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Interplay between Collagen Hydrolysates and the Ability of FKBP35 from *Plasmodium knowlesi* in Preventing Insulin Aggregation

(Interaksi antara Kolagen Hidrolisat dan Keupayaan FKBP35 daripada Plasmodium knowlesi dalam Mencegah Pengagregatan Insulin)

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ABSTRACT

FK506-binding protein 35 (FKBP35) from *Plasmodium knowlesi* (Pk-FKBP35) is a potential target for combating the surge in simian malaria cases. While previously speculated to hinder protein synthesis aggregation within parasite cells, this study seeks to experimentally validate the capacity of Pk-FKBP35 to avert protein aggregation. Additionally, it aims to examine the influence of collagen hydrolysates (CH) on this ability. Initially, Pk-FKBP35 was overexpressed in *Escherichia coli* BL21(DE3) and subsequently purified. To assess its capacity for preventing aggregation, a dithiothreitol (DTT)-induced insulin aggregation assay was conducted and observed via SDS-PAGE. The findings showed a concentration-dependent inhibition by Pk-FKBP35 against DTT-induced insulin aggregation. At the concentration of 0.75 mg/mL of Pk-FKBP35, the amount of soluble insulin was increased to about 5-fold higher. Interestingly, in the presence of FK506, Pk-FKBP35's ability to prevent insulin aggregation remains intact. Since FK506 is known to specifically bind to the catalytic domain of Pk-FKBP35, this suggests that the region responsible for the protein's aggregation prevention activity is independent from the catalytic domain. Moreover, when coupled with CH derived from bovine, bone broth, fish, and swine, Pk-FKBP35's effectiveness in preventing DTT-induced insulin aggregation was attenuated, albeit to varying degrees. Notably, swine and bone broth CH exhibited superior inhibition of aggregation prevention compared to bovine and fish CH. This study validates Pk-FKBP35's capability to impede protein aggregation, showcasing a promising potential for inhibition by CH, particularly those sourced from swine and bone broth.

Keywords: Collagen hydrolysate; FKBP35; peptidyl-prolyl cis-trans isomerase (PPIase); *Plasmodium knowlesi*; zoonotic malaria

ABSTRAK

Protein pengikat-FK506 daripada *Plasmodium knowlesi* (Pk-FKBP35) dipercayai berpotensi untuk dibangunkan sebagai ubat anti-malaria bagi melawan kes zoonotik malaria yang semakin membimbangkan. Walaupun sebelum ini dikatakan dapat menghalang penggumpalan semasa sintesis protein dalam sel parasit, kajian ini bertujuan untuk mengesahkan secara uji kaji keupayaan Pk-FKBP35 untuk mencegah penggumpalan protein. Selain itu, kajian ini juga bertujuan untuk meneliti pengaruh hidrolisat kolagen (CH) terhadap keupayaan tersebut. Dengan itu, Pk-FKBP35 diekspresikan secara berlebihan di dalam *E. coli* (BL21) dan ditulenkan. Keupayaan menghalang penggumpalan protein diperhatikan menggunakan insulin sebagai model substrat yang dinyahaslikan menggunakan ditiotreitol (DTT) dan dipantau secara visual melalui elektroforesis gel natrium dodesil sulfat-poliakrilamida (SDS-PAGE). Keputusan menunjukkan bahawa Pk-FKBP35 berupaya menghalang penggumpalan insulin secara kebergantungan kepekatan. Bilangan insulin yang larut meningkat lima kali ganda dengan kehadiran 0.75 mg/mL Pk-FKBP35. Menariknya, kehadiran FK506 tidak menjejaskan kemampuan Pk-FKBP35 untuk mencegah penggumpalan insulin. Memandangkan FK506 diketahui melekat secara khas pada domain katalitik Pk-FKBP35, ini menunjukkan bahawa kawasan yang bertanggungjawab untuk aktiviti pencegahan penggumpalan protein adalah berasingan daripada domain katalitik tersebut. Dalam masa yang sama, kehadiran CH daripada lembu, tulang, ikan dan khinzir berupaya mengurangkan pencegahan penggumpalan insulin yang dinyahaslikan menggunakan DTT dengan kadar yang berbeza. CH khinzir dan tulang mencegah keupayaan Pk-FKBP35 lebih baik berbanding CH daging dan ikan. Kajian ini membuktikan Pk-FKBP35 mempunyai keupayaan mencegah penggumpalan protein dan keupayaan ini boleh direncatkan dengan kehadiran CH.

