Malaysian Journal of Microbiology, Vol 19(6) Special Issue 2023, pp. 718-726
DOI: http://dx.doi.org/10.21161/mjm.230020

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Heterologous expression and purification of calmodulin from *Plasmodium knowlesi* using codon-optimized synthetic gene

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ABSTRACT

Aims: Calmodulin (CaM) is vital for the survival of *Plasmodium knowlesi*, a simian malaria parasite that infects both macaques and humans in Southeast Asia. To advance antimalarial drugs development targeting this protein, it is imperative to produce ample quantities of pure CaM for further research. Hence, this study aims to establish a robust strategy for the heterologous expression and purification of CaM from *Plasmodium knowlesi* (Pk-CaM).
Methodology and results: First, we optimised the gene sequence of Pk-CaM for *Escherichia coli* expression, chemically synthesised it and integrated it into the pET28a plasmid. The optimised gene displayed a 45.15% GC content and a 0.81 codon-adaptation index, making it highly compatible with *E. coli*. Pk-CaM expression was assessed under various conditions, with the best results achieved at a post-induction temperature of 20 °C for 16 h, yielding a fully soluble protein. Subsequently, we purified the protein using Ni^{2+}-NTA affinity chromatography and size-exclusion chromatography (SEC), obtaining 15 mg from 1 L of culture. The folding properties of purified Pk-CaM were analysed using far-UV circular dichroism (CD) spectroscopy, revealing a predominance of helical structures, both with and without Ca^{2+} ions. Binding to Ca^{2+} ions induced structural changes, increasing the helical content compared to when Ca^{2+} ions were absent.
Conclusion, significance and impact of study: The optimal conditions for the recombinant expression and purification of Pk-CaM in a correctly folded and functional form were successfully established in this study. This achievement provides a solid foundation for conducting further comprehensive research in the pursuit of novel antimalarial drugs.

Keywords: Antimalarial drug, calmodulin, gene synthetic, *Plasmodium knowlesi*

INTRODUCTION

A potential and fatal infectious disease known as malaria has impacted the whole world. This disease is caused by *Plasmodium* parasites including *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*. Among all the plasmodium parasites, *P. knowlesi* is known to have the highest prevalence in Malaysia especially in Sabah and Sarawak (Aftab *et al.*, 2023). Another study by Hussin *et al.* (2020) stated that the largest number of *P. knowlesi* cases in Malaysia came from Sabah and Sarawak with a total of 9902 cases. Notably, the first case of *P. knowlesi* infection was detected in 1965 in a US national working in Malaysia (Chin *et al.*, 1965). *P. knowlesi* is transmitted through the bite of the *Anopheles leucosphyrus* group, a species of mosquito that is commonly found in forested regions of Southeast Asia (Silvester *et al.*, 2017). The most susceptible group of people to this disease are males, adults, and forest-related workers while less severe cases are among children (Naserrudin *et al.*, 2022). Furthermore, as mentioned by Davidson (2019), the utilisation of land by humans leads to intricate changes in the transmission dynamics of the parasite among humans, mosquito vectors and macaque hosts. This phenomenon contributes to the increasing prevalence of *P. knowlesi* in Sabah and Sarawak. In addition, *P. knowlesi* has the shortest asexual reproduction cycle among all malaria parasites that infect humans which causes parasitemia levels to rise quickly (Singh and Daneshvar, 2013).

The plasmodium primarily targets and damages red blood cells and liver cells upon entering the bloodstream of humans (Oaks *et al.*, 1993). Although the precise mechanisms responsible for initiating the invasion process are not thoroughly understood, it is recognized that the presence of optimal calcium levels is essential for

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

accomplishing a successful invasion of the parasites (Moreno and Docampo, 2003; Soldati *et al*., 2004). The regulation of the invasion process may be significantly influenced by calcium-dependent signalling pathways that are dependent on calcium. According to Ghosh and Jana (2021), the change in calcium ion concentrations can be detected by a protein called calmodulin (CaM) which further undergoes binding of proteins by accepting calcium ions (Ca^{2+}) at its four binding sites. CaM is a tiny Ca^{2+} dependent protein with 148 amino acids, which is also known as a calcium-binding messenger protein. It is a highly conserved, multifunctional protein that is found in all eukaryotic cells (Lai *et al*., 2015). Alaimo *et al*. (2014) described CaM as a protein that plays a crucial role in the regulation of various essential processes such as apoptosis, ion transport, and metabolic homeostasis. When Ca^{2+} ions interact with CaM, the protein undergoes a structural alteration leading to the exposure of hydrophobic regions. This exposure facilitates the binding of inhibitors and similar molecules (Alaimo *et al*., 2014).

