

Cloning and Expression of Voltage-dependent Anion Channel 2 (VDAC2) from Electrically Stimulated Chicken

Nurhidayah Ahmad Hassan¹, Azura Amid^{1*} and Hamzah Mohd Salleh^{1,2}

¹Bioprocess and Molecular Engineering Research Unit, Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia, Gombak, P.O. Box 10, 50728 Kuala Lumpur, Malaysia.

²International Institute for Halal Research and Training (INHART), International Islamic University Malaysia, Gombak, P.O. Box 10, 50728 Kuala Lumpur, Malaysia.

(Received: 21 August 2013; accepted: 25 October 2013)

VDAC2 protein identified to be overexpressed in stunned chicken is a potential biomarker in differentiating stunned chicken since the expression was proportional to the voltage applied. To facilitate research, the gene encoding VDAC is cloned, expressed and optimized in BL21-AI *Escherichia coli*. cDNA was synthesized from mRNA of stunned chicken's skeletal muscle and cloned using recombination reaction. All positive clones were identified by PCR and restriction enzyme digestion as well as DNA sequencing. Recombinant VDAC2 protein was purified by Ni-NTA spin-column. Western blot performed using polyclonal anti-N terminal human VDAC2 and anti-His tag revealed a 34 kDa protein, confirming the expression of recombinant VDAC2.

Keywords: *Gallus gallus*, electrical stimulation, *Escherichia coli*, recombinase reaction.

Voltage-dependent anion channel (VDAC) is a protein of the porin family¹ which is the most abundant proteins found in the outer mitochondrial membrane (OMM) and it is responsible for transportation of metabolites between cell organelle and the cytosol²⁻⁴. Structurally, VDAC is a porin-type β -barrel diffusion pore formed by one polypeptide with a molecular weight of about 30 kDa⁵. There are three isoforms of VDAC which are VDAC1, VDAC2 and VDAC3, each of a molecular mass of 30-35 kDa, 30-32 kDa and 31 kDa respectively⁶⁻⁸. VDAC1 is found abundantly in chicken brain mitochondria whereas the lowest amount of VDAC is VDAC2⁹. Physiologically, VDAC functions as the primary pathway movement of adenine nucleotides through the mitochondrial outer membrane thus controlling the traffic of these essential compounds

to and from the mitochondria as well as the entry of other substrates into a variety of metabolic pathways¹⁰. By controlling metabolite traffic across the OMM, VDAC plays an important role in energy production. As such, down-regulation of VDAC1 expression would result in decreased energy production and consequently affect cell vitality^{11,12}. Besides its function as a transport protein, VDAC is responsible in mitochondria-mediated cell death or apoptosis. There is evidence that mitochondrial porin and truncated Bid (tBid) upon complex formation can induce VDAC closure leading to reduced metabolite exchange and mitochondrial dysfunction^{13,14}.

VDAC genes have been cloned from *Saccharomyces cerevisiae*^{15,16}, *Neurospora crassa*¹⁷ and *Drosophila melanogaster*^{18,19}. In mammals, VDAC cDNAs and genes have been cloned from several species such as human, rat, mouse and in each, there appear to be three different genes encoding distinct, expressed VDAC isoforms⁶. They found that each of the three mammalian VDAC genes, at least as represented

* To whom all correspondence should be addressed.
Tel.: +603-6196 4429; Fax: +603-61964442;
E-mail: azuraamid@iiu.edu.my

by the mouse isoforms, appear to function in much the same way as the yeast VDAC1 and other characterized VDAC proteins²⁰. Comparison of the fungal proteins indicates that the amino acid sequence of VDAC is not highly conserved in which the two fungal proteins share only 30% identity, although the two types of channels have very similar function.

Gateway cloning is a multistep recombinational cloning method which eliminates the need for classical restriction enzyme and ligase (REaL) cloning for the transfer of genes between vectors. Entry clones contain *attL* sites, while expression clones contain *attB* sites. The *att1* and *att2* sites are unable to recombine with each other, and thus produce the unique order of the recombination reactions that maintains the reading frame and eliminates the need for directional screening²¹. Since VDAC2 has the lowest amount in the original host, thus recombinant VDAC2 is more favorable than direct extraction and purification of native protein counterpart for several reasons. With the availability of more cloning techniques, smaller quantities were required to produce indefinite amount of the protein. Other advantages are the expression systems can be manipulated to obtain highest amount of protein and lastly it facilitates the purification processing through fusion proteins. The challenge is to find the best fit from a variety of expression vector and host, assay system, purification protocol and characterization of the recombinant product. Moreover, molecular cloning of VDAC2 from *Gallus gallus* using Gateway technology has not been documented yet in any literature thus this cloning work is novel.

Recent study showed that VDAC2 level is elevated in the samples of muscles of chickens stunned at 0.75 A, 70 V, 5 s from 2D proteomic profiles. It was confirmed at the transcriptional level whereas the gene coding for VDAC2 exhibit up-regulation up to 59.87 fold²². Thus, VDAC2 is a potential biomarker to detect whether the chicken has been electrically stunned in pre-slaughtering process. Therefore, recombinant protein obtained from this study can be used for antibody production and further facilitates the development of a suitable detection kit. Research on chicken's VDAC protein is still lacking compared to other organisms and this study provide initial

characterization of this protein which will be further studied extensively.