Kata kunci: FKBP35; hidrolisat kolagen; isomerase cis-trans peptidil-prolil (PPIase); *Plasmodium knowlesi*; zoonotik malaria

INTRODUCTION

Malaysia has set the National Strategic Plan for Malaria Elimination to achieve a malaria-free status by 2020. Nevertheless, while no more cases reported for human indigenous malaria (*Plasmodium falciparum*, *P. vivax*, *P. malariae,* and *P. ovale)* since 2008, zoonotic malaria cases caused by *P. knowlesi* have been on the rise, particularly in the Borneo region (Chin et al. 2020; Muhammad et al. 2022). Globally, the escalating cases of *P. knowlesi* are also observed in other countries in the regions of South-East Asia (SEA), Eastern Mediterranean, and Western Pacific region. Since 2020, the World Health Organization (WHO) included *P. knowlesi* malaria in the annual World Malaria Report, signifying the significance of monitoring and addressing this zoonotic malaria as a serious concern.

P. knowlesi is transmitted by mosquito bites from monkey to monkey, from monkey to human, from human to human, and from human back to monkey (Amir et al. 2018; Chin et al. 1968; Hansen et al. 2013). This explains why reported cases of *P. knowlesi* cases have been reportedly active in states with high forest coverage in Malaysia such as Sabah, Sarawak, Kelantan, Perak, and Pahang (Amir et al. 2018). While the treatment for *P. knowlesi* case is available, Rasmussen, Alonso and Ringwald (2022) reported that some malaria parasites, particularly *P. falciparum* and *P. vivax*, have demonstrated resistance to existing antimalarial medications in certain geographic regions. *P. knowlesi*, on the other hand, has never been evidenced to have drug resistance, yet, is likely to have it in the future. Fatih et al. (2013) also advise exercising caution due to the clinical failure of mefloquine treatment for *P. knowlesi.* This issue adds to the complexity of completely eradicating malaria. Furthermore, there is a paucity of research on antimalarial drugs targeting *P. knowlesi*. Accordingly, emphasizes the need to investigate novel therapeutic targets for the development of potent antimalarial drugs specifically aimed at Pk-FKBP35.

Earlier, Bell, Wernli and Franklin (1994) and Monaghan and Bell (2005), reported that immunosuppressant drug FK506 was found to inhibit malaria parasites with no resistance effect. This drug works by inhibiting FK506-binding protein (FKBP) of *Plasmodium*, a member of peptidyl-prolyl *cis-trans* isomerase (PPIase) family proteins, which responsible for catalyzing the slow isomerization of cis-prolyl peptide bonds in cis-proline containing proteins. This protein is often considered a rate-limiting step in protein folding (Fanghanel & Fischer 2004). Indeed, Rajan and Yoon (2022) identified PPIase proteins in malaria parasites as promising targets for antimalarial drugs, especially in light of the emergence of drug-resistant strains. However, the use of FK506 is not feasible due to its side effect of suppressing the immune response of the patient. Consequently, exploring alternative compounds that inhibit FKBP35 to replace FK506 is crucial for discovering new antimalarial drugs without inducing resistance and side effects (Atack et al. 2020). Building on this assumption, comprehensive studies on the structure and function of FKBP35 serve as a strategic foundation for further drug discovery endeavors.

This protein is expressed in all malaria parasites, including *P. knowlesi* (Goh et al. 2018), which leads to the possibility of the development of universal inhibitors targeting this protein across different malaria parasite species. The biological roles of FKBP35 in malaria parasites also involve its ability to prevent protein aggregation during protein synthesis events. This is based on the properties of other multidomain FKBPs which usually exhibit a dual function of catalyzing the cis-proline isomerization and protein aggregation prevention (Budiman et al. 2011; Goh et al. 2018). Structurally, FKBP35 is confirmed to be a multidomain protein that consists of an FK506-binding domain (FKBD) and tetratricopeptide repeated domains (TPRD). While the FKBD domain is known as a catalytic domain responsible for PPIase catalytic activity, the TPRD has been confirmed to play a role in dimeric structure formation (Goh et al. 2018; Gwarteh-Kwansah et al. 2018; Yoon et al. 2007). While the ability of FKBP35 to catalyze slow isomerization of cis-proline bonds was confirmed, no report is available on the ability of this protein in aggregation prevention. Confirmation of this ability should open new druggable sites for screening drug candidates.

