Chapter 4 Discussion

Adiponectin is an adipocyte protein mainly secreted by abdominal fat tissue. Since adiponectin identification a lot of studies has shown that adiponectin exhibits anti-inflammatory, anti-atherogenic, and anti-diabetic properties (Hotta *et al.,* 2000; Matsuzawa *et al.,* 2004) (see Chapter 1, Figure 1.2). Most of these studies were based on the observations of adiponectin levels that were associated with serum lipids and indexes of insulin resistance (Xita *et al.,* 2005). Therefore, recombinant adiponectin is a promising molecule that has the potential to be used in human therapy of human afflictions especially for some of metabolic syndromes. However, several different types of adiponectin molecules have been identified in the blood plasma such as trimers, hexamers and oligomers. Studies have also shown that adiponectin activity differs for these molecules (Wang *et al.,* 2008). Recombinant adiponectin produced by mammalian cells was found to be more active and has the ability to form high molecular weight isoforms compared with adiponectin produced by *E. coli* (Avides *et al.,* 2008). However, producing high quantity of recombinant adiponectin by mammalian cells expression system will be impractical because of high costs and complexity of mammalian cells expression system. Therefore, considering the fact that adiponectin is potentially useful for the therapy of metabolic syndromes, studies that elucidate the type of bioactive molecule, defines the adiponectin signaling pathway and describes the method to produce efficient amount of bioactive recombinant adiponectin becomes the focus in this study.

In the present study we compared adiponectin expression by yeast *P. pastoris* as eukaryotic expression system and adiponectin expression by *E. coli* as prokaryotic expression system to study the advantages of each expression system concerning

successful expression of adiponectin isoforms, the amount of recombinant protein, adiponectin bioactivity and the cost effectiveness of each expression system. In addition we investigated the possible mechanisms of adiponectin effect through the study of the effects of adiponectin administration on the expression of some related genes.

4.1. *In vitro* gene construction

Previous studies described human *adiponectin* gene expression in *E. coli* which involved RNA isolation from human abdominal fat tissue followed by cDNA preparation to clone in *E. coli* (Hu *et al.*, 2003). Another study described that porcine adiponectin expression in *E. coli* had been carried out by the same method of RNA isolation and cDNA preparation (Jacobi *et al.*, 2004). This method for gene preparation was time consuming and costly as they involved samples collection, RNA isolation and cDNA preparation. In the present study, the *adiponectin* gene was obtained by PCR-based overlapping extention procedures. Extension of overlapping gene fragments by PCR is a simple and cost effective technique for gene construction *in vitro*. Overlapping gene fragments can be generated by normal PCR and then used in overlapping PCR as template DNA to generate a full-length gene (Heckman & Pease, 2007). In this study *adiponectin* gene was constructed by an overlap-extension PCR through three steps as described by Wurch *et al.* (1998) as follows: The first step is a conventional PCR reaction that was achieved by two sets of primers to amplify the two exons of *adiponectin* (Figure 4.1.A). The second PCR step consists of the fusion of the PCR fragments generated in the first step using the complementary extremities that included six extra-overlapped bases of the primers (Figure 4.1.B). The third step corresponds to the PCR amplification of the

fusion products (Figure 4.1.C). In our study, six overlapped bases was sufficient to fulfill the fusion product compared with previous study that used 12 overlapped bases to join each two fragment (Wurch *et al.*, 1998).

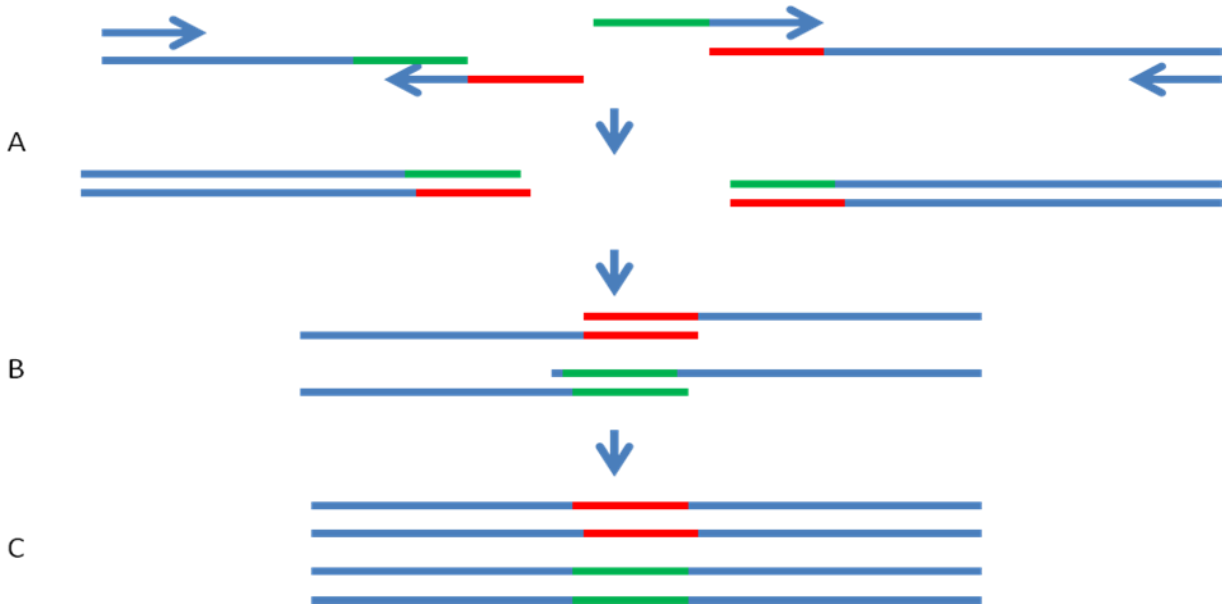

Figure 4.1: *In vitro* construction of *adiponectin* gene by overlap- extension PCR. The red and green colours stand for the complementary regions between primers and DNA fragments.