Antagonists that bind directly to CaM lead to hinder the activity of CaM. The key characteristics of these antagonists include soluble lipids, having ionic interactions with CaM and containing specific geometric structures (Sunagawa *et al*., 2006). A study by Matsumoto (1987) reported that the invasion of erythrocytes by merozoites and the maturation of schizonts can be prevented by the inhibition of CaM antagonists including (N-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide hydrochloride) W7, trifluoperazine (TFP), and calmidazolium chloride (R24571) compounds. This implies that targeting CaM could be an effective approach for the development of antimalarial drugs. Besides, CaM has also been linked to various pathways associated with cancer and rare mutations in CaM have been recognized as a contributing factor to cardiac arrhythmias in humans (Grant *et al*., 2019). While extensive studies have been conducted for CaM from *P. falciparum*, none report, to date, for CaM from *P. knowlesi* (Pk-CaM).

Comprehensive studies on structure and function of Pk-CaM should provide insight into addressing this protein as a viable target for novel antimalarial drug development. While current treatment for *P. knowlesi* case is available, it is believed that this parasite is gaining resistance towards the currently available drug. The attempts to find alternative drugs to inhibit *P. knowlesi* is therefore unavoidable. Such attempts, nevertheless, require availability of Pk-CaM in high purity and amount, either for biochemical assays or structural analysis, which feasible to be obtained through protein recombinant approach. The current study describes the first attempt to produce Pk-CaM through heterologous expression using *E. coli* as a host cell. In this attempt, the expression system utilised a synthetic-codon-optimised gene and expressed under different conditions. The success of Pk-CaM to be produced recombinantly in this study opens wide possible studies pertaining to the structure and function of this protein for further antimalarial drug discovery.

MATERIALS AND METHODS

Gene optimization, synthesis, and expression system construction

The gene sequence of calmodulin from *P. knowlesi* referred to as Pk-CaM was obtained from PlasmoDB database (transcript ID: PKNH_0420800.1). The initial steps involved optimising the gene sequence for efficient expression in *E. coli* through codon optimization. Following that, the optimised sequence was chemically synthesised utilising the resources provided by GeneScript (New Jersey, USA). The gene was then inserted into the pET28a plasmid with the ampicillin resistance gene and *Nde*I and *Xho*I as the restriction sites, yielding an expression system for recombinant Pk-CaM, namely pET28-PkCaM.

Protein expression

The methodology for over-expressing recombinant Pk-CaM was adopted from the research conducted by Goh *et al*. (2018) utilising *E. coli* BL21 (DE3) as the chosen host cell, with some modifications. The pET28-PkCaM was first transformed into *E. coli* BL21 (DE3) cells using a heat shock method. The positive transformant was then pre-cultured in Luria-Bertani (LB) broth medium containing 35 mg/mL kanamycin at 37 °C overnight. The pre-culture was then transferred to a larger volume of LB broth culture (up to 2% of pre-culture volume) and then kept incubated at 37 °C until OD600 reached 0.6. The expression of Pk-CaM was then induced by the addition of 1 mM isopropyl-D-1-thiogalactopyranoside (IPTG) followed by a prolonged incubation (post-induction incubation) at varied temperatures and time (Table 1). The cells were then harvested through centrifugation at 10,000× *g* for 10 min. In total, 4 expression conditions were tested in this study.

Cell lysis

Cell lysis was performed according to Razali *et al*. (2020) with slight modification. Firstly, the harvested cells were washed and resuspended in 20 mM phosphate buffer (pH 8.0) and 100 mM NaCl. The lysis was then done through sonication in ice. The soluble fraction was then collected from the cell debris by centrifugation at 35,000× *g* for 30 min at 4 °C, further collected and used for purification steps.