MATERIALS AND METHODS

Polyclonal anti-N-terminal human VDAC2 was purchased from Aviva (USA) and anti-His tag was purchased from Millipore (USA). Alkaline phosphatase-conjugated goat anti-rabbit IgG was obtained from Biorad (USA). Female broiler chickens (*Gallus gallus*) aged 35 days and weighing approximately 1.5 kg were collected from a farm in Kuang, Selangor, Malaysia.

Samples preparation

The chickens were electrically stunned with fabricated waterbath stunner at 0.75 Ampere, 70 Volt for a duration of 5 s before being slaughtered. The control samples were not electrically stimulated. Each sample was prepared in triplicates. The muscle tissue was dissected from the chicken's thigh and immersed in RNAlater solution (Biobasic, Canada) to preserve the RNA. All the apparatus were treated with RNase free water and sterilized.

VDAC2 gene isolation

VDAC2 gene fragment was amplified by the reverse-transcription PCR method. The forward and reverse primers used for *Gallus gallus* VDAC2 gene isolation (GeneBank accession number: NM_204741.1) were 5'-CACCATGGCGATTCC TCCATCATA-3' (forward) and 5'-CCCTTC TCTGCAGCAGTTTC-3' (reverse) using Primer3 (v.0.4.0) software (<http://frodo.wi.mit.edu/>). The PCR reaction was performed with the proofread ACCUENZYME™ DNA polymerase (Promega, USA) in three steps reaction: (1) 94°C predenaturation for 2 min, (2) 29 amplification cycles of 94°C for 30 s denaturation, 54°C for 30 s primer annealing, then 72°C for 1 min extension, and (3) 72°C for 10 min. The 881 base pair (bp) fragment was generated. A total of 10 µl of the PCR product was used for the analysis on 1.5% agarose gel containing ethidium bromide, visualized under UV and then documented using gel documentation system. DNA fragment was gel purified using gel purification kit (Qiagen, Germany). Finally, the purified DNA fragment was sent for sequencing and the obtained sequence was analyzed and compared with Gene Bank sequence using BLAST software (<http://www.ncbi.nlm.nih.gov/>).

Cloning of VDAC2 gene into entry vector

The VDAC2 gene was cloned according to the manufacturer's procedure (Invitrogen, USA). One μl of purified VDAC2 DNA fragment, 1 μl salt, 3 μl sterilized water and 1 μl entry vector, pENTR-TEV-D-TOPO (Invitrogen, USA) were gently mixed by swirling the tip without pipetting up and down and incubated for 30 min at 20-25°C, room temperature. Then, 2 μl mixture from the TOPO reaction was transformed into One Shot Top Ten chemically competent *E. coli* cells (Invitrogen, USA) by heat-shock for 30 s at 42°C and immediately transferred back onto ice. Then 250 μl of room temperature SOC medium was added followed by incubation at 37°C for 1 h and shaken horizontally at 200 rpm. Next, 20 μl and 100 μl of the cultured cell was spread onto LB agar supplemented with 50 $\mu\text{g}/\text{ml}$ kanamycin and incubated at 37°C overnight. Two volumes are required to ensure that one plate will have a well space colony. The white colony selection was used to identify the positive transformants. Finally, positive transformants were confirmed by colony PCR, double digestion by restriction enzyme and sequencing.

Construction of the expression plasmid

Once the positive entry clone have been obtained, an LR recombination reaction was performed using Gateway® LR Clonase™ II enzyme mix (Invitrogen, USA) was performed to transfer the gene of interest from the entry vector into the destination vector pDEST17 (Invitrogen, USA) for successful gene expression. However, before cloning into an expression host *E. coli* BL21-AI, the recombinant destination vector has to be subcloned first into *E. coli* DH5 α for maintenance and propagation of the recombinant plasmid. The transformation and selection procedure were similar to the previous one except that the host used was *E. coli* strain DH5 α and 100 $\mu\text{g}/\text{ml}$ ampicillin was used as selection for positive transformants.

Expression and purification of VDAC2 protein

The destination vector containing VDAC2 gene was transformed into an expression host, *E. coli* strain BL21-AI cells (Invitrogen, USA). The positive transformants were cultured into 5 ml LB broth supplemented with 100 $\mu\text{g}/\text{ml}$ ampicillin and incubated in an incubator shaker at 37°C, 250 rpm until OD₆₀₀ reached 0.6-1.0. Then 5 ml of the mid-log phase culture was inoculated into 95 ml

fresh media with ampicillin. Once the OD₆₀₀ reached 0.2-0.4, the culture was induced with 0.2% L-arabinose. At each 0, 1, 2, 3, and 4 h of incubation time, 1 ml of the cells culture was taken and analyzed by 12% SDS-PAGE. Later, the induced cells were harvested by centrifugation at 5,000 x g for 15 min and resuspended in 700 μl buffer A (6 M GuHCl, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, pH 8) and 3 Units/ml benzonase. The supernatant was collected by centrifuging the lysate at 12,000 x g for 30 min at room temperature. VDAC2 protein was purified under denatured conditions. Ni-NTA spin column (Qiagen, Germany) was pre-equilibrated with 600 μl buffer B (7 M urea, 0.1 M NaH₂PO₄, 0.01M Tris-HCl, pH 8) before applying supernatant to the column, then centrifuged at 1600 rpm for 5 min. The column was washed four times with 600 μl buffer C (8 M urea, 0.1 M NaH₂PO₄, 0.01M Tris-HCl, pH 6.3) and finally eluted with 200 μl buffer E (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, pH 4.5). The purified protein was observed by SDS-PAGE stained with silver nitrate.