It has been published that human *adiponectin* gene spans 16 kb on chromosome locus 3q27 (Saito *et al.*, 1999; Takahashi *et al.*, 2000). Additionally, *adiponectin* gene was also identified in a cDNA clone of about 4.5 kb that contains a 732 bp open reading frame coding a 243 amino acid of adiponectin protein (Hu *et al.*, 1996; Maeda *et al.*, 1996). This results are approximately consistent with our gene construct after overlapping exon one with exon two. As a result of combining exon one (203 bp) with exon two (531 bp), the full length adiponectin with native signal peptide was 734 bp and encodes 244 amino acids.

Adiponectin amino acids can be grouped in a signal peptide and three domains includes variable domain, collagenous domain and globular domain (Scherer *et al.*, 1995; Nakano *et al.*, 1996). In our study, after translation the nucleotide sequence of adiponectin construct to amino acids, the total adiponectin amino acids was 244 residues. This consists of a native signal peptide of 18 amino acids long, followed by a 22 amino acids variable region, 65 amino acids of collagenous domain and 136 amino acids that represent globular domain. Also, consistent with previous finding the construct of *adiponectin* without native signal peptide starts from the same codon determined by Hu *et al.* (2003) which encodes the full length (230 amino acids) of adiponectin after eliminating the leader peptide.

In order to clone and express *adiponectin* in *Pichia pastoris*, two constructs of *adiponectin* with and without signal peptide were amplified with forward and reverse primers that carried different restriction enzymes recognition sites (*Eco*RI and *Not*I) in order to clone it with the correct orientation into a yeast expression vector. The adiponectin construct was amplified using the forward primers without restriction

enzyme site and the reverse primer harbouring *Hind*III restriction site to clone and express in *E. coli* expression vector. In this case *adiponectin* fragment will harbour blunt and sticky ends achieving the correct orientation into *E. coli* expression vector.

In yeast, published studies indicated that the yeast signal peptide was more efficient than the heterologous protein native signal peptide, suggesting that signal peptide replacement is an efficient way to enhance the recombinant protein expression level (Zhu *et al.*, 2009). However, another study highlighted the need for individually optimized signal peptides for every heterologous secretion target (Brockmeier *et al.*, 2006). Therefore, in order to get successful expression of *adiponectin* in *P. pastoris* and *E. coli*, we constructed *adiponectin* gene with and without signal peptide selecting the successful one in the expression experiments.

4.2. Expressions of adiponectin in prokaryotic and eukaryotic expression systems

The development of expression systems is important to fulfill the increasing requirement for production of new recombinant proteins by effective and efficient means. The most commonly used expression systems are based on the prokaryote *Escherichia coli*, which is genetically and microbiologically well studied. Basically, there are some important requirements need to be available in the expression system such as the host cell genetic background and the characteristic of the expression plasmid characteristics. The genetic factors of the expression plasmid include transcriptional promoter, an antibiotic resistance marker, origin of replication, translation initiation regions and terminators of transcription and translation (Baneyx, 1999; Jonasson *et al.*, 2002). The choice of a

suitable expression system depends largely on the biochemical and biological properties of the protein of interest, as well as on the nature of the planned experiments and the amount of recombinant protein required (Geisse *et al.*, 1996).

4.2.1. Expression of adiponectin by *E. coli*

The expression of recombinant proteins in *E. coli* is a rapid, simple and inexpensive method. Many expression vectors are available with different N- and C-terminal tags and many host cell strains can be used for different applications. The convenient protein expression system should be able to produce high yield and good quality of the recombinant protein.

The total amount of recombinant protein depends on some factors affect on protein expression and purification. One of these factors is the promoter strength that is used in the expression vector. In our study *adiponectin* gene was cloned downstream from the strong *tac* promoter and the *mal*E signal peptide to give high expression of the *adiponectin* in pMAL™ expression system. It has been shown that *tac* promoter is one of the strongest promoters that can be induced by the most common inducer isopropyl-beta-dithiogalactopyranoside (IPTG) (Hanning & Makrides, 1998). Another factor affecting protein yield is the location of the recombinant protein expression in the host cell. Principally, the nascent recombinant polypeptide can be directed to three different locations in bacterial cell namely the cytoplasm, the periplasm and the cultivation medium. Some advantages and disadvantages are related to the direction of a recombinant protein to a specific cellular compartment. For example, the high yield can

be achieved by expressing the recombinant protein in the bacterial cytoplasm. However, the formation of disulfide bonds is apparently different within the two distinct compartments of *E. coli,* namely the reducing cytoplasm and the oxidizing periplasm (Villaverde & Carrio, 2003). Especially in the case of disulfide bonded proteins, inclusion body formation can be anticipated if the protein is produced in the bacterial cytoplasm, as formation of disulfide bonds usually does not occur in this reducing cellular compartment leading to incorrect folding and the formation inclusion bodies (Lilie *et al.,* 1998). It has been shown that disulfide bond formation is segregated in *E. coli* and is actively catalyzed in the periplasm by the Dsb system (Rietsch and Beckwith, 1998).

In this study we chose the pMAL™-p4 vector to express adiponectin by *E. coli* as periplasm secreted protein to enhance disulfide bonds formation and to avoid protein aggregation as inclusion bodies. As mentioned previously (see section 1.1.2) adiponectin protein contains two Cys amino acids that are important for correct assembly of adiponection molecules. The different types of adiponectin isoforms can be especially formed through the disulfide bond formation by Cys-39 in the adiponectin variable domain (Waki *et al.,* 2003). The functional importance of disulfide bond formation in adiponectin protein was shown by the expression of mutant adiponectin with a substitution of Cys by Ser at codon 39 which resulted in the failure to form isoforms higher than trimers (Fruebis *et al.*, 2001; Onay-Besikci *et al.*, 2004).