Protein purification

Purification was applied by Ni^{2+}-NTA affinity chromatography and size-exclusion chromatography using an ÄKTA Pure liquid-chromatography system (GE Healthcare, Chicago, IL, USA) following the protocol in Budiman *et al*. (2009) with some modifications. Initially, the column was equilibrated with a 20 mM phosphate buffer (pH 8.0) solution containing 100 mM NaCl. The soluble fractions were first filtered using a 0.22 µm filter

Malays. J. Microbiol. Vol 19(6) Special Issue 2023, pp. 718-726
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Table 1: Expression conditions of Pk-CaM.

Condition table	Host cell	OD600	Post-induction incubation	
			Temperature (°C)	Time (h)
1	*E. coli* BL21 (DE3)	0.6	37	3.5
2	*E. coli* BL21 (DE3)	0.6	25	16
3	*E. coli* BL21 (DE3)	0.6	37	16
4	*E. coli* BL21 (DE3)	0.6	20	16

Table 2: Gene optimisation of the Pk-CaM gene.

Parameters	Original Pk-CaM gene	Optimised Pk-CaM gene
GC content (%)	37.12	45.15
Codon adaptation index (CAI)	0.61	0.81
Distribution of codons based on usage frequency in *E. coli* (%)		
0-10	0	24
11-20	0	2
21-30	0	0
31-40	0	11
41-50	0	0
51-60	0	3
61-70	0	0
71-80	9	4
81-90	9	6
91-100	82	47

before being loaded onto the column at the flow rate of 1 mL/min. A linear gradient of 500 mM imidazole in 20 mM phosphate buffer (pH 8.0) containing 100 mM NaCl was used for the elution of bound proteins.

The concentration of the purified protein was determined using the NanoDrop™ 2000 (Thermo Fisher Scientific, Waltham, MA, USA). This determination was made by measuring the absorbance at 280 nm, where a solution with a concentration of 0.1% (1 mg/mL) exhibited an absorbance of 0.047, as calculated according to the method described by Goodwin and Morton (1946).

SDS-PAGE

The expression, solubility and purity of Pk-CaM protein were confirmed and analysed using 15% SDS-polyacrylamide gel electrophoresis following the method by Laemmli (1970) with slight modification. Gel electrophoresis was conducted at a constant voltage of 200 V for approximately 50 min. The gel was further stained with Coomassie Brilliant Blue (CBB) dyes and destained using a destain solution (15% MeOH, 10% acetic acid). After destaining, 50 mL of distilled water was added to the gel and left overnight for continual destain. The gel was further visualised using a Gel DocTM XR+ imager (Bio-Rad, Hercules, CA, USA).

Circular dichroism (CD)

CD spectra were measured using a J-815 automatic spectropolarimeter (JASCO Co., Japan). The protein was prepared under two different conditions. In the first condition, the purified Pk-CaM sample was treated with 2 mM $CaCl_2$ in 20 mM sodium phosphate (pH 8.0) through dialysis at 4 °C overnight. Meanwhile, in the second condition, Pk-CaM was just dissolved in 20 mM sodium phosphate (pH 8.0) with no $CaCl_2$. Both were prepared at the concentration of 0.2 mg/mL. The CD spectroscopy was then utilised to measure the spectrum of far-UV at the wavelength of 200-260 nm. The average amino acid molecular mass of 110 was used to compute the mean residue ellipticity θ, which has units of $deg \cdot cm^2 \cdot dmol^{-1}$ (Budiman *et al.*, 2012).