Protein detection with anti-VDAC2 and anti-His tag by Western Blotting

Proteins separated as described above were transferred onto nitrocellulose membranes using Mini Trans-Blot® Electrophoretic Transfer Cell (Biorad, USA) at 100 V, 4°C for 1 h. Membrane was blocked by incubation (2 h, 4°C) with 5% non-fat dried milk in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5). Then, the membrane was probed with 1:1,000 dilutions of primary antibodies, then washed extensively with TBST and incubated with 1:10,000 dilutions of alkaline phosphatase conjugated secondary antibody; both primary and secondary antibodies were diluted in TBST. The colour was developed using Sigma Fast BCIP/NBT tablet (Sigma Aldrich, USA) which was solubilized in 10 ml distilled water.

RESULTS

VDAC2 gene isolation

The VDAC2 gene was successfully amplified and showed a fragment with a size of 881 bp fragment (Fig. 1a). The sequence from purified PCR product was searched using BLAST (<http://www.ncbi.nlm.nih.gov/>) and it has 97% homology with the VDAC2 of *Gallus gallus* sequence in database (GeneBank accession number:

NM_204741.1). The sequencing result from the purified PCR product confirmed that the start codon ATG exist and was in frame but not so for the stop codon. This truncated sequence probably caused from highly concentrated DNA. It can have a highly detrimental effect on the electrophoresis of

sequencing reaction products. They cause the capillary to become blocked, inhibiting the current and causing the reaction products to pass through the capillary slower than normal. Nevertheless the stop codon TAA can be found in the sequence of cloned expression pDEST17 vector (Fig. 3b).

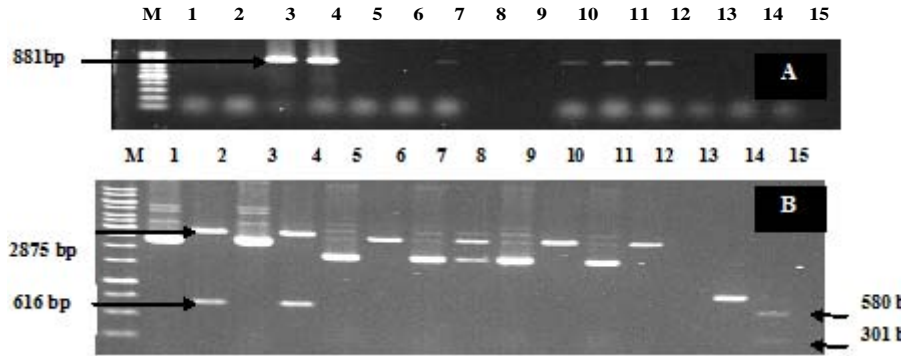


Fig. 1. The PCR and restriction enzyme analysis of entry clone. **A** There were six colonies of entry clone which have the VDAC2 gene and these colonies were further investigated by restriction enzyme analysis. Lane M is the 100bp DNA ladder, the rest lanes are colony PCR product amplified the VDAC2 gene from the clones of recombinant entry vector **B**. Lane M is the 1 kb marker, lane 1, 3, 5, 7, 9, 11 and 14 represent circular uncut plasmid which will run faster than the cut plasmid. Lane 2, 4, 6, 8 and 10 are recombinant entry vector digested with *MfeI* and *XhoI*. Lane 15 is the purified PCR product digested with *MfeI* that gave a total fragment is of 881 bp

```

ATGGCGATTCCATCATATGCAGACCTGGCAAACTGCCAGAGATATC
M A I P P S Y A D L G K S A R D I
TTCACAAAAGGATATGGGTTGGGCTGGTGAACTGGATGTGAAGACAAAATCTGCAAGT
F N K G Y G F G L V K L D V K T K S A S
GGAGTGGAAATTCACAACATCTGGTTCATCGAACACAGACACTGGAAAAGTGAATGGAAGC
G V E F T T S G S S N T D T G K V N G S
TTGGAGACCAAAATACAAGTGGGCTGAGTATGGGCTGACTTTCACAGAAAAGTGGAAACACA
L E T K Y K W A E Y G L T F T E K W N T
GATAAACAATCGGAAACAGAAAATGCAATGAAAGATCAGATTGCCAAAAGGCTTGAAGTTG
D N T L G T E I A I E D Q I A K G L K L
ACGTTTGATACAACTTCTCACCAAATACAGGAAAGAAAAGTGGTAAAAATTAAGTCAGCA
T F D T T F S P N T G K K S G K I K S A
TATAAACGTGAATGCCATAAACCTAGGTTGTGACGTGACTTTGATTTTGTGGGCTGGCA
Y K R E C L N L G C D V D F D F A G P A
ATCCATGGTTTCAAGCGGCTTTGGTTATGAAGGATGGCTAGCGGGCTATCAAATGACTTTT
I H G S A V F G Y E G W L A G Y Q M T F
GATAAGTCCAAAATCAAATGACAAGGAATAACTTCTCTGTGGTTACAAGACTGGAGAC
D S A K S K L T R N N F S V G Y K T G D
TTCCAGCTTCACACCAATGTCATGATGGTTCCGGAATTTGGCGGTTCAATTTACCAGAAA
F Q L H T N V N D G S E F G G S I Y Q K
GTGAGTGACAATCTTGAGACTGCTGTAAACCTGGCATGGACAGCTGGTAGCAACAGCACT
V S D N L E T A V N L A W T A G S N S T
CGCTTTGGAATTCAGCTAAAATACAAGTTGGATTCCACCGCTTCTATCTCTGCAAAAAGTG
R F G I A A K Y K L D S T A S I S A K V
AACAACCTAGTCTAGTTGGAGTGGGTTACACCCAGACCCGAGGCCAGGTGTGAAGCTT
N N S S L V G V G Y T Q T L R P G V K L
ACTTTGCTGCCCTGATAGATGAAAGAGCATCAATGCTGGTGGCCATAAACTTGGTCTT
T L S A L I D G K S I N A G G H K L G L
*
GGCTGGAATGGAGGCCTAAAAGTGAGAAACTGCTGCTAAGAGGATGNACCCAGCTTT
G W N W R P K S E K L L L R G X T Q L
    
```

