before being directly correlated to human therapy. Despite the limitation, establishing zebrafish as a model for human disease research can outweigh the cost and also would be faster than solely relying on mammals as models. Although zebrafish are not meant to replace existing mammal models of disease studies, experiments which require a large number of animals may be best performed in zebrafish (Guyon *et al.*, 2007).

2.5 Introduction to proteomics

A natural extension of *genomics* research is the characterisation of the gene products, which is protein. This has spawned the research area of *proteomics*, which is the study of the entire suite of proteins from a genome (Zhou, 2004). Proteomics originally referred to the study of the proteome, utilising technologies of large-scale protein separation and identification. The word proteome is a coined term to the concept of genome and is used to describe the complete set of proteins that is expressed and modified after expression by the entire genome of a cell. Thus, proteome analysis is actually a complement to DNA-sequence and RNA-expression analyses (Takahashi *et al.*, 2003).

In general, the proteomics approach begins with the analysis of the protein components in a proteome obtained from certain cells or tissues, and thus identifies and quantifies each of the proteins that constitute the proteome. This analysis is called “protein profile analysis” and the term “expression proteomics” means a field of research of protein profile analysis. Thus, by means of “expression proteomics” we will be able to identify a protein cluster that has changed in the cellular response to a given environment change. In proteomics, the functional relationships among proteins are interpreted from the identified protein cluster. Therefore, proteomics also provide the tools to scale up conventional biochemical approaches, such as those for large-scale protein-interaction analysis (Takahashi *et al.*, 2003; Mocellin *et al.*, 2004).

With the help of emerging genomics and proteomics technologies, it has become possible to gather enormous amounts of information about biological systems within very small time frames. The common application of both emerging technologies play a vital role in the progression of science and our biological understanding of many organisms and fish is no exception (Corthals *et al.*, 2000).

Post genomics technologies have provided fish biologist the opportunity to gain greater insight into the cellular and molecular mechanisms involved in the regulation of key physiological processes. Genomics and proteomics technologies are expected to prove the importance of fish physiology in three main areas: firstly, as a comparative means of analysing gene evolution; secondly, as a way of undertaking a global analysis of cell and tissue signaling pathways involved in pathology of disease, stress physiology and flesh quality; and finally, as a way of rapidly identifying novel proteins, and fish homologues of known proteins which have shown to play significant roles in other animal groups.

With the completion of the human genome, it is clear that emphasis will soon switch from data gathering to interpretation. The zebrafish is also useful as an experimental model for functioning genomics and therapeutic development application. Zebrafish embryos offer a system that rapidly provides efficacy, toxicity and specificity data within an *in vivo* setting (Nasevicius and Ekker, 2001). Proteomics techniques are already being used in fish biology. For example, Pineiro *et al.* (2001) reported the use of proteomics in the characterisation and partial sequencing of species-specific sarcoplasmic polypeptides in a marine fish of high commercial value.

Proteomics analysis is most commonly accomplished by the combination of two-dimensional electrophoresis (2DE) and mass spectrometry (MS). The interfacing of one- and two-dimensional polyacrylamide gel electrophoresis with N-terminal sequencing and mass spectrometry have gained wide acceptance particularly in areas of protein identification using database-searching approaches and characterisation of post-translational modification of proteins.

By linking such a technique to an imaging software, an automated spot picker and protein identification techniques such as matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry based peptide mapping, it is now possible to compare the protein profiles of two tissues before and after a tissue response, and to identify the proteins that are different in each case (Parrington and Coward, 2001).

2.5.1 Isoelectric focusing (IEF) and two-dimensional electrophoresis (2DE)

The separation and isolation of individual proteins and peptides from complex mixtures is a major problem and is the objective of protein chemistry.

Although several chromatography techniques are the preferred choice for large-scale procedures, polyacrylamide gel electrophoresis (PAGE) offers the best results for purifying small amounts of polypeptides. Gel electrophoresis methods (PAGE or capillaries) have superior resolving power, selectivity, speed and relatively mild operating conditions. Furthermore, these methods are suitable for the analysis of both the hydrophilic and hydrophobic proteins and peptides (Michalski and Shiell, 1999).