RESULTS AND DISCUSSION

In this investigation, the gene responsible for Pk-CaM was subjected to codon optimization and chemically synthesised to facilitate its expression in the prokaryotic organism *E. coli*. The major method for recombinant protein production in genetic synthesis is heterologous expression, which requires codon optimization (Fu *et al.*, 2020). Accordingly, the gene encoding Pk-CaM was optimised in its codon for compatibility with *E. coli* as a host cell. The optimization as shown in Table 2 resulted in changes in GC content of Pk-CaM from 43.84 to 52.56%, which is considerably acceptable for *E. coli*. Wang *et al.* (2012) indicated that adjustment in the GC content of the gene during heterologous expression highly contributed to the improvement of the expression level of the protein. In addition to changes in GC content, the Codon Adaptation Index (CAI) of the newly synthesised protein Pk-CaM was also adjusted from 0.32 to 0.96 to be preferable in the target host (*E. coli*) (Table 2). In addition, more than 80% of the codon in optimized gene has the usage frequency of more than 90%, which was much higher than that of

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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Human    -ADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQ
Pf-CaM   MADKLTEEQISEFKEAFSLFDKDGDGT---KELGTVMRSLGQNPTEAELQ
Pk-CaM   MADKLTEEQISEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQ
          **:******:****************   ********************

Human    DMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGY
Pf-CaM   DMINEIDTDGNGTIDFPEFLTLMARKLKDTDTEEELIEAFRVFDRDGDGY
Pk-CaM   DMINEIDTDGNGTIDFPEFLTLMARKMKDTDTEEELIEAFRVFDRDGDGY
         *****:*:*************:****:****:***::*******:**:**

Human    ISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTAK
Pf-CaM   ISADELRHVMTNLGEKLTNEEVDEMIREADIDGDGQINYEEFVKMMIAK
Pk-CaM   ISADELRHVMTNLGEKLTNEEVDEMIREADIDGDGQINYEEFVKMMIAK
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Figure 1: Amino acid sequence alignment of Pk-CaM with human CaM or Pf-CaM. Non identical residues are highlighted by grey boxes.

unoptimized gene (42%). The lowest the codon usage frequence of optimized gene was 70%, while (Table 2). Nikbakht *et al.* (2014) reported that this codon issue is the main challenge in heterologous expression. Generally, the greater the amount of the gene containing codons that are rarely used by the expression host, the lower the heterologous protein will be expressed (Gustafsson *et al*., 2004), however, the expression level can be improved via gene/codon optimization by changing the DNA sequence without altering the translated amino acid sequence. Apart from both issues, the AT rich fragment was also removed from Pk-CaM as the fragment is believed to contribute to the early termination during gene translation (Wang *et al*., 2011). Altogether, we believed that the optimised synthetic gene of Pk-CaM is considerably preferable for *E. coli*'s expression system. The changes on DNA sequence of Pk-CaM did not alter its amino acid sequence. Figure 1 showed amino acid comparison between Pk-CaM and its homologous protein from human (h-CaM) or *P. falciparum* (Pf-CaM), which showed high similarity among the proteins. The similarity between Pk-CaM and h-CaM or Pf-CaM was 89.86% and 99.32%, respectively.

Further, when the optimised synthetic gene of Pk-CaM was expressed in *E. coli* under different conditions, a well-expressed level was only observed under condition 4 (post-induction incubation of 20 °C for 16 h) (Figure 3). At this condition, a remarkable band corresponding to the size of Pk-CaM (21 kDa) was observed after IPTG induction. This indicates that 1 mM IPTG is sufficient to express this protein under this condition. Interestingly, the other three conditions did not yield any band corresponding to Pk-CaM size after IPTG induction (Figure 2a-c), which suggested that the expression of this protein did not occur under these conditions. As indicated by Fakruddin *et al.* (2013), while *E. coli* is the most frequently used host to produce recombinant proteins, there are some challenges in the success of production of these proteins under *E. coli*. Brown (1995) indicated that codon bias vastly hampers recombinant protein expression in *E. coli*. This, nevertheless, is unlikely applicable for Pk-CaM as the gene was optimised to meet the requirement for *E. coli*. It is believed that the inability of *E. coli* to express Pk-CaM under conditions 1-3 is due to metabolic load as described by Neubauer *et al.* (2003). Under this condition, *E. coli* metabolism was heavily burdened by the presence of foreign genes to express. This resulted in the cells expressing only essential genes to express for their survival. It is possible also that the failure of expression under conditions 1-3 was due to a low level of IPTG. This study did not carry out any optimization of IPTG concentration. Other recombinant proteins were successfully induced at this concentration; therefore, it was chosen as the common concentration (Larentis *et al*., 2014). It should be noted that IPTG concentration is regarded as a crucial element in the success and efficiency of recombinant protein expression. Reduced IPTG concentration could result in expression failure because the repressor molecule might not be fully removed from the operon. On the other hand, it has been observed that a higher IPTG concentration hinders cell growth, similar to what has been reported in other recombinant systems (De León *et al*., 2004; Manderson *et al*., 2006; Islam *et al*., 2007; Beigi *et al*., 2012). Furthermore, IPTG is quite costly, which means its utilisation in large-scale industrial production of recombinant proteins should be minimised to ensure an efficient production process.