Fig. 2. DNA and deduced amino acid sequences resulted from the sequencing of purified pDEST17-VDAC2 plasmid. Nucleotides that are double underlined indicate binding sites for forward and reverse primers. Capital and italic alphabets show nucleotide codon for each amino acid sequence, whereas "*" refers to stop codon. Underlined amino acids indicate a 319-amino acid predicted to be the chicken VDAC2 protein

Identification and analysis of the VDAC2 clones

The purified PCR product was cloned into pENTR-TEV-D-TOPO entry vector and the positive clones were confirmed by PCR and double restriction enzyme analysis (Fig. 1). Six colonies that contain the VDAC2 gene was treated with two restriction enzymes, *MfeI* and *XhoI*. Meanwhile, the purified PCR product used as blunt end fragment in TOPO cloning was also digested with *MfeI*. *MfeI* will cut the VDAC2 fragment at position 301 bp starting from the ORF of VDAC2 thus producing two fragments with a size of 580 bp and 301 bp (lane 15 in Fig. 1b). On the other

hand, two fragments with a size of 2875 bp and 616 bp, (lanes 2 and 4 in Fig. 1b) showed that the VDAC2 was successfully inserted into the entry vector nevertheless in the reverse orientation. The purified recombinant plasmid from pDEST17 in *E. coli* DH5 α cells was analyzed as described previously using PCR, double digestion with restriction enzyme (result not shown) and finally sequenced. In contrast to the entry clone, the insert of the VDAC2 in destination vector was oriented correctly. The orientation of the VDAC2 gene was verified by the sequencing result (Fig. 2). Therefore, the expression of VDAC2 can be carried

