CONSTRUCTION AND EXPRESSION OF HUMAN *ADIPONECTIN* IN PROKARYOTIC AND EUKARYOTIC EXPRESSION SYSTEMS AND THE STUDY OF ITS EFFECT ON SELECTED BLOOD PARAMETERS AND EXPRESSION OF RELATED GENES

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Abstract

Adiponectin is one of the most bioactive substances secreted by adipose tissue which is involved in protection against metabolic syndrome, artherosclerosis and type II diabetes. Research into the use of adiponectin as a promising drug for metabolic syndromes requires production of this hormone in high quantities. This may be achieved using recombinant DNA technology, which would also allow the production of different molecular forms as well as providing greater input in terms of understanding its signalling pathway. This study was mainly targeted towards producing adiponectin hormone as a recombinant protein by *P. pastoris* (P-ADP) as a cheap and convenient eukaryotic expression system for potential application in pharmaceutical therapy. For comparison, adiponectin was also expressed using the *E. coli* (E-ADP) as a traditional prokaryotic expression system. Following successful expression, the relative bio-properties of P-ADP was assessed *in vivo* in comparison with E-ADP. Additional studies of the effect of P-ADP on the expression of the genes encoding glucagon, insulin and leptin receptors were carried out.

Adiponectin gene was constructed *in vitro* by splicing its two exons using overlap-extension PCR. Full length *adiponectin* was amplified by PCR and cloned into pMAL™-p4 vector for expression in *E. coli* as periplasmic secreted protein. The fusion protein was purified by amylose column after digestion with factor Xa. To express *adiponectin* in *P. pastoris*, the full length *adiponectin* was amplified by cloning into pGEM-T vector and then sub-cloning into pPICZαA vector to be expressed as extracellular secreted protein. The 6xHis-tagged recombinant adiponectin was purified by one step affinity chromatography using Nickel column. SDS-PAGE and western blot were used to detect and analyse the recombinant proteins and Bradford assay was used for protein quantification. Three experiments were designed to assess and compare the effects of E-ADP and P-ADP on blood glucose and lipid profile using ICR mice as a

model system. Real-Time PCR was used to examine the changes in the regulation of glucagon, insulin and leptin receptors after administration with P-ADP. The expression of target genes was normalized with *β-actin* as endogenous gene and the data was statistically analysed based on *∆ct* values and RQ values using t-test. The results showed that *adiponectin* gene was successfully constructed *in vitro* by overlap-extension PCR and expressed by *E. coli* as a soluble periplasm protein and by *P. pastoris* as a soluble extracellular protein. *P. pastoris* expression system was successful in producing high molecular weight of adiponectin molecules and relatively high quantity of recombinant protein (0.1 mg/ml) as compared with *E.coli* (0.04 mg/ml). The optimum conditions of adiponectin production by *P. pastoris* were 0.5% of methanol induction every 12 hours for 60 hours at 30°C. E-ADP and P-ADP were biologically active in the lowering of blood glucose and triglyceride and increasing high density lipoprotein. The ability of P-ADP in lowering blood glucose was significantly higher than E-ADP. However, there was no significant difference on the effect on lipid profile. P-ADP significantly down-regulates glucagon receptors and up-regulates leptin receptors, whilst there was no significant effect on insulin receptors. Our results suggest that *P. pastoris* expression system is better in producing high quantity, high biological activity and easily purified recombinant adiponectin comparing with *E. coli* expression system that can be used in large scale production of adiponectin as potential drugs for metabolic syndromes.

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Abbreviations

3T3-L1	Cell line derived from 3T3 cells used in biological research on adipose tissue.
°C	degree Celsius
µg	microgram
µg/ml	microgram per millilitre
µl	microlitre
µM	micro Molar
3T3-F442A	preadipocyte cell lines
ADPs	adiponectin DNA fragment with signal peptide sequence
ADPws	adiponectin DNA fragment without signal peptide sequence
AMPK	5' adenosine monophosphate-activated protein kinase
AOX	Alcohol oxidase
APS	ammonium persulfate
TEMED	N, N, N', N'-tetramethylethylenediamine
Arg	Arginine
BMGY	Buffered Glycerol-complex Medium
BMMY	Buffered Methanol-complex Medium
bp	base pair
BSA	bovine serum albumin
BW	Body weight
$CaCl_2$	calcium chloride
cDNA	complementary DNA

Abbreviation	
CDS	coding sequence
CHO	Chinese hamster ovary
CHOL	total cholesterol
Ct	Cycle time
CV	coefficient of variation
Cys	cysteine
DEPC	Diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphate
e.g	for example
E-ADP	recombinant adiponectin expressed in *E. coli*
EDTA	ethylenediaminetetraacetic acid
ERp44	endoplasmic reticulum folding assistant protein
et al.	*et alii* (and other people)
EtBr	ethidium bromide
FLD1	formaldehyde dehydrogenase gene
g/L	gram per litre
Glu	Glutamic acid
Gly	Glycine
HDL	high density lipoprotein
His	Histidine
HMW	High molecular weight
hrs	hours
i.e.	that is
IGF-1	Insulin like growth factor 1
Ile	Isoleucine

Abbreviation	
IPTG	isopropyl-beta-dthiogalactopyranoside
kb	kilo bases
kDa	kilo dalton
KOAc	potassium acetate
lbs/sq.in.	pounds per square inch
LB	Luria-Bertani broth
LDL	low-density lipoproteins
LMW	Low molecular weight
LSLB	low salt Luria-Bertani broth
M	molar
MBP	maltose binding protein
mg/L	milligram per litre
mg/ml	milligram per millilitre
Mg^{2+}	magnesium ion
min	minute
mM	milli Molar
mRNA	messenger RNA
Mut^{-}	methanol utilization minus
Mut^{+}	methanol utilization plus
Mut^{S}	methanol utilization slow
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
Ni	nickel
nm	nanometer
O_2	oxygen

Abbreviation	
OD_{600}	optical density at 600nm
P-ADP	recombinant adiponectin expressed in *P. pastoris*
PAGE	polyacrylamide gel electrophoresis
PAOX1	promoter from the alcohol oxidase 1 gene
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pfu	*Pyrococcus furiosus*
PPARγ	peroxisome proliferator-activated receptor γ
Pro	proline
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	revolutions per minute
RQ	relative quantification
RT	reverse transcription
RT-PCR	Real-Time PCR
s	second
sdH_2O	sterile distilled water
SDS	sodium dodecyl sulfate
SEM	standard error means
Ser	Serine
SNPs	single nucleotide polymorphisms
Stdev	standard deviation
Taq	*Thermus aquaticus*
TBE	Tris Borate EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline-Tween 20

TCA	trichloroacetic acid
TE	Tris-EDTA
TG	triglyceride
Tm	Melting temperature
TNF-α	Tumor necrosis factor-alpha
TZDs	thiazolidinediones
U	unit
UV	ultraviolet
V	volume
X-Gal	bromo-chloro-indolyl-galactopyranoside
YPD	Yeast Extract Peptone Dextrose