Further, when the solubility of expressed Pk-CaM under condition 4 was checked with SDS-PAGE (Figure 3), the band corresponding to Pk-CaM was only observed under the supernatant fraction. This indicated the expression was fully soluble under condition 4 (post-incubation temperature of 20 °C for 16 h). As reported by Sorensen and Mortensen (2005), recombinant proteins that have been overexpressed in bacterial cells may aggregate, which leads to insolubility, due to either high folding intermediate concentrations accumulating or

ISSN (print): 1823-8262, ISSN (online): 2231-7538

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DOI: http://dx.doi.org/10.21161/mjm.230020

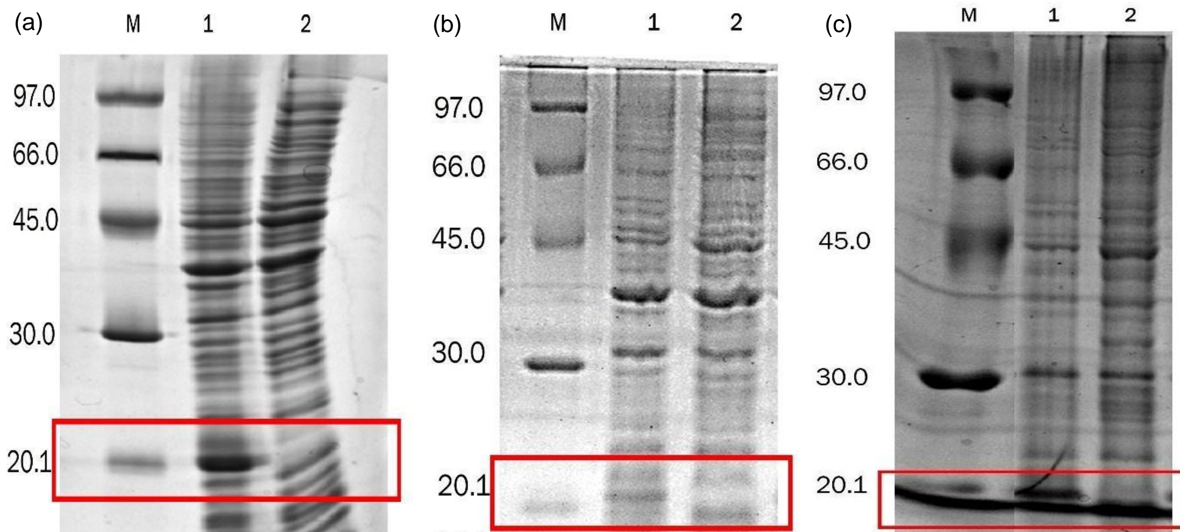

Figure 2: Expression check of Pk-CaM under 15% SDS-PAGE under condition 1 (a), condition 2 (b) and condition 3 (c). M: Protein marker (units in kDa); Lane 1: The cell before IPTG induction; Lane 2: The cell after IPTG induction; The band corresponding to Pk-CaM is indicated by a red box. Details of the conditions are stated in Table 1.