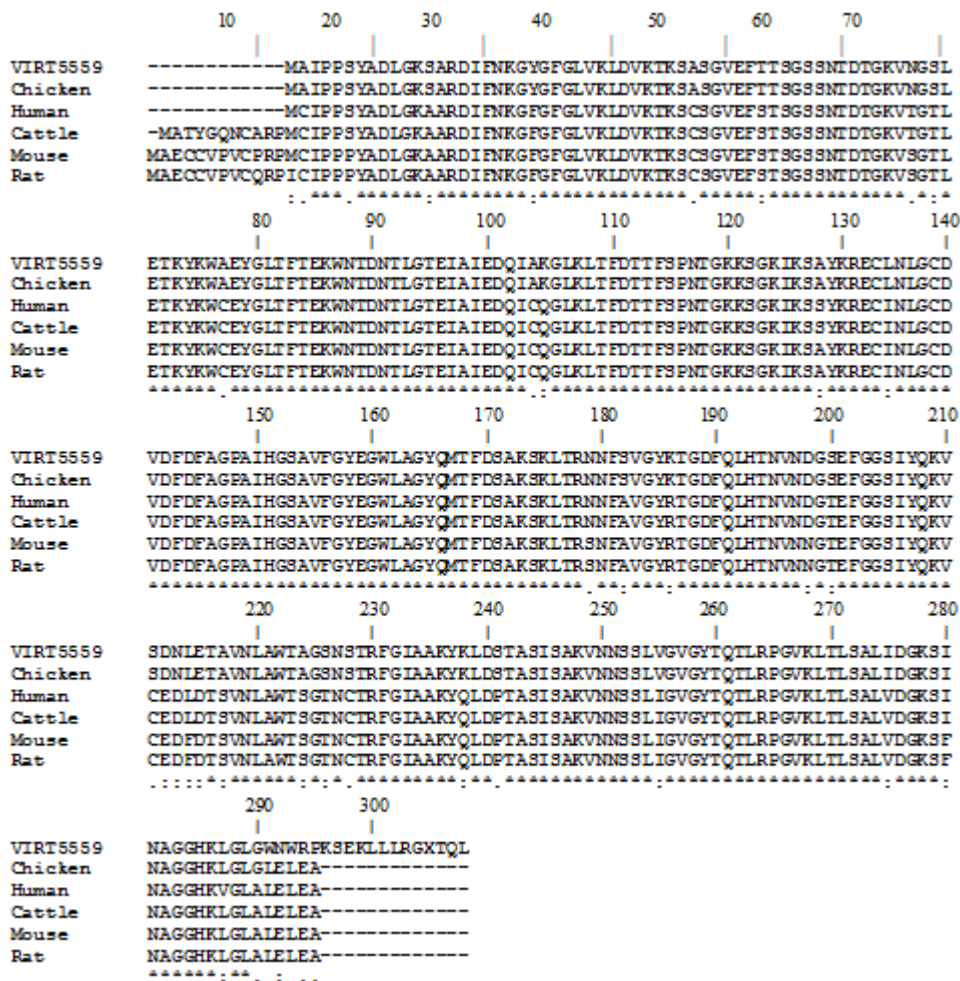


Fig. 3. Comparison of VDAC2 amino acid sequences (VIRT5599) among five species. The VDAC2 sequences of chicken (NP_990072.1), human (CAG33245.1), cattle (AAI02905.1), mouse (NP_035825.1) and rat (NP_112644.1), were retrieved from NCBI protein database (<http://www.ncbi.nlm.nih.gov/>) and aligned using clustal analysis. "*" indicates an identical amino acid residue, whereas "-" indicates deletion of an amino acid, ":" indicates strongly similar amino acids and "." indicates weakly similar amino acids.

out because it is important that the gene is in frame for functional protein translation. The 319-amino acid peptide molecular weight is estimated to be 31.8 kDa and theoretical pI of 9.24. The deduced amino acid was translated from the DNA sequencing analysis of purified pDEST17 expression plasmid using Expsy tool Software (<http://web.expasy.org/>).

The sequenced was compared with the database and the result is 99% similar with VDAC2 *Gallus gallus* (GeneBank accession number: NM_204741.1). This means the cloning plasmid

was constructed successfully. Moreover, aligning the protein generated by the Expsy tool (given FASTA>VIRT5559) showed that the amino acid is very likely to be the VDAC protein from *Gallus gallus* (chicken) with similarity score of 98.23% (Fig. 3). CLUSTAL W alignment analysis of this VDAC2 protein showed that it has quite high similarity with other organisms such as 87% identical to human (CAG33245.1), 85% identical to cattle (AA102905.1), and 83% identical to both mouse (NP_035825.1) and rat (NP_112644.1), respectively.

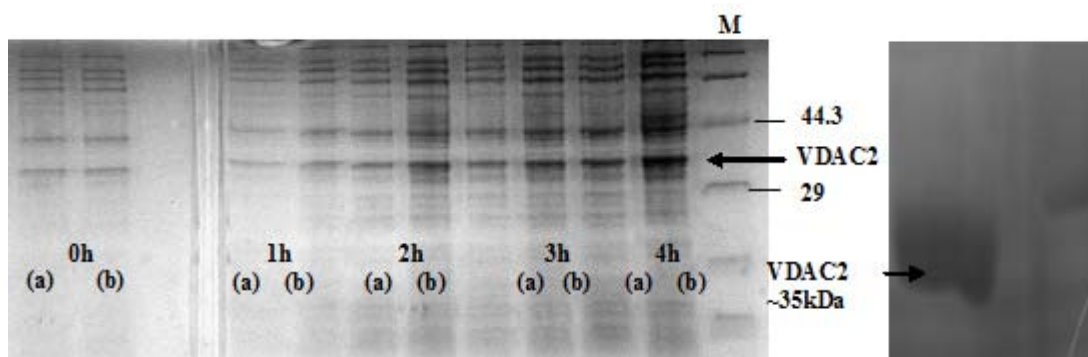


Fig. 4. The SDS-PAGE of the VDAC2 expression and purification. **A** The expression of VDAC2 starting from 0 h, initial induction time until 4 h after induction. The culture was splitted into uninduced and induced which were represented at lane (a) and (b), respectively. The expression of VDAC2 increased with time in the presence of L-arabinose inducer. **B** The purified VDAC2 on SDS-PAGE stained with silver nitrate.

After confirming that VDAC2 gene was inserted correctly in pDEST17 destination vector, the plasmid was transformed into BL21-AI *E. coli* cells for protein expression.

Expression of VDAC2

The VDAC2 cloned in pDEST17 was expressed as histidine tagged fusion protein. The molecular mass of the peptide is estimated to be 34.6 kDa by taking into consideration the addition of nucleotide sequence of ATG initiation codon upstream of the GST and 6xHis tags codon. VDAC2 was successfully expressed with an increasing intensity after 4 h of induction with L-arabinose (Fig. 4a). On the other hand, the uninduced culture may contains other unidentified proteins with similar size to VDAC2 or the VDAC2 was not efficiently repressed since it is not as intense in the induced condition is faint in contrast to more intense VDAC2 bands, in which the culture was induced with 0.2% (w/v) L-arabinose. By varying the concentration of L-arabinose, the expression

of protein can be modulated. Subsequently, the recombinant protein was purified using metal affinity column chromatography (Fig.4b) and the size confirmed by immunoblotting using anti-His antibody (data not shown).