4.2.1.1. Adiponectin protein solubility

There are different strategies to produce soluble recombinant protein by *E. coli*. For example, reducing the expression rate by lowering the temperature after induction (Schein & Noteborn, 1988) or using low concentrations of IPTG for induction (Winograd *et al.*, 1993) will allow the developing polypeptide to fold into the native state. Also there are other alternate approaches to increase protein solubility such as co-expressing of chaperones (Nishihara *et al.*, 1998) or truncate long multi-domain proteins into separate domains as mentioned in chapter one (Himanen *et al.*, 2001; Wybenga-Groot *et al.*, 2001). In our study we found out that the method of fusion protein can be used in producing sufficient amount of soluble eukaryotic recombinant protein by *E. coli.*

Adiponectin gene was inserted down-stream from the *mal*E gene of *E. coli*, which encodes maltose-binding protein (MBP) resulting in expression of an MBP-adiponectin fusion protein. MBP has unique properties in that it is an affinity tag that enhances the solubility of fused recombinant proteins, by assisting in protein folding and, in its periplasmic expression version, promoting disulfide bond formation (Hammarstrom *et al.*, 2002; Shih *et al.*, 2002). In addition, *E. coli* MBP has also shown as a more effective solubility tag compared with solubility enhancing proteins like S-transferase (GST) and thioredoxin (Trx) (Kapust & Waugh, 1999; Braun *et al.*, 2002).

The cleavage of fusion proteins can be achieved by enzymatic or chemical methods. The enzymatic method provides highly specific restriction, reducing the possibilities of unwanted digestion. For this purpose factor Xa, thrombin, and enterokinase are the commonly used enzymes. In addition, specific cleavage of fusion proteins can also be achieved by chemical reagents such as cyanogen bromide and

hydroxylamine (LaVallie *et al.*, 2001). In this study we used pMAL™ expression vector that included a sequence encoding the four-amino acids recognition site for the specific protease factor Xa cleavage. Before digestion with factor Xa the expected size for MBP-adiponectin fusion protein was approximately 48kDa as detected by SDS-PAGE (Figure 3.9) and western blot (Figure 3.10). After digestion with factor Xa the adiponectin protein band appeared in the expected size of 30kDa (Figure 3.11). In order to confirm that the expected size of MBP-adiponectin fusion protein was approximately 48kDa in the crude extract, after digestion with Factor Xa the fusion protein band had disappeared and new band with the expected size about 40 kDa appeared (Figure 3.12). According to SDS-PAGE analysis data (see Figure 3.12), the expected size of adiponectin protein was detected in the crude and purified extracts after digesting both with factor Xa. In addition, the same result was detected by western blot using anti-adiponetin anti-body. These result indicated that the recombinant adiponectin was expressed in soluble form in *E. coli* periplasim. It has been shown that the protein solubility is a good indicator of correct folding as determined by functional binding (Molloy *et al.*, 1998). Our results suggest that adiponectin has been expressed in this study without misfolding. A similar observation was published by Martinez *et al.* (1995) when recombinant human phenylalanine hydroxylase (hPAH) was produced in *E. coli* using the pMAL expression vectors. The recombinant hPAH, recovered in soluble forms, revealed a high specific activity even in crude extracts and was detected as a homogeneous band by Western-blot analysis using affinity-purified polyclonal rabbit anti-(rat-PAH) antibodies (Martinez *et al.*, 1995). To further corroborate our observation, recombinant adiponectin protein was subjected to SDS-PAGE under non-denaturing conditions as shown in Figure 3.35. The

recombinant protein resolved into different molecules as a result of oligomerization, which can be inferred that the recombinant protein folding is similar to that of the native one. This finding was also consistent with that of Ghakshusmathi & Varadarajan (2002) when they used SDS-PAGE without denaturing condition to determine the proper folding of the protein extracted from inclusion bodies compared with the native folding protein (Ghakshusmathi & Varadarajan 2002). The re-folding process was performed to increase adiponectin solubility by exposing the recombinant adiponectin to 6M of guanidine hydrochloride and dialysis against the column buffer. The buffer was changed every two hours three times in order to reduce the time of exposure to intermediate concentration (2 to 4M) of guanidine hydrochloride to avoid incomplete folded protein aggregation (Kelly and Price, 1991).

4.2.1.2. Adiponectin protein purification

It is well established that protein fusion tags are competent tools for purifying proteins from crude extracts. The yield, solubility, and the purity of the recombinant proteins are mainly influenced by the affinity tag systems (Litchy *et al.*, 2005). The fusion MBP-recombinant protein is ready for purification by affinity chromatography column containing polysaccharides such as amylose (Litchy *et al.*, 2005). In this study, recombinant adiponectin protein was expressed as fusion protein (MBP-adiponectin) in a soluble form by *E. coli* and purified to homogeneity using amylose resin column. The results of this study showed that 1 liter of culture yielded approximately 40 mg of recombinant adiponectin after the purification process. It has been published that more than 63 proteins of 95 eukaryotic proteins expressed at soluble levels of greater than 1

mg/liter as N-terminal MBP fusions. Additionally, the recombinant proteins at lower expression levels than 1 mg/liter were high molecular weight proteins, high content of hydrophobic amino acids and low complexity domains (Dyson *et al.*, 2004). Collectively, in this study, MBP-adiponectin was purified by amylose resin column due to high affinity of MBP for amylose and at the same time maintains or enhances adiponectin ability to be soluble as mentioned previously.