In the pursuit of discovering drugs for malaria, therapeutic peptides emerge as a promising avenue, offering potential alternatives to FK506. Moreover, as the search for a safer option intensifies, peptides become appealing due to their reduced immunogenicity (Wang et al. 2022). Since 1921, which marked the creation of the first therapeutic peptide, insulin, significant advancements have been made, leading to the approval of more than 80 peptide-based drugs globally. As a result, the exploration and development of medications based on peptides have become a prominent focal point in pharmaceutical research (Wang et al. 2022). Helton and Kennedy (2020) proposed that peptide-based therapeutics could serve as an alternative to small molecule inhibitors for antimalarial treatment. According to Kurniaty et al. (2023), these antimalarial peptides initially came from plants or animals in their single peptide form. However, investigating peptide mixtures obtained through hydrolysis processes could offer a promising avenue for developing and testing new antimalarial peptide libraries.

Collagen hydrolysates (CH) serve as excellent peptide sources as this was produced through hydrolysis of collagen by proteases. Hydrolysis of proteins, including collagen, by certain proteases results in the production of peptide fragments due to the disruption of their peptide bonds (Afiyah, Arief & Budiman 2015). In some cases, peptide fragments generated from this hydrolysis may exhibit biological activities depending on the nature of the source proteins (Adiyoga et al. 2022; Hanifah, Arief & Budiman 2016). In particular, collagen is known to be rich in proline

which leads to the production of proline-rich peptides upon hydrolysis. Fanghanel and Fischer (2004) reported that the inhibitors of FKBP are structurally mimicking proline residues which are therefore able to have competitive binding with the proline substrate to the catalytic site. The exploration of CH as a potential antimalarial agents introduces innovative therapeutic approaches, thereby expanding the available repertoire against zoonotic malaria infections. By exploring alternative inhibitors, the study addresses the critical concern of drug-resistant strains, a pertinent issue in malaria treatment. Moreover, the potential cost-effectiveness of these drug candidates offers a promising avenue, aligning with global efforts to deliver affordable healthcare solutions, particularly in regions heavily burdened by malaria. At a molecular level, the study contributes valuable biological insights by elucidating the interaction between Pk-FKBP35 and CH. This provides an understanding of underlying molecular mechanisms around the aggregation prevention ability of Pk-FKBP35. This study aims to investigate the ability of Pk-FKBP35 to prevent protein aggregation *in vitro* and examine the effect of CH on this ability. Preventing protein aggregation is a known characteristic of chaperone proteins; therefore, if Pk-FKBP35 is confirmed to have this ability, it may be considered to exhibit chaperone-like activity. To explore this, insulin was used as a model substrate to study aggregation prevention, with its aggregation visually observed using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Insulin is widely employed as a model substrate in protein folding studies because of its tendency to form various oligomeric and supramolecular structures through self-assembly or aggregation (Bumagina et al. 2010; Das, Shah & Saraogi 2022). The aggregation of insulin is easily induced by the reduction of its disulfide bridges, which connect its A and B chains, leading to their dissociation and the formation of amorphous aggregates (Bumagina et al. 2010). Additionally, Das, Shah and Saraogi (2022) highlighted that insulin is a suitable model substrate for chaperone assays due to its simplicity, availability, and ease of access. For the first time, this research confirms that Pk-FKBP35 not only exhibits traditional peptidyl-prolyl cis-trans isomerase (PPIase) activity but also prevents DTT-induced aggregation of insulin, thus mimicking the function of a chaperone protein in preventing client protein aggregation. The study further explores the effects of CH from various sources on Pk-FKBP35's ability to prevent insulin aggregation, suggesting that CH may possess promising antimalarial properties that warrant further investigation.

MATERIALS AND METHODS

PREPARATION OF COLLAGEN HYDROLYSATE AND FK506

The collagen hydrolysate (CH) utilized in this study originated from various sources including bovine (Halavet, Turkey), bone broth (Vital Protein®, USA), fish, and swine (Tamachan, Japan). All CH were procured commercially from ACCVA Solutions Sdn. Bhd. Subsequently, these CH were prepared by dissolving them in autoclaved distilled water to attain the required concentrations. Meanwhile, FK506 (InvivoGen, France) was dissolved using dimethylsulfoxide (DMSO) to the required concentrations.