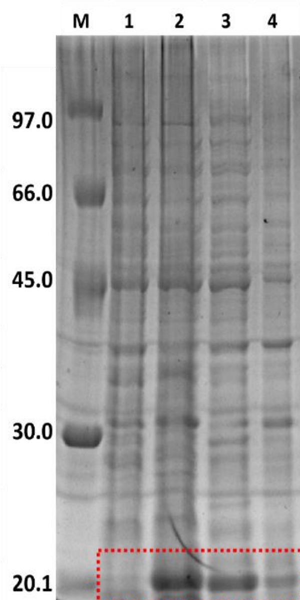

Figure 3: Expression check of Pk-CaM under 15% SDS-PAGE. M: Protein marker (units in kDa); Lane 1: The cell before IPTG induction; Lane 2: The cell after IPTG induction; Lane 3: Soluble fraction of the cell obtained after the sonication; Lane 4: Insoluble fraction of the cell obtained after the sonication. The band corresponding to Pk-CaM is indicated by a red box. Expression profile under condition 4.

ineffective molecular chaperone processing. This indicated that post-induction temperature at 20 °C had successfully lowered the concentration of accumulation of folding intermediates. The strategy in reducing temperatures during protein expression for obtaining higher solubility was also applied to other recombinant proteins, including human interferon α-2, subtilisin E, ricin A chain, bacterial luciferase, Fab fragments, β-lactamase, rice lipoxygenase L-2, soybean lipoxygenase L-1, kanamycin nucleotidyltransferase and rabbit muscle glycogen phosphorylase (Vasina and Baneyx, 1997).

Further, Pk-CaM under the expression condition 4 was successfully purified using a combination of Ni^{2+}-NTA chromatography and size exclusion chromatography techniques. To note, the gene of Pk-CaM was cloned into pET28 which allows this protein to have 6His-tag for purification purposes. As shown in Figure 4a, the elution profile of Pk-CaM from Ni^{2+}-NTA chromatography resulted in a single peak that eluted at about 87% of concentration buffer B (corresponding to 435 mM imidazole). Nevertheless, the SDS-PAGE of the eluted fractions showed that the presence of contaminant bands was quite serious (Figure 4b), which implied that the protein was unable to be purified only using a single step of Ni^{2+}-NTA purification. While purification targeting 6His-tag is relatively sensitive, there were many reports indicating that indigenous proteins from *E. coli* were also able to bind to the column, particularly for proteins containing some His residues in exposed position (Bornhorst and Falke, 2000). Accordingly, the contamination is often found under Ni^{2+}-NTA affinity chromatography. A typical purity level obtained from this purification step was reported to be up to 95%, depending on the type of protein and expression condition (Janknecht *et al.*, 1991).

Accordingly, an additional step of purification was done through size exclusion chromatography which resolved the proteins based on their sizes (Walls and Loughran, 2010). As shown in Figure 5a, a single elution peak was obtained from the column at an elution volume of about 81 mL. Purity checks under SDS-PAGE as

ISSN (print): 1823-8262, ISSN (online): 2231-7538

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DOI: http://dx.doi.org/10.21161/mjm.230020