Protein purification

VDAC2 can only be purified under denatured conditions since native condition failed

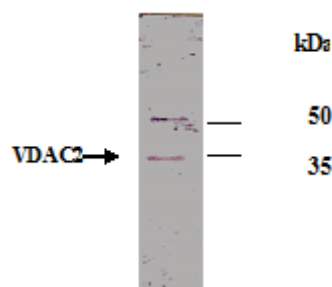


Fig. 5. The Western blot showed the 6 x His tagged VDAC2 band at 35 kDa

to purify the protein (result not shown). Nevertheless, this partial purification showed that recombinant VDAC2 was expressed. The *E. coli* expression system contains a series of Gateway-adapted destination vectors designed to facilitate high-level, inducible expression of recombinant proteins in *E. coli*. As for pDEST17 vector, it allows production of N-terminal with 6xHis tagged protein for detection and purification of recombinant fusion proteins. Ni-NTA spin column is based on the remarkable selectivity of a unique Ni-NTA resin for recombinant proteins carrying a small affinity tag consisting of 6 consecutive histidine residues, the 6xHis tag. Using silver staining to observe the expression of the VDAC2, a band with a size of ~35 kDa showed very high intensity as shown in Fig. 4b.

VDAC2 identification by Western blot

The recombinant protein from *E. coli* was extracted and transferred onto nitrocellulose membrane for Western blotting. The antibody used is against polyclonal human N-terminal VDAC2 (Fig. 5). This is to identify the tagged VDAC2 protein is successfully expressed and the size of the recombinant VDAC2. In order to validate the appearance of native VDAC2 in over stunned chicken as observed by Abu Samah et al. (2011), a Western blot was carried out on the chicken's sample from its skeletal muscle which have been electrically stimulated under different current but the time was set constant for all. Another sample which came from chicken without being stunned was made as control reaction. From the results obtained, all the samples including control showed a band of VDAC2 (result not shown). Thus, native VDAC2 protein was also detected in non-stunned chicken. Therefore, it is essential to quantify the VDAC2 protein expressed in both control and electrically stimulated sample but the lack of commercial antigen and antibody of chicken VDAC2 has cause hindrance.

There were two bands observed by Western blot with a size of ~35 kDa and ~50 kDa respectively. The 35 kDa band appears to be in agreement with the predicted size of protein fusion from the deduced amino acid obtained from the pDEST17 DNA sequence translation. However, the identity of the 50 kDa band is still elusive.

DISCUSSION

With respect to native protein antigens, the binding affinity of most antibodies is influenced by conformational determinants, and antibodies may not bind the same protein in a denatured state²⁴. Since polyclonal antibody generally is less specific compared to monoclonal antibody, therefore, it is not surprise to obtain more than a single band from Western blot analysis. VDAC2 is one isoform of VDAC and monoclonal antibody would be more likely to identify single member of protein families. As such it is highly recommended to use the monoclonal anti-VDAC2 to confirm the identity of the protein. Nevertheless the expression of recombinant VDAC2 has been justified from all the evidence. Moreover, there is evidence that VDAC2 may has its two populations with different peptide size. Hinsch *et al.* reported from its immunoblot study, two bands spotted against anti-VDAC2 P2/45 antibodies from an extract of bovine sperm sample²⁵. Another study from Menzel *et al.*²⁶, where Western blot analysis with antibodies monospecific for each isoform of VDAC in MS peptide sequencing showed that the main protein resolved in electrophoresis was VDAC2 with minor contaminations of the other isoforms. This result raised the intriguing hypothesis of the expression of two VDAC2 sub-isoforms, with different polypeptide lengths, and with a pattern of similar post-translational modification. The first hypothesis is that it is caused by alternative splicing that could add N-terminal sequences with different lengths to the β -barrel forming structure. The second was that VDAC2 can be phosphorylated in various situations that can change the isoelectric point of a protein. Aside of bovine VDAC2, there is also evidence of 2 populations of mouse VDAC2 isoform expressed in yeast. Selectivity measurements support the 2-populations hypothesis when the conductance and selectivity of individual channels were measured simultaneously (in the presence of a salt gradient) and most of the observations naturally grouped into 2 populations of distinct conductance and selectivity. The presence of two populations of VDAC2 channels might arise from some cellular regulatory process that results in longlasting changes in the properties of the protein²⁰.

In spite of the low level of sequence identity between human and chicken VDAC sequences, the overall pattern of putative transmembranes, sided β sheets is preserved, suggesting that the human proteins can assume a conformation similar to chicken VDAC channel. Moreover, immunocytochemical studies using antibodies directed to the NH₂-terminal 19 residues of human VDACL have indicated that antibody cross-reacting molecules are present in the plasma membrane as well as the mitochondria²⁷. The identification of two human VDAC genes which encode proteins that are identical in 13 positions over this region raises the possibility that such antibodies recognize both isoforms. Thus, although both proteins can be directed to the mitochondria in yeast, in human cells one form may also be preferentially expressed at the plasma membrane. Thus, using a polyclonal antibody of human VDAC2 but specific to N-terminal to identify the recombinant VDAC2 is justified.

The electrically stimulated sample produced amplified VDAC2 gene thus confirmed that VDAC2 was transcribed in electrically stimulated chicken's muscle tissue. Stimulation of high current and voltage will cause an over expression of hypoxia caused by iron deprivation²⁸. Consequently hypoxia induces membrane lipid peroxidation and that membrane lipid peroxidation results in the activation of VDAC-mediated transport²⁹. Recently, using a novel "patch within a patch" technique, it has been possible to record what may correspond to mitochondrial channel activity within cells. Here, mitochondria located within the presynaptic terminals of the squid giant axon were patch-clamped in a region of the terminal that contained mitochondria, but no other obvious intracellular membranes as assessed by electron microscopy. The results indicated that in unstimulated cells, there was little electrical activity in these membranes, but channel activity increased dramatically during and following an action potential in the surrounding neuron⁶.