OVER-EXPRESSION AND PURIFICATION PK-FKBP35

The overexpression and purification of Pk-FKBP35 were conducted following the procedures outlined by Goh et al. (2018), with some modifications. The transformed *E. coli* (BL21) cells derived from aforementioned study (Goh et al. 2018), were cultured in Luria-Bertani (LB) broth supplemented with 35 µg/mL of kanamycin at 37 °C for 16 h. Subsequently, once the optical density at 600 nm (OD600) reach 0.6 to 0.7, protein expression was initiated by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The culture was then maintained at 25 °C for an additional 16 h. Cell harvesting was performed by centrifugation at 6,500 rpm for 10 min by using Avanti J-E Centrifuge (Beckman Coulter, USA). The resultant pellet was reconstituted using a 10:1 ratio of 20 mM phosphate buffer (pH 8.0) with 150 mM NaCl and subsequently sonicated with a Q700 Sonicator (QSonica, USA). Sonication settings were configured for 10 s on, 30 s off, over 6 min, at an amplitude of 30%. The soluble fraction was collected following centrifugation at 35,000 rpm with an Optima L-100K ultracentrifugation (Beckman Coulter, USA) for 30 min at 4 °C.

Pk-FKBP35 was purified through a two-step process involving affinity chromatography and size-exclusion chromatography. An affinity chromatography column (HiTrap TALON, Cytiva, Marlborough, USA) with a 1 mL capacity was utilized. The column was pre-equilibrated with a buffer containing 20 mM phosphate (pH 8.0), 150 mM NaCl, and 5 mM imidazole. Elution was conducted with a linear gradient of 300 mM imidazole. The eluted fraction from this step was subsequently concentrated to a final volume of 200 µL and loaded onto a Superdex 200 column (GE Healthcare, Amersham, UK) for size-exclusion chromatography. The size-exclusion chromatography was performed using a buffer composed of 20 mM phosphate (pH 8.0), 100 mM NaCl, and 5% Tween 20.

The protein's expression and purity were evaluated using 15% SDS-PAGE, following the protocol outlined by Laemmli (1970). Protein concentration was determined through UV absorption measurements, with an absorbance at 280 nm of 0.1% (1 mg/mL) equivalent to 0.73, calculated as per the approach of Goodwin and Morton (1946).

DTT-INDUCED INSULIN AGGREGATION

The methodology for this assay was derived from Budiman et al. (2012) with some amendments. This assay used dithiothreitol (DTT) to induce insulin aggregation.

The reaction mixture includes 20 mM phosphate buffer (pH 8.0), 2 mg/mL insulin, 200 mM DTT, and 0.3 mg/mL Pk-FKBP35 in the absence or presence of various concentrations of CH. The CH to Pk-FKBP35 concentration ratio was set as 1:1, 1:10, 1:50, and 1:100. The reaction was started once DTT was added to the reaction mixture. Then incubation was done for 30 min at room temperature (25 °C) while shaking at 250 rpm using a thermoshaker (Thermo Fisher Scientific, USA). After the incubation, it was further centrifuged for 10 min at 13,000 rpm to separate the aggregation pellet and the supernatant. For control purposes, Pk-FKBP35 with autoclaved Milli-Q water was designated as 100% in relative soluble insulin.

The supernatant was collected from the reaction mixture after the separation and subjected to 17% SDS-PAGE gel. This concentration was used to allow better separation for small size protein like insulin (5.8 kDa) as also suggested by Okita et al. (2017). The gel was viewed by Gel Doc XR imaging system (Biorad, USA) with Image Lab software (v.6.0.1). The gel contrast was set at default to minimize bias during quantification. The band intensity from the gel was quantified by using ImageJ (Schneider, Rasband & Eliceiri 2012) software. Subsequently, the relative band intensity which corresponds to the soluble insulin is calculated using Microsoft Excel 2016.

PPIase ACTIVITY

The catalytic activity of PPIase was assessed using a protease coupling assay based on the method described by Fischer et al. (1989). The reaction mixture consisted of 35 mM HEPES buffer (pH 7.8), 25 µM Suc-ALPF-*p*Na (substrate), 0.2 mg/mL of Pk-FKBP35, 0.2 mg/mL insulin, and with or without the presence of 2 mM DTT. For the control, insulin was omitted and replaced with an equivalent volume of autoclaved Milli-Q water. The mixture was incubated for 3 min, followed by the immediate addition of chymotrypsin to a final concentration of 11.4 µM. The isomerization rate was monitored by measuring the changes in the concentration of *p*-nitroanilide (*p*Na) at 390 nm absorption using a microplate reader (Infinite® 200 PRO, Tecan Life Sciences, Männedorf, Switzerland).