4.2.2. Adiponectin expression by *Pichia pastoris*

Production of recombinant protein by eukaryotic expression systems has been used frequently as therapeutics and research tools as well. The mammalian cell lines are used as eukaryotic expression systems include transfected adherent CHO cells and nonadherent lymphoid cell lines. Moreover, there is another equivalent choice such as infection of insect cells by recombinant baculoviruses. These types of expression systems produce good quality of eukaryotic recombinant protein with post- translational modifications but the most offer quantity that is still less than desireble (Geisse *et al.*, 1996).

Yeast as a eukaryotic organism has the ability to produce glycosylated recombinant proteins while exhibiting almost similar codon preferences as higher organisms (Romanos *et al.*, 1992). Likewise, *Pichia pastoris* has the advantages of eukaryotic expression system like post-translational modifications, fast growth on economic media, with little risk of contamination with oncogenic or viral DNAs and endotoxins (Romanos *et al.*, 1992). Moreover, the methylotrophic yeast *Pichia pastoris* is

noted for its high productivity of recombinant proteins in batch and continuous fermentation (Cregg & Higgins, 1995; Faber *et al.,* 1995). Therefore, this yeast species is potentially competent to efficiently produce recombinant proteins encoded by eukaryotic genes.

4.2.2.1. Production of adiponectin as extracellular secreted protein

Previous studies have indicated that the expression levels of recombinant proteins in *Pichia pastoris* as intracellular protein was low in quantity (1-2% of the total cell proteins). This is due to the fact that when methanol is used as the inducer for protein expression, the enzymes for methanol metabolism are synthesized *de novo* and some of them are translocated into peroxisomes. Remarkably, peroxisomes can fill most of the cellular volume and AOX1 enzyme alone can account for up to 35% of the total soluble protein (Wegner & Harder, 1986). On the other hand, *P. pastoris* is efficient in protein secretion and there are low levels of endogenous proteins compared to other yeasts. Therefore, recombinant protein produced through the secretory pathway constitutes the majority of the total protein in the culture medium (Barr *et al.*, 1992). The present study was therefore aimed to express human adiponectin protein as extracellular protein using pPICZαA vector that contains the *Saccharomyces cerevisiae* α factor signal peptide for efficient extracellular secretion.

The *adiponectin* gene was amplified *in vivo* using *E. coli* cloning vector pGEM-T to facilitate the second cloning in pPICZαA expression vector. The gene was amplified with and without native signal peptide however; the sequencing data showed that *adiponectin* fragment with its native signal peptide has two chromatography peaks

starting with *adiponectin* sequence (Figure 3.25). This observation was consistent with agarose gel electrophoresis results obtained from colony PCR screening (Figure 3.23). As expected, batch fermentation of this recombinant yeast did not yield recombinant *adiponectin* (Figure 3.31). On the other hand, *adiponectin* construct without its native signal peptide was successfully expressed to yield the desired recombinant product (Figure 3.30). The sequencing and agarose gel electrophoresis results of colony PCR screening indicated the correct cloning in the yeast expression vector. The position of *adiponectin* in the expression vector downstream of *Saccharomyces cerevisiae* α factor signal peptide facilitated in producing relatively high amount of extracellular recombinant protein (0.06-0.111 mg/ml). This amount of recombinant adiponectin is a reasonably good quantity compared with recombinant adiponectin produced by *E. coli* in our study (0.04 mg/ml) or in other studies that was 0.04-0.08 mg/ml produced by *E. coli* and 0.01 mg/ml produced by baculovirus (Avides *et al.*, 2008).

4.2.2.2. Optimization of protein expression

There are many factors affecting the yield of recombinant protein produced by *Pichia pastoris* including time of harvesting, methanol concentration, temperature, and pH. A time course expression study was applied in this study to determine the optimal conditions of recombinant protein production in flask fermentation.

The time of protein harvesting is one of the important factors to balance between protein production and the start of protein degradation due to protease activity in the culture media. In this study, SDS-PAGE and western blot data showed that sixty hours after starting of methanol induction was the best time for protein harvesting. It appeared

that after sixty hours of methanol induction there was evidence of degradation in the recombinant protein (see Figure 3.33). This is also shown in the measure of the concentration of recombinant protein at subsequent time points (Table 3.1).

One of the most essential factors in *P. pastoris* expression system is the methanol concentration. In this time course optimization, we added 0.5% as a final concentration of methanol every 12 hours. The key point in methanol induction is keeping the exact intervals between each point time of methanol induction because the high levels of this inducer substrate will also be toxic to the cells. Therefore, it is very important to monitor and control this variable factor (Zhang *et al.*, 2000). On the other hand, the low levels of methanol may not be enough to initiate the AOX transcription (Cereghino & Cregg, 2000). It has been shown that positive influence on recombinant protein production can be achieved by constant methanol concentration during the induction phase (Hellwig *et al.*, 2001).

The batch fermentation was carried out at a constant temperature (30°C) and pH (6.0) throughout the course of expression as recommended in the *Pichia pastoris* Expression Instruction Manual. However, the culture environment may be varied according to the recombinant protein characteristics, for example alkaline *β*-mannanase produced at pH 7.0 was 6.7 times of that at pH value of 6.0 and the highest *β*-mannanase activity of 32.2 IU/ml in culture supernatant was achieved at 120 h of cultivation (pH 7.0) (He *et al.*, 2008).

As a combined observation, results of this study suggested that the best expression condition for recombinant adiponectin by *Pichia pastoris* was 0.5% of methanol induction administrated every 12 hours for 60 hours at 30°C and pH 6.

4.2.2.3. Protein solubility

High-level expression of recombinant human adiponectin was achieved in *Pichia pastoris* fermentation cultures as an α-factor signal peptide fusion, based on the intended *in vitro* construction of *adiponectin* gene. *Adiponectin* gene was cloned upstream of 6x His tag to facilitate recombinant protein purification by Nickel column. Most of the recombinant proteins that were produced by *Pichia pastois* were in soluble form (Liu *et al.*, 2007).

The essential parameter that was used in this study to assess the solubility of recombinant adiponectin is the examination of oligomerization formation. This investigation was carried out by separating adiponectin protein in SDS-PAGE blot under non-denaturing conditions. The recombinant adiponectin separated to different molecular weight molecules ranging from the expected size of adiponectin monomer (30kDa) to above 200kDa molecules (see Figure 3.35). These oligomers cannot form without disulfied bond formation at Cys 39 in the variable domain of adiponectin (Fruebis *et al.*, 2001; Waki *et al.*, 2003; and Onay-Besikci *et al.*, 2004). The results suggest that *Pichia pastoris* has the ability to produce recombinant adiponectin in a soluble form with good sulphur bond formation and oligomerization.

4.2.3. Comparison between *adiponectin* expression in *E. coli* and *P. pastoris*

Expression results obtained in this study showed that recombinant human *adiponectin* gene constructed *in vitro* was successfully expressed in *E. coli* and *P. pastoris.* Moreover, it was also evident that the recombinant proteins produced by each of

these expression systems were in a soluble form. Interestingly, the recombinant adiponectin that were produced by *P. pastoris* (P-ADP) was able to form oligomers at higher molecular weight compared to the recombinant adiponectin produced by *E. coli* (E-ADP). Previous studies have indeed shown that the bacterially expressed recombinant adiponectin displayed a complete lack of differentially modified isoforms and failed to form trimers and larger multimers (Richards *et al.*, 2006). A relevant study presumed that the ability of adiponecin to self–association depended on post-translational modification that is not possible in *E. coli* which produced mainly adiponectin in trimers form as opposed to adiponectin produced by baculovirus which was able to form multimers (Avides *et al.*, 2008). In the present study, E-ADP was more amenable to form timers, and to a lesser extent, multimers compared with P-ADP which was able to easily self–associate into multimers (see Figure 3.35). As mentioned previously, the ability of adiponectin molecules to assemble in multimers depends on the sulphur bond formation by Cys 39 and glycosylation of five conserved lysine residues within the collagenous domain (Wang *et al.*, 2004). Interestingly, we observed that in the *E. coli* expression system that allows periplasm production of E-ADP was able to assemble into low molecular weight oligomers. On the other hand, the *P. pastoris* expression system still superior in producing recombinant adiponectin with higher molecular weight oligomers. These observations suggest that *P. pastoris* offers more advantages such as eukaryotic post-translational modifications especially glycosylation and disulfide bond formation, as well as efficient secretion pathway of recombinant protein (Romanos *et al.*, 1992).

In the prokaryotic expression system, the production of soluble E-ADP required binding adiponectin protein with tag protein such as maltose binding protein (MBP)

which is necessary for purification as well. However, it is essential to remove the MBP by digestion with factor Xa, and second step of purification is required to eliminate MBP from the final product. This process required time and cost, and would also result in protein loss and degradation through refolding and purification processes especially in the large scale production. Protein expression in *P. pastoris* has the advantage of not requiring a native tag protein to enhance solubility, and the purification process can be easily achieved by one step Nickel column purification, based on the presence of 6xHis tag in the carboxyl terminus. Moreover, *E. coli* cytoplasm and periplasm contain a lot of endogenous proteins that would require high quantities of solutions for washing steps whereas *P. pastoris* has little endogenous proteins secreted extracellular. Finally, the total amount of recombinant P-ADP (60 mg/L) was higher than E-ADP (40 mg/L), which rendered the eukaryotic expression system more advantageous.

4.3. Adiponectin bioactivity

Recent research showed that recombinant adiponectin may have various therapeutic potential for metabolic syndrome, including type II diabetes, atherosclerosis, and cardiovascular diseases. However, adiponectin in the blood circulation accounted for 0.01% of total plasma proteins, which is higher than the concentrations of other hormones (5–30 μg/ml) under normal physiological conditions. Moreover, its levels are maintained within a very narrow range (Scherer *et al.*, 1995). Additionally, adiponectin exists in the blood as different molecular forms, trimers, hexamers, and high-molecular weight (HMW) versions (Wang *et al.*, 2008). Therefore, these fundamental points

(protein amount and isoforms formation) have to be taken into consideration in the effort to produce recombinant adiponectin for pharmacological purposes.

In the present study, we produce recombinant human adiponectin by two different expression systems resulting in different amounts of the recombinant protein and isoforms. The bioactivity study was focused on comparison between the effect of the recombinant adiponectin produced by *P. pastoris* (P-ADP) to that produced by *E. coli* (E-ADP) on blood glucose and lipid profile. As mentioned, results obtained in this study suggested that *P. pastoris* is able to produce good amount of proper folded and glycosylated adiponectin that can form different isoforms. Therefore, we carried out other experiments focusing on investigating P-ADP bioactivity through administration of low and high doses of this protein, and its potential in lowering blood glucose after oral glucose challenge.

4.3.1. Effects of E-ADP and P-ADP on blood glucose

In this study, an experiment was designed to examine the effects of E-ADP and P-ADP proteins on blood glucose level and lipid profile. Mice were fed high sucrose/ fat diet and injected intraperitonialy with equal doses of recombinant E-ADP and P-ADP. Our results have demonstrated that the two types of recombinant adiponectin were biologically active in lowering blood glucose to that levels. Previous studies described that the injection of recombinant full-length adiponectin produced from mammalian cells abolishes hyperglycemia in several diabetic animal models, including obese ob/ob, non obese diabetic and streptozotocin-treated mice (Berg *et al.*, 2001). A related study has

shown the ability of recombinant adiponectin produced by different host cells in lowering blood glucose *in vivo*. For example, full-length adiponectin and its C-terminal globular domain were expressed in *E. coli* and used alloxan- treated rats (Hu *et al.*, 2003). The recombinant adiponectin has an ability to transiently abolish hyperglycemia. Additionally, recombinant adiponectin were then used to immunize a rabbit to obtain anti serum that can be used to detect human adiponecin in blood serum by western blot analysis. This finding demonstrated that the recombinant adiponectin produced by *E. coli* was able to produce specific antibodies due to its correct folding (Hu *et al.*, 2003).

More recently, recombinant globular domain of human adiponectin produce by *P. pastoris* was biologically active in lowering blood glucose in mice (Liu *et al.*, 2007) but to date no report has been published demonstrating the effect of full- length recombinant adiponecin expressed by *P. pastoris* on blood glucose and lipids. In our study, we successfully produced biologically active full-length human adiponectin by *P. patoris* as well as by *E. coli* as expression systems to use in comparing the biological activity. The potential of P-ADP in lowering blood glucose is significantly higher than E-ADP when the mice were administrated with same doses of each recombinant protein. We presume that the superiority of P-ADP over E-ADP is due to the ability of *P. pastoris* as eukaryotic organisms to process the recombinant protein in a fashion that is more appropriate compared to the *E. coli* system. This argument is supported by observation of high molecular weight multimers (HMW) formation in P-ADP compared to E-ADP as mentioned previously. Despite the importance of having Cys 39 as a backbone of HMW adiponectin formation (Waki *et al.*, 2003), the availability of appropriate post-translational modification mechanism is also important in ensuring successful formation

of HMW adiponectin molecules. This would include some essential processes, such as, prolylhydroxylation which is important to maintain the integrity of collagen fibrils and possibly affecting the tertiary structure of collagen domain. Another important process is the glycosylation of lysine residues, as it had been previously shown that replacement of lysine residues with arginine residues resulted in the adiponectin molecules having impaired function and are unable to form the HMW isoforms associated with insulin-sensitizing activity (Wang *et al.*, 2002 & 2006). We can thus conclude that the soluble E-ADP with correct folding but without the necessary post-translation processes would yield recombinant protein that possess low biological activity compared with P-ADP that is able to be processes into HMW isoforms.

4.3.2. P-ADP potential in lowering blood glucose

In the previous section we showed that P-ADP exhibited higher biological activity than E-ADP based on its formation of high molecular weight (HMW) isoforms. Subsequently, we performed further analysis for P-ADP bioactivity, which included comparing the effects of administering high and low doses of the adiponectin, as well as oral glucose tolerance test. The data of these experiments showed that both high dose and low dose of P-ADP significantly decreased blood glucose. However, there was a significant difference between the high and low doses of P-DAP in lowering blood glucose. The same high dose of P-ADP used in oral glucose tolerant test supported our finding. We observed that high dose of P-ADP exhibited a very significant decrease in blood glucose after oral glucose challenge. It was evident in published clinical data that

the HMW of human adiponectin multimeric forms strongly correlated with the improvement of insulin sensitivity and glucose tolerance than total adiponectin levels (Hara *et al.*, 2006). We hypothesise that adiponectin multimers can serve as a reserve pool of other adiponectin molecules such as trimers. This pool however, does not include monomers since adiponectin monomer molecules cannot be secreted by mammalian cells unless assembled into trimeric forms (Waki *et al.*, 2003). The high level of blood glucose can act as the reducing factor for Cys 39 sulphur bonds in multimers to release trimers. This hypothesis can be supported by findings of Waki *et al.* (2003) when they demonstrated that certain mutant adiponectin gene could be associated with hypoadipoctinemia. These mutant adiponecin molecules were not able to complete their assembly into trimers, resulting in impaired multimerization and consequently impaired secretion, which are known to be among the cause of a diabetic phenotype or hypoadiponectinemia in subjects having these mutations (Waki *et al.*, 2003). Another supporting findings demonstrated that serum high-molecular weight (HMW) adiponectin (but not total adiponectin) decreased rapidly after glucose loading in subjects with normal glucose tolerance or impaired fasting glucose; and the decrease of HMW adiponectin may be associated with an increase of serum insulin (Ozike *et al.*, 2009). We may conclude that P-ADP decreased blood glucose significantly higher than E-ADP because *P. pastoris* was better at producing high molecular weight adiponectin compared to *E. coli.* There is growing evidence to demonstrate that only eukaryotic expression system is capable of forming the high molecular weight of isoforms of recombinant proteins while prokaryotic expression system can only form the low molecular weight adiponectin (Avides *et al.*, 2008). Although the common explanation for this observation is the lack

in the translation modifications such as glycosylation that is required for proper structure formation and oligomerization, another possible reason for this phenomenon is the collagen domain were not able to fold properly in the prokaryotic system (Hug & Lodish, 2005).

4.3.3. Effects of E-ADP and P-ADP on lipid profile

In the present study, mice were injected with equal doses of P-ADP and E-ADP immediately after feeding with high sucrose /fat diet. After four hours from the feeding and treatment, the mice were sacrificed and blood serum was collected to analyze the lipids profile. The data from these experiments showed that recombinant adiponectin (P-ADP and E-ADP) significantly decreased triglycerides (TG) and increased high density lipoprotein (HDL) whilst, there were no significant effects on total cholesterol (TCOH) and low density lipoprotein (LDL).

Previous studies have shown that the injection of endogenous bovine adiponectin into mice potently enhanced lipid clearance after a high fat meal and chronic administration of this protein for a period of two weeks significantly reduced hepatic lipid accumulation in high-fat fed mice (Wang *et al.*, 2004). These results provide direct evidence that endogenous bovine adiponectin is a physiological hormone that can regulate lipid metabolism. It has also been shown that recombinant adiponectin from *E. coli* has the ability to decrease the level of plasma free fatty acids caused either by administration of high fat test meal or by intra-vein injection of intra-lipid (Fruebis *et al.*, 2001). Another related study had explained that recombinant globular adiponectin from

P. pastoris was biologically active in accelerating the clearance of free fatty acid in animal models (Liu *et al.,* 2007). Results from our study shown consistent finding, the recombinant adiponectin potentially lowered TG levels in blood plasma.

It has also been previously shown that chronic administration of recombinant full-length adiponectin produced by mammalian cells can ameliorate dyslipidemia and insulin resistance in animal models (Xu *et al.,* 2003). This activity of the recombinant adiponectin may be due to increased *β*-oxidation of fatty acid in liver and muscle through PPARα activation, and thus decreasing triglyceride content in the liver and muscle (Yamauchi *et al.*, 2001 & 2003). Our results concur with these reports, as they indicated that both P-ADP and E-APD are able to positively influence lipid profile through decrease of TG and increase of HDL. It is noteworthy that, clinical studies have indeed shown that serum adiponectin was negatively correlated with serum triglycerides and positively with HDL-cholesterol (Xita *et al.*, 2005). Furthermore, patients with reduced adipose tissue mass have markedly increased adiponectin circulating levels (Delporte *et al.*, 2003; Housova *et al.*, 2005).

The results of our study also showed that there is no significant difference between P-ADP and E-ADP in terms of their effects on lipid profile. Previously, we mentioned that the difference between P-ADP and E-ADP was perhaps due to the difference of post- translational modification resulting in HMW isoforms in P-ADP but not E-ADP. We hypothesize however, that HMW adiponectin isoforms are not capable of direct influence on lipid profile since the loading of oral fat in healthy subjects was not able to change total serum adiponectin or HMW of adiponectin isoforms (Ozeki *et al.*, 2009).

4.4. Effect of P-ADP administration on related gene expression

Thus far, results in the present study provided strong support for P-ADP exhibiting better bioactivity compared to E-ADP. We continued our investigation onto looking at the effect of adiponectin administration on the expression of selected genes, namely the glucagon, *insulin* and *leptin* receptors. These receptors have a strong relationship with glucose and lipid metabolism in the human body (Fruebis *et al.*, 2001). In this study, we hypothesized that P-ADP administration may exert an effect on the regulation of the level of these receptors, which in turn, will the affect specific hormonal activities on the target tissues. The variable hormone effectiveness on its target tissue can come through the changes in its levels or the changes in its receptors regulation or both of them. Previous studies provided evidence that the administration of the globular domain of adiponectin produced by *E. coli* had no effect on glucagon, insulin and leptin levels (Fruebis *et al.*, 2001).

This study was carried out based on mRNA quantification of transcripts from the genes encoding glucagon, insulin and leptin receptors. In brief, as mentioned in chapter two and three, the control group and treatment group (four mice in each) were injected with saline and P-ADP respectively. After four hours, the mice were sacrificed and the tissues were snapping frozen to extract total RNA. Then, cDNA was prepared to use in quantitative Real-Time PCR (RT-PCR). According to the protocol of relative quantification RT-PCR, the expression of the genes of interest was normalized with β-actin as the endogenous gene. The data was statistically analysed based on Δct values and RQ values (Table 3.3, 3.4 and 3.5) as described by Bookout & Mangeladorf (2003).

4.4.1. Effect of P-ADP administration on the expression of glucagon receptors

The results of this study have shown that P-ADP treatment significantly down regulated glucagon receptors in the liver and kidney whilst there was no significant effect on the glucagon receptors in the fat tissue. The difference on the expression of glucagon receptors in the skeletal muscle however, was not clear enough for any conclusion to be made. The results of this experiment indicate that P-ADP was able to reduce mRNA level of glucagon receptors especially in the liver and kidney. This finding could be one of the main causes of lowering blood glucose after P-ADP administration. Meanwhile, lowering of blood glucose levels after administration of adiponectin occur without a significant increase in insulin levels or a decrease in glucagon levels (Fruebis *et al.*, 2001). Interestingly, patients with poorly controlled type II diabetes have increased rates of endogenous glucose production, which can be attributed to increased rates of gluconeogenesis (Hundal *et al.*, 2000). Another study explained that adiponectin promotes elevated phosphorylation of hepatic AMP-activated protein kinase (AMPK) through inhibition of the expression of hepatic gluconeogenic enzymes such as phosphoenolpyruvate carboxylase and glucose-6-phosphatase that lead to inhibition of endogenous glucose production (Berg *et al.*, 2001; Combs *et al.*, 2001;Yamauchi *et al.*, 2002). We suggest it might be possible in the first instance, adiponectin signals of down-regulation of glucagon receptors to reduce the physiological role of glucagon in hepatic glucose production. Subsequently, the expression of hepatic gluconeogenic enzyme will be decreased leading to inhibition of endogenous glucose production.

4.4.2. Effect of P-ADP administration on the expression of insulin receptors

The experiments of gene expression in our study also revealed that P-ADP administration did not change the gene expression of insulin receptors. This result indicates that adiponectin's influence on blood glucose uptake might be taking effect through a different mechanism and insulin receptors may not be directly involved in this mechanism. It has been previously shown that adiponectin receptors (AdipoR1 and AdipoR2) mediate its effect on glucose uptake through increased AMPK level (Kadowaki *et al.*, 2006). Pharmacological studies explained that rosiglitazone administration increases glucose uptake in obese mice significantly, and at a lesser extent in obese mice lacking the *adiponectin* gene (Nawrocki *et al.*, 2006). It was also shown that adiponectin mediates the effect of rosiglitazone by increasing AMPK activity in the liver and muscle when adiponectin knockout mice did not respond to rosiglitazone while the wild type mice significantly responded through increase AMPK activation (Yamauchi *et al.*, 2002).