Swidzincki *et al.* showed that in *Arabidopsis thaliana*, the protein increased during heat and senescence-associated death but the corresponding transcript did not show any change³⁰. Thus, the PCR is not a suitable indicator for a potential detection kit development. In addition, VDAC is an important membrane protein

that is responsible in ion transport and can be found in many organisms. Unless the application of real time PCR, the increase in the gene level can be an indicator for electrically stimulated chicken.

The sequencing analysis confirms that the VDAC2 gene has been successfully transferred into the destination vector in a correct position despite the position of the gene in the entry clone was in reverse. However, the *attB1* and *attB2* sites cannot be identified in the result. The *attB* amino acid sequence does not interfere with transcription or translation. No effect of the *attB* sites on expression levels in *E.coli*, insect and mammalian cells have been observed³¹. It is believed that there may be certain mutation-prone hotspots within the *attL* sites that happen in *E.coli*. However, some of these hotspots do not interfere with the recombination reaction nor do they cause a shift in the reading frame if recombined into a N-terminal tagged destination vector. It is not known why these hotspots occur. But when it does, it does not get transferred into the destination vector but remains within the backbone of the entry clone³². The ~10 kDa increment in size of recombinant VDAC2 compared to native VDAC2 (results not shown) is solely caused by the addition of *attB1* site and 6xHis-tagged fusion protein. In N-terminal or N+C-terminal fusions, the translational signals and the fusion protein sequences are provided by the destination vector and will be upstream of the *attB1* site. Consequently, the 25-bp *attB1* site becomes part of the coding sequence and inserts 8 amino acids between the fusion domain and the protein encoded by a gene.

CONCLUSION

The present study showed that recombinant VDAC2 has been successfully constructed in pDEST17 expression vector and the protein was expressed by BL21-A1 *E. coli* cells. This study also revealed the possibility that chicken VDAC2 may has two populations with different sizes. Since the protein was partially purified, a further purification process must be optimized to achieve a pure antigen for antibody development. Moreover, this study can provide the cloning technique used to obtain recombinant chicken VDAC2 since many studies

focused on VDAC from other organisms. It is possible that VDAC2 antigen is considered as a potential biomarker to target on electrically stimulated chicken. Ultimately, a rapid test detection kit which can detect an over-stunned chicken that cause the death of the chicken before slaughter can be developed from this milestone.

ACKNOWLEDGMENTS

The authors would like to thanks the International Islamic University Malaysia for research facilities and the Ministry of Science, Technology and Innovation Malaysia for providing NSF scholarship to Sr. Nurhidayah for her MSc programme.

REFERENCES

- Saccone, C., Caggese, C., D'Erchia, A.M., Lanave, C., Oliva, M., Pesole, G. Molecular clock and gene function. *Journal of molecular evolution*. 2003; **57** Suppl 1:S277-85.
- Bernardi, P., Broekemeier, K.M., Pfeiffer, D.R. Recent progress on regulation of the mitochondrial permeability transition pore; a cyclosporin-sensitive pore in the inner mitochondrial membrane. *Journal of bioenergetics and biomembranes*. 1994; **26**:509-17.
- Colombini, M., Blachly-Dyson, E., Forte, M. VDAC, a channel in the outer mitochondrial membrane. *Ion channels*. 1996; **4**: 169-202.
- Mannella, C.A. Minireview: on the structure and gating mechanism of the mitochondrial channel, VDAC. *Journal of bioenergetics and biomembranes*. 1997; **29**: 525-31.
- Benz, R. Permeation of hydrophilic solutes through mitochondrial outer membranes: review on mitochondrial porins. *Biochimica et biophysica acta*. 1994; **1197**: 167-96.
- Blachly-Dyson, E., Forte, M. VDAC channels. *IUBMB life*. 2001; **52**: 113-8.
- Colombini, M. VDAC: the channel at the interface between mitochondria and the cytosol. *Molecular and cellular biochemistry*. 2004; **256-257**: 107-15.
- Lemasters, J.J., Holmuhamedov, E. Voltage-dependent anion channel (VDAC) as mitochondrial governor—thinking outside the box. *Biochimica et biophysica acta*. 2006; **1762**:181-90.
- Poleti, M.D., Tesch, A.C., Crepaldi, C.R., Souza, G.H., Eberlin, M.N., de Cerqueira Cesar, M. Relationship between expression of voltage-dependent anion channel (VDAC) isoforms and type of hexokinase binding sites on brain mitochondria. *Journal of molecular neuroscience : MN*. 2010; **41**:48-54.
- Blachly-Dyson, E., Zambronicz, E.B., Yu, W.H., Adams, V., McCabe, E.R., Adelman, J., et al. Cloning and functional expression in yeast of two human isoforms of the outer mitochondrial membrane channel, the voltage-dependent anion channel. *The Journal of biological chemistry*. 1993; **268**:1835-41.
- Abu-Hamad, S., Zaid, H., Israelson, A., Nahon, E., Shoshan-Barmatz, V. Hexokinase-I protection against apoptotic cell death is mediated via interaction with the voltage-dependent anion channel-1: mapping the site of binding. *The Journal of biological chemistry*. 2008; **283**:13482-90.
- Shoshan-Barmatz, V., Keinan, N., Zaid, H. Uncovering the role of VDAC in the regulation of cell life and death. *Journal of bioenergetics and biomembranes*. 2008; **40**:183-91.
- Kuwana, T., Mackey, M.R., Perkins, G., Ellisman, M.H., Latterich, M., Schneider, R., et al. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell*. 2002; **111**:331-42.
- Rostovtseva, T.K., Antonsson, B., Suzuki, M., Youle, R.J., Colombini, M., Bezrukov, S.M. Bid, but not Bax, regulates VDAC channels. *The Journal of biological chemistry*. 2004; **279**: 13575-83.
- Forte, M., Adelsberger-Mangan, D., Colombini, M. Purification and characterization of the voltage-dependent anion channel from the outer mitochondrial membrane of yeast. *The Journal of membrane biology*. 1987; **99**: 65-72.
- Mihara, K., Sato, R. Molecular cloning and sequencing of cDNA for yeast porin, an outer mitochondrial membrane protein: a search for targeting signal in the primary structure. *The EMBO journal*. 1985; **4**:769-74.
- Kleene, R., Pfanner, N., Pfaller, R., Link, T.A., Sebald, W., Neupert, W., et al. Mitochondrial porin of *Neurospora crassa*: cDNA cloning, in vitro expression and import into mitochondria. *The EMBO journal*. 1987; **6**:2627-33.
- Messina, A., Neri, M., Perosa, F., Caggese, C., Marino, M., Caizzi, R., et al. Cloning and chromosomal localization of a cDNA encoding a mitochondrial porin from *Drosophila melanogaster*. *FEBS letters*. 1996; **384**: 9-13.
- Ryerse, J., Blachly-Dyson, E., Forte, M., Nagel, B. Cloning and molecular characterization of a