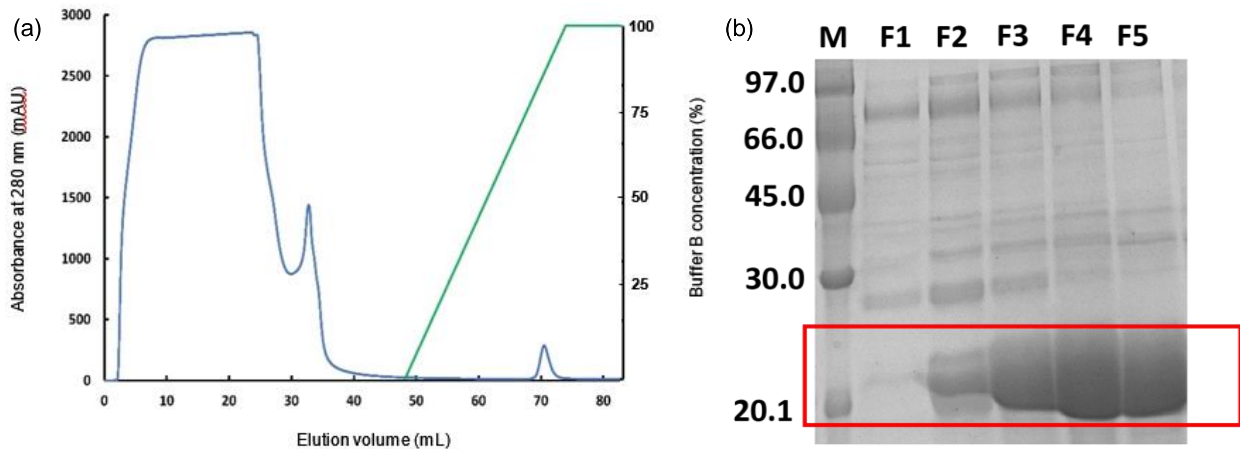

Figure 4: (a) Elution profile of Pk-CaM under Ni^{2+}-NTA chromatography. Blue solid line indicates the changes of absorbance at 280 nm (mAU), while the green solid line indicates the percentage of B buffer used during elution; (b) 15% SDS-PAGE of the fractions (F1-F5) under eluted peak from Ni^{2+}-NTA chromatography. M: Protein marker (units in kDa).

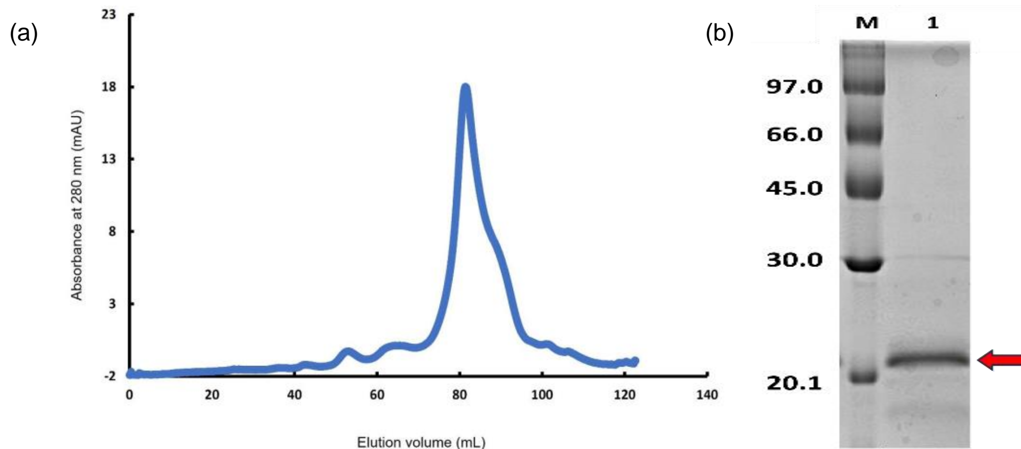

Figure 5: (a) Elution profile of Pk-CaM under size exclusion chromatography; (b) Purification check of Pk-CaM under 15% SDS-PAGE after size exclusion chromatography. M: Protein marker (units in kDa). Lane 1: Purified Pk-CaM.

shown in Figure 5b indicated that the single band corresponding to Pk-CaM was observed with no obvious contamination bands appearing. This indicated that the step size exclusion chromatography had successfully purified this protein to a high purity level. The yield of pure protein was then calculated to be about 15 mg of pure Pk-CaM from a 1 L culture. To note, there is no such standard for how much yield of recombinant protein produced by *E. coli* to be considered as high yield. The level might depend on the type of proteins, expression vector, and conditions. Morão *et al.* (2022) reported the yields of up to 946 mg of protein expressed per 1 L culture of *E. coli*, with an average of 60-70 mg. Meanwhile, Huleani *et al.* (2021) reported that the use of *E. coli* to produce diagnostic proteins often yielded only 10-20 mg per 1 L culture, which is considerably good. To note, preliminary bioassay and structural analysis involving Pk-CaM is sufficient. Further improvement in its purity level is feasible through a bioreactor approach. To note, only CaM of *P. falciparum* (Pf-CaM) was reported to be produced through a recombinant approach using *E. coli* cells (Juhász *et al.*, 2020). Nevertheless, detailed data on the yield and solubility of Pf-CaM from *E. coli* host cells. To note, the gene of Pf-CaM was also obtained through a synthetic approach yet was expressed using a different expression vector (pET-3d) and purified under phenyl-sepharose chromatography.