DATA ANALYSIS

The data are presented as the mean ± standard error from three independent replicates. Differences among the means were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Means with a P value greater than 0.05 are considered not significantly different, while those with a P value less than 0.05 or 0.01 are considered significantly different and highly significant, respectively.

RESULTS AND DISCUSSION

Over-expression of Pk-FKBP35 was conducted to obtain a target protein for the inhibition assay. The production of recombinant proteins finds applications in various aspects such as vaccines, pharmaceuticals, antibodies, and biochemical analysis (Nosaki & Miura 2021). Earlier, Goh et al. (2018) has developed the method for the production of this protein. Therefore, this study utilized a similar approach as reported by Goh et al. (2018), in which the expression system of Pk-FKBP35 for the over-expression was constructed using a synthetic-codon optimized DNA to ensure its compatibility with the host cells (*E. coli*).

The over-expression of Pk-FKBP35 was detected under 15% SDS-PAGE as depicted in Figure 1. As shown, a thicker band with an apparent size of about 37 kDa was shown in the cells induced by 1 mM IPTG (Lane 2), compared to the cells without IPTG (Lane 1). This band is believed to be Pk-FKBP35 as the size is only less than 1 kDa different to the theoretical size of Pk-FKBP35 (36.6 kDa) based on its calculated amino acid sequence (Goh et al. 2018). The slight discrepancy of around 1 kDa might be attributed to the presence of a 6His-tag at the N-terminal of the protein. A thicker band observed in the presence of IPTG indicated that Pk-FKBP35 was successfully over-expressed following induction with 1 mM IPTG. Marbach and Bettenbrock (2012) reported that IPTG, a molecular analog of allolactose, is a common inducer for protein expression under this system, which removes a repressor from the lac operon to induce gene expression.

Pk-FKBP35 was successfully purified using affinity chromatography coupled with size-exclusion chromatography. As shown in Figure 2(A), Pk-FKBP35 obtained from affinity chromatography still contains contaminant proteins as observed under SDS-PAGE. This implies that the protein requires further purification steps. While affinity chromatography using the TALON column is specific for recombinant protein containing histidine-tagged, the possibility of having protein contaminants is still open as some indigenous *E. coli* proteins may have exposed His residues which might be able to attach to the column resin. This notion corroborates with Robichon et al. (2011) and Völzke et al. (2023) which mentioned that binding of these contaminant proteins to the metal-chelating resins could be attributed to their inherent metal-binding abilities or the presence of histidine residue clusters on their surfaces. Additionally, the occurrence of these contaminants was a result of the upregulation of native bacterial proteins in response to stress conditions, suggesting the bacteria's adaptation to produce larger amounts of foreign material (Parsy et al. 2007; Tan et al. 2022).

Further, when size-exclusion chromatography was employed as an additional step to purify, a single band of Pk-FKBP35 was observed under SDS-PAGE (Figure 2(B)), which indicated that the purification has yielded Pk-FKBP35 with high purity. To note, Goh et al. (2018) also required a two-step purification to obtain Pk-FKBP35, which confirmed that acceptable purity of this

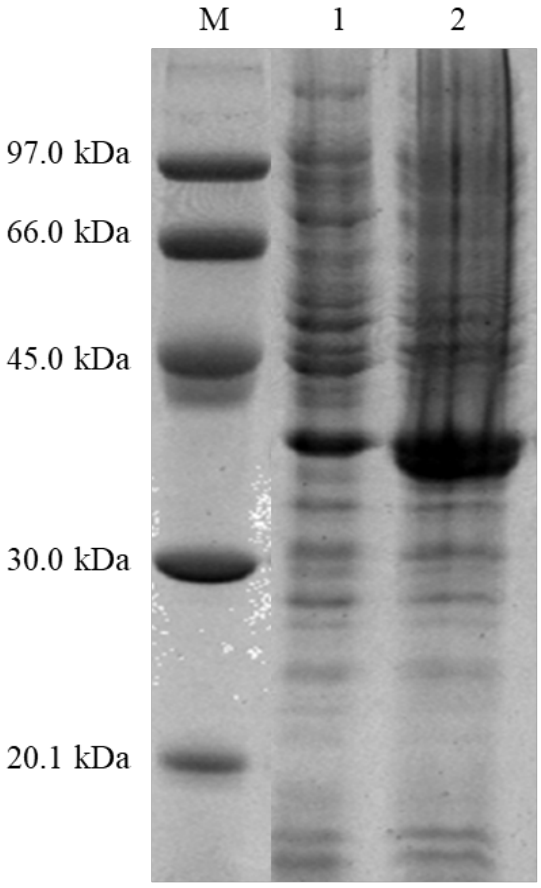


FIGURE 1. SDS-PAGE of over-expression of Pk-FKBP35. Lane 1 corresponds to Pk-FKBP35 before induction by IPTG, lane 2 indicates after induction by IPTG