- voltage-dependent anion-selective channel (VDAC) from *Drosophila melanogaster*. *Biochimica et biophysica acta*. 1997; **1327**: 204-12.
20. Xu, X., Decker, W., Sampson, M.J., Craigen, W.J., Colombini, M. Mouse VDAC isoforms expressed in yeast: channel properties and their roles in mitochondrial outer membrane permeability. *The Journal of membrane biology*. 1999; **170**:89-102.
 21. Esposito, D., Garvey, L.A., Chakiath, C.S. Gateway cloning for protein expression. *Methods Mol Biol*. 2009; **498**: 31-54.
 22. Abu Samah, N., Amid, A., Yusof, F. Over expression of Voltage Dependent Anion Channel 2 (VDAC2) in muscles of electrically stunned chickens. *IJUM Engineering Journal*. 2011; **12**:
 23. Guzman, L.M., Belin, D., Carson, M.J., Beckwith, J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *Journal of bacteriology*. 1995; **177**: 4121-30.
 24. Nelson, P.N., Westwood, O.M., Jefferis, R., Goodall, M., Hay, F.C. Characterisation of anti-IgG monoclonal antibody A57H by epitope mapping. *Biochemical Society transactions*. 1997; **25**: 373S.
 25. Hinsch, K.D., De Pinto, V., Aires, V.A., Schneider, X., Messina, A., Hinsch, E. Voltage-dependent anion-selective channels VDAC2 and VDAC3 are abundant proteins in bovine outer dense fibers, a cytoskeletal component of the sperm flagellum. *The Journal of biological chemistry*. 2004; **279**:15281-8.
 26. Menzel, V.A., Cassara, M.C., Benz, R., de Pinto, V., Messina, A., Cunsolo, V., et al. Molecular and functional characterization of VDAC2 purified from mammal spermatozoa. *Bioscience reports*. 2009; **29**: 351-62.
 27. Stuber, D., Matile, H., Garotta, G. System for high-level production in *Escherichia coli* and rapid purification of recombinant proteins: Application to epitope mapping, preparation of antibodies and structure-function analysis. *In Lefkovits I, Pernis B Immunological Methods*. 1990;4, Academic Press, New York:121-52.
 28. Behn, C., Araneda, O.F., Llanos, A.J., Celedon, G., Gonzalez, G. Hypoxia-related lipid peroxidation: evidences, implications and approaches. *Respiratory physiology & neurobiology*. 2007; **158**:143-50.
 29. Stark, G. Functional consequences of oxidative membrane damage. *The Journal of membrane biology*. 2005; **205**:1-16.
 30. Swidzinski, J.A., Sweetlove, L.J., Leaver, C.J. A custom microarray analysis of gene expression during programmed cell death in *Arabidopsis thaliana*. *The Plant journal : for cell and molecular biology*. 2002; **30**:431-46.
 31. Simpson, J.C., Wellenreuther, R., Poustka, A., Pepperkok, R., Wiemann, S. Systematic subcellular localization of novel proteins identified by large-scale cDNA sequencing. *EMBO reports*. 2000; **1**: 287-92.
 32. Sasaki, Y., Sone, T., Yoshida, S., Yahata, K., Hotta, J., Chesnut, J.D., et al. Evidence for high specificity and efficiency of multiple recombination signals in mixed DNA cloning by the Multisite Gateway system. *Journal of biotechnology*. 2004; **107**:233-43.