Further, when CD spectroscopy was utilised to confirm the folding status of Pk-CaM, the result showed far-UV CD spectra of Pk-CaM, in the absence of Ca^{2+} ion helical structure. This is in good agreement with Kawasaki *et al.* (2019) that indicated the calmodulin structure is heavily dominated by helical structure. Interestingly, the far-UV CD spectrum of Pk-CaM in the presence of Ca^{2+} ion also showed a similar pattern of the domination of helical structure, yet with a slightly deeper spectrum. This

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

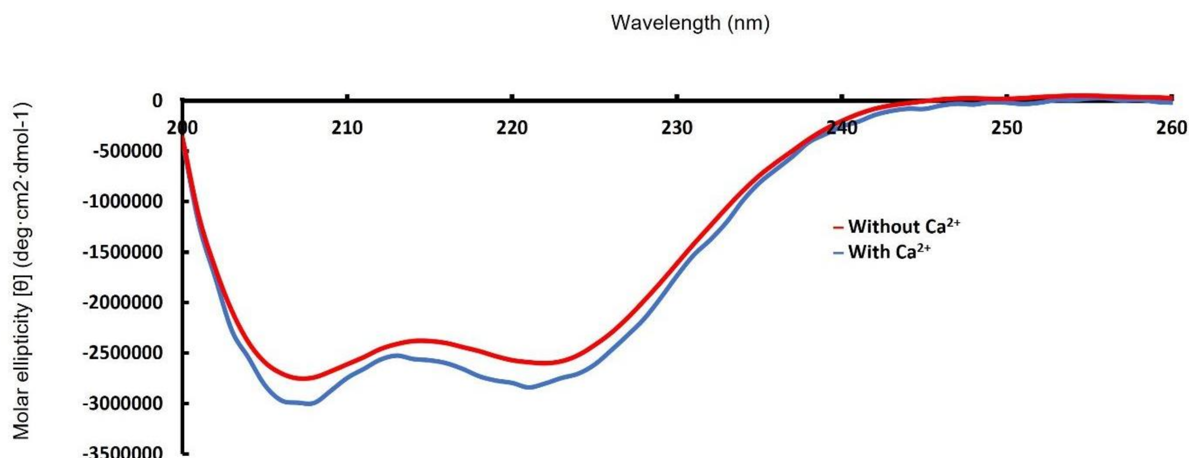

Figure 6: Far-UV CD spectra of purified Pk-CaM treated with and without Ca^{2+} ions.

might indicate that upon binding to Ca^{2+} ions, Pk-CaM adopted some structural changes, yet remained dominated by helical structures. As reported by Spyrakis *et al.* (2011), both Ca-bound or free-forms of CaM were dominated by helical structure. Indeed, the Ca-bound form of CaM slightly has more helical structure as the linker connecting the N-lobe and C-lobe was folded into helical structures upon binding to the Ca^{2+} ion. This is also supported by Juhász *et al.* (2020) who observed additional helix formation on CaM upon binding to Ca^{2+} ion. Accordingly, it is acceptable to have a deeper far-UV CD spectrum for Pk-CaM in the presence of Ca^{2+} ion as an indication there was more helical content in its structure. Interestingly, Juhász *et al.* (2020) also indicated that the far-UV CD spectrum of CaM from humans and *P. falciparum* were found to be slightly deeper in the presence of Ca^{2+} ion than that of in the absence of Ca^{2+} ion.

CONCLUSION

This research study demonstrates the successful expression of the calmodulin protein from *P. knowlesi* through the implementation of a codon-optimised synthetic gene which resulted in a fully soluble and pure protein under *E. coli* host cell with the yield of 15 mg per 1 L culture of *E. coli*. Purified Pk-CaM was confirmed to be properly folded which is dominated by alpha-helical structures based on CD spectra. The study also showed that the presence of Ca^{2+} ions has induced structural changes in this protein which not only indicated that the purified protein is properly folded but also fully functions in Ca^{2+}-binding. Furthermore, the success of this study should contribute to the further availability of Pk-CaM for further studies in attempts to discover drugs targeting this protein for eradicating the *P. knowlesi* case.

ACKNOWLEDGEMENTS

This study is supported by the Ministry of Higher Education, Malaysia, under the Fundamental Research Grant Scheme no FRGS/1/2022/SKK10/UMS/02/1 (UMS Code: FRG0566-1/2022).

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538