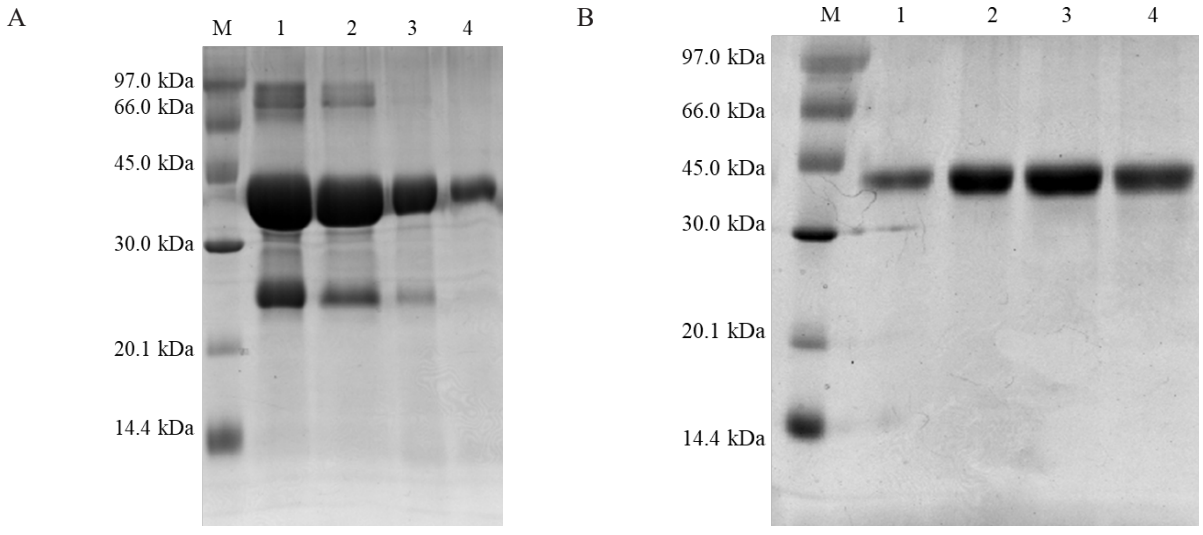


FIGURE 2. SDS-PAGE of Pk-FKBP35 after purification. (A) Affinity chromatography; (B) Size-exclusion chromatography. M corresponds to a low molecular weight marker kit (Cytiva, Marlborough, USA), while lanes 1 to 4 correspond to the eluted fraction after each chromatography

protein requires more than one step of chromatography. The purity of Pk-FKBP35 obtained from this purification was considerably high (>90%). This purity level is more than enough for use in bioactivity assay. According to Huynh and Partch (2015), the purity level of proteins for biochemical assay or drug screening is about 75%.

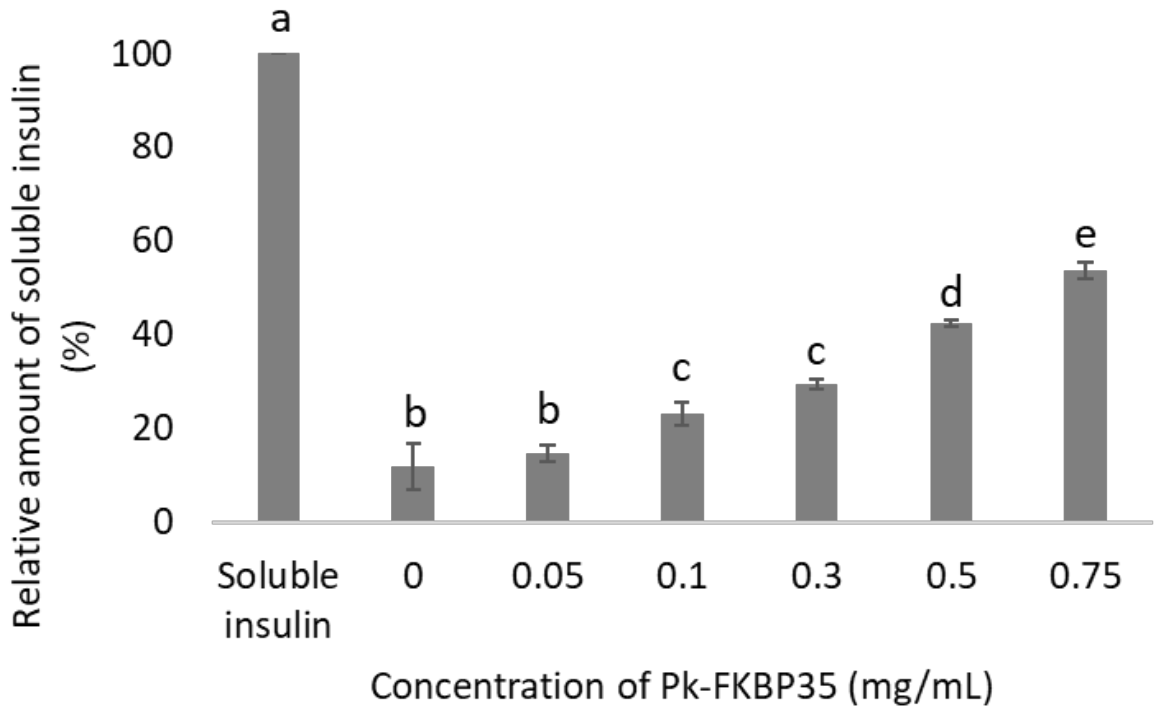
The ability of Pk-FKBP35 to prevent aggregation of insulin was then examined using SDS-PAGE. This method is considerably much simpler and more efficient as compared to the spectroscopy method as previously reported (Budiman et al. 2012). Nevertheless, both methods rely on the properties of insulin to aggregate and form precipitation upon the reduction of its S-S bond between A and B chains by DTT (Bumagina et al. 2010). In the case of Pk-FKBP35 is capable of preventing aggregation, the amount of precipitated insulin upon reduction is expected to be less than in the absence of Pk-FKBP35. Alternatively, the amount of soluble insulin (a non-aggregated form of insulin) should be higher in the presence of Pk-FKBP35. Figure 3 shows the relative amount of soluble insulin upon the reduction by DTT in the presence of Pk-FKBP35 remarkably increased in a concentration-dependent manner ($P<0.05$). This indicated that the presence of Pk-FKBP35 was able to prevent DTT-induced aggregation of insulin. This notion suggested that this protein exhibited chaperone function which phenotypically is characterized by the ability to prevent client protein aggregation (Budiman et al. 2011). This molecular capability exhibited by Pk-FKBP35 is commonly observed in the FKBP family protein, especially in the case of multidomain FKBP proteins (Budiman et al. 2011; Furutani et al. 2000; Kang et al. 2008; Tong & Jiang 2016). The activity is distinguished from its PPIase activity, in which the PPIase activity refers to the ability to accelerate a slow isomerization of cis-prolyl bond, while chaperone function is the ability to aid in the proper folding of other proteins during or after their synthesis and facilitate the refolding process following partial denaturation, preventing random protein aggregation (Budiman et al. 2011; Lund & Ellis 2008; Rasmussen, Alonso & Ringwald 2009). It is important to highlight that the chaperone function of FKBP35 of *P. falciparum* has been documented in previous studies (Kang et al. 2005; Monaghan & Bell 2005; Thommen et al. 2023). Nevertheless, its capability to experimentally prevent DTT-induced insulin aggregation has not been demonstrated.

The mechanism by which Pk-FKBP35 prevents DTT-induced insulin aggregation is believed to follow the earlier proposal for FKBP22 from *Shewanella* sp. SIB1 (Budiman et al. 2012). Under this mechanism (Figure 4), DTT firstly reduces S-S bond connecting the A and B-chains of insulin which leads to the separation of these two chains. As indicated by Hua and Weiss (2004) and Rasmussen, Alonso and Ringwald (2009), the stability of both A and B chains is compromised in the absence of their connection through the disulfide bond. Therefore, the separation of both chains by DTT leads these chains to aggregate. Noteworthy, the course of subunit aggregations, as reported by Das, Shah and Saraogi (2022) is through an intermedia step. At this point, Pk-FKBP35 selectively binds to the intermediate state of the subunit, presumably through hydrophobic interaction, which allows both subunits to re-fold and reform their S-S bond and remain soluble (folded). In this mechanism, Pk-FKBP35 has no ability to bind to non-reduced insulin which is also in good agreement with FKBP22. Therefore, it is plausible to observe that the effect of Pk-FKBP35 to prevent DTT-induced insulin aggregation is in a concentration-dependent manner as a higher concentration of Pk-FKBP35 allows more binding to the intermediate state of insulin for the protection against DTT.

Further, when the ability of Pk-FKBP35 was tested in the presence of its specific inhibitor, no remarkable changes in the amount of insulin by the increase of FK506's concentration (Figure 5). As shown in Figure 5, no differences were observed in the relative amount of soluble insulin in the presence of various concentrations of FK506 ($P>0.05$), indicating that FK506 does not affect the ability of Pk-FKBP35 to prevent aggregation. This corroborates with the independent ability of FKBP22 from *Shewanella* sp. SIB1 from FK506 (Budiman et al. 2020). Earlier, FK506 was reported to selectively bind to and inhibit the catalytic domain (FKBD) of Pk-FKBP35 (Goh et al. 2018). Accordingly, FKBD is unlikely to be involved in the prevention of DTT-induced insulin aggregation. Hence, the non-catalytic domain (TPRD) of this protein was predicted to be responsible for the aggregation prevention ability. This implies that PPIase activity and the ability of aggregation prevention are structurally independent.

To confirm that PPIase activity and aggregation prevention ability were not associated, the PPIase activity of Pk-FKBP35 in the presence of various concentrations of reduced and non-reduced states of insulin (Figure 6) was examined. As shown in Figure 6, the PPIase activity of Pk-FKBP35 was not affected by insulin ($P>0.05$), with or without DTT, up to 1 mg/mL concentration. This indicated that insulin did not interfere with the region responsible for PPIase catalytic activity (FKBD) and bind to distant sites from FKBD. It is therefore plausible to propose TPRD as a binding site for insulin as the domain is relatively distant from FKBD and has no effect on PPIase catalytic activity.

Earlier reports also demonstrated that PPIase activity and chaperone function of multidomain FKBPs are proposed to be regulated by different structural regions (Alag et al. 2013, 2010; Mckeen et al. 2008; Wochnik et al. 2005). The catalytic domain is solely responsible for catalytic activity, while the non-catalytic domain is responsible for chaperone function (binding to the intermediate state and preventing aggregation of client proteins). Interestingly, TPRD of Pk-FKBP35 was earlier reported to be in dimeric form, while FKBD is stable and active in its monomeric form (Goh et al. 2018; Silvester et al. 2017). This leads to the



Different letters above the bars indicate statistically significant difference at P<0.05

FIGURE 3. The relative amount of soluble insulin (%) against different concentrations of Pk-FKBP35 (mg/mL). Soluble protein indicates a protein without DTT. The 0 mg/mL indicates insulin with DTT in the absence of Pk-FKBP35

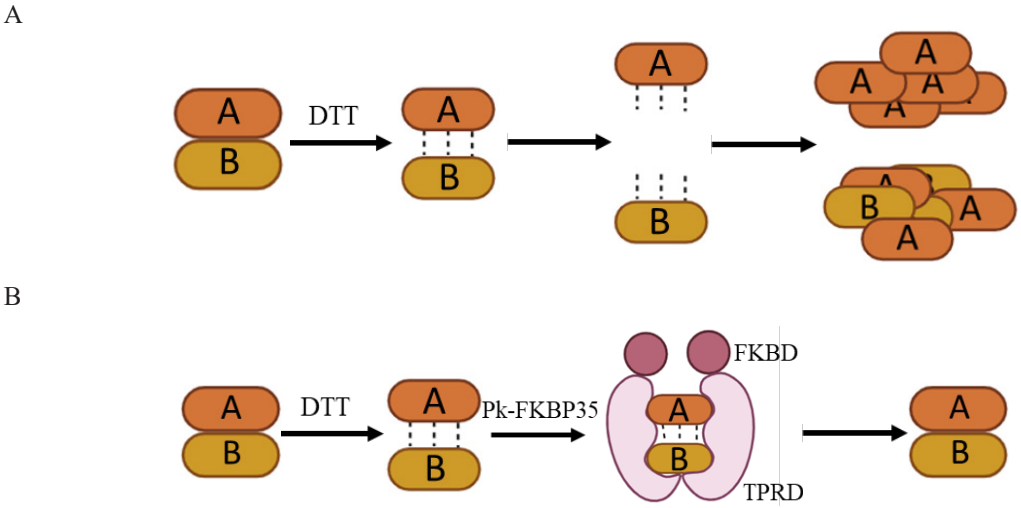