4.4.3. Effect of P-ADP administration on the expression of leptin receptors

Leptin is a hormone produced by fat tissue and influences body weight, food intake and manages the energy homeostasis. It affects on the high brain centres especially on the hypothalamus by negative feedback loop mechanisms (Halaas *et al.*, 1995; Friedman & Halaas, 1998). It has been found that the combination between body mass index (BMI) and leptin levels is the most prognostic indicators of insulin resistance

syndrome (Chu *et al.*, 2000). In parallel, other studies have demonstrated that leptin concentration has a strong relationship with obesity and a possible role in insulin resistance suggesting that the variation in leptin levels may be one the main causes of insulin resistance (Zimmet *et al.*, 1996 & 1998).

In our study we found that P-ADP administration significantly up-regulates the expression of leptin receptors in the mouse liver, fat tissue and kidney. These results may indicate that adiponectin can affect synergistically with leptin on blood glucose uptake and lipid profile. Our results supports previous finding by Yamauchi and his co-workers that explained insulin sensitivity was enhanced by a combination of physiological doses of leptin and adiponectin (Yamauchi *et al.*, 2001). Moreover, recombinant leptin increased insulin sensitivity by promoting fatty acids oxidation (Shimabukuro *et al.*, 1997). Another related study showed that leptin can activate AMPK indirectly through the central nervous mechanism (hypothalamus - sympathetic nervous axis) or can activate AMPK directly through a direct effect on the target organs (Minokoshi *et al.*, 2002).

We hypothesize that the administration of recombinant adiponectin decreased blood glucose level by direct effect on AMPK level (Kadowaki *et al.*, 2006) or indirect effect through up- regulation of leptin receptors, which would then exert their effect on AMPK level as mentioned above. Another possible mechanism for lowering blood glucose as suggested by this study is the effect of recombinant adiponectin administration on down-regulation of glucagon receptors and subsequent inhibition of further endogenous glucose production. Also, recombinant adiponectin induces fatty acids β-oxidation by affecting directly on the PPARα activation and thus decreasing triglyceride content in the tissues (Yamauchi *et al.*, 2001; 2003) or indirectly causes an effect through

up-regulation of leptin receptors and subsequently on the increase of fatty acids oxidation (Shimabukuro *et al.*, 1997).

4.5. Summary and conclusion

In this study, we constructed human *adiponectin* gene *in vitro* by overlap-extension PCR. We found out that this method was simple and useful for gene construction especially in reducing cost and time for tissue collection, RNA isolation and cDNA preparation. However, in cases where a gene has high number of exons, more PCR steps will be needed to overlap these exons. In such cases, however, obtaining cDNA through conventional methods would probably be less laborious compared with overlap-extension PCR. The constructed human *adiponectin* gene was cloned and expressed successfully in *E. coli* as the prokaryotic expression system. The pAML™ expression system was useful in producing soluble recombinant adiponectin by way of producing a fusion protein with native *E. coli* protein (MBP). Tests on biological activity also showed that the recombinant adiponectin was biologically active in the lowering of blood glucose and have positively affects the lipid profile. We can summarize the advantages of *E. coli* over yeast as protein expression system as follows:

- The expression course in *E. coli* (4 hours) was shorter than the expression course in yeast (60 hours)
- In *E. coli* expression, one simple media (LB broth) was used for growth and induction phases whereas in yeast expression two complex media were used for growth phase (BMGY) and induction phase (BMMY)

- One time induction by IPTG was performed in the expression by *E. coli*, however, in yeast the induction by methanol was performed each 12 hours for 60 hours as optimum time for protein harvesting.
- The antibiotic that used in *E. coli* expression system (Ampicillin) was cheaper than what used in the yeast expression system (Zeocin™).

In another part of our study, the constructed adiponectin gene was cloned and expressed successfully in yeast (*P. pastoris).* The pPICZαA expression system was used to produce recombinant adiponectin as extracellular protein. The presence of *AOX1* promoter and *Saccharomyces cerevisiae* α factor signal peptide helped in producing a relatively good amount of extracellular recombinant protein (0.06-0.111 mg/ml). The soluble bioactive recombinant adiponectin successfully lowered blood glucose and positively changed the lipid profile. Additionally, *P. pastoris* expression system offers several advantages over *E. coli* expression system, and we can summarize these advantages as follows:

- The recombinant adiponectin produced by *P. pastoris* (P-ADP) was significantly more active in lowering blood glucose comparing with that produced by *E. coli* (E-ADP).
- *P. pastoris* was able to produce recombinant adiponectin forming multimers whereas *E. coli* was unable to produce recombinant adiponectin higher than trimer. The fact that *E. coli* was able to produce soluble adiponectin protein but without the required post-translational modification steps rendered E-ADP less efficient than P-ADP.

- P-ADP was soluble and biologically active without the need to be fused with another protein to enhance its solubility, as in the case of E-ADP expressed in *E .coli*. Subsequently, the purification of P-ADP was achieved in one step using Nickel-column that has an affinity to C-terminal 6xHis tag. In *E. coli* expression, the fusion MBP requires further digestion with Factor Xa followed by two steps purification to remove the MBP.
- The amount of P-ADP (0.06-0.111 mg/ml) was higher that E-ADP (0.04mg/ml) which could be due to the low amounts of extracellular secreted endogenous protein in *P. pastoris* compared with periplamic *E. coli* proteins.