Proteins were separated by electrophoresis in polyacrylamide gels for more than 40 years. In 1975, work done by O'Farrell showed that a combination of IEF and reducing SDS-PAGE was able to display a massive number of proteins. This work represented the start of 2DE and begins a new age for the analytical protein separation.

IEF was then achieved using synthetically derived amphoteric compounds, carrier ampholytes in the seventies. These low molecular mass compounds were migrated to their respective p*I*s to create a pH gradient in an electric field during the IEF. In the eighties, the technology was expanded by the introduction of "immobilised pH gradient" (IPG). With this, the pH gradient is formed by copolymerisation of the buffering compounds (Immobiline^{TM}) with acrylamide and cross-linker into the polyacrylamide matrix.

In the mid 1990s, in-gel sample rehydration throughout the entire IPG strip was introduced. For the in-gel rehydration method, the sample was mixed with the re-swelling solution or most commonly known as rehydration buffer. This method has eliminated sample streaking and precipitation at the application point and consequently it allows more proteins to be loaded. Recently, IPG strips with narrow pH range are also available commercially, for example, 1 pH unit/18cm. The greater

distance displayed by proteins in this narrow pH range IPG strips increases the resolving power (Corthals *et al.*, 2000).

Currently, proteomics research commonly involves a sequent two-step procedure which is 2DE (protein separation) and MS (analysis). Two-dimesional polyacrylamide gel electrophoresis (2D-PAGE) has been the main method used in separation of thousands of proteins. The 2D-PAGE is basically separated into two major steps; IEF, separates protein due to their isoelectric points (p*I*) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), separates the proteins according to their size (Wittke *et al.*, 2004).

An important aspect in ensuring a high quality protein separation is during the sample preparation step. Samples to be separated cannot be too diluted or high in salt content as these factors interfere with the electrophoretic process, resulting in poor resolution of proteins. Various steps in protein purification result in diluted protein fractions and often containing high salt concentrations which must be effectively removed prior to electrophoresis. Desalting can be achieved using a range of desalting columns, ultrafiltration using spin column technology or dialysis. Alternative methods commonly used for sample concentration are precipitation with ammonium sulphate, acetone, TCA/deoxycholate (Ozols, 1990) and phenol/ether (Sauve *et al.*, 1995), or ultrafiltration and concentration on a hydrophobic resin (Ziegler *et al.*, 1997; Michalski and Shiell, 1999).

2.6 Organelle proteomics

Although promising, the information gathered from large-scale proteomics shows that the whole cell or tissues extract might be too complex for the available technologies and is not suitable for studies of low abundance proteins. There are

several limitations for complex sample analysis. Firstly, although the total number of the gene product in a given cell is around 10,000, the effective number is likely to be several fold higher that owing to the splice variants and post-translational modifications. Secondly, in a highly complex sample with a high variation in the level of protein expressions, the low abundance proteins are inevitably masked by those in high abundance (Brunet *et al.,* 2003).

Therefore, researchers suggested an extra step which is prefractionation on protein mixture which contains thousands of different protein species prior to 2DE. By prefractionation, one simplifies a complex protein mixture by reducing the number of proteins to be loaded into the gel matrix. This directly diminishes the poor quality spot resolution associated with IPG strip over-loading and minimising the spot fusion as well as co-migration. An additional advantage of sample prefractionation is that it helps in purifying away high abundance proteins so that the low abundance proteins can be visualised.

The sample fractionation or enrichment rule should be simple, preferably small to micro-scale, to minimise the loss of proteins during the fractionation process. The fewer the steps in the prefractionation process, the higher the yield tends to be. Purification of specific clusters of protein via subcellular fractionation provides two important benefits; the protein diversity and complexity is reduced as only one cluster or subset of proteins of the entire proteome is selected and one preselects for biologically associated proteins.

The latter is very much important for researchers whose studies focus on a framework of adaptors, anchoring and scaffolding proteins that function in maintaining the kinases and phosphatase in certain subcellular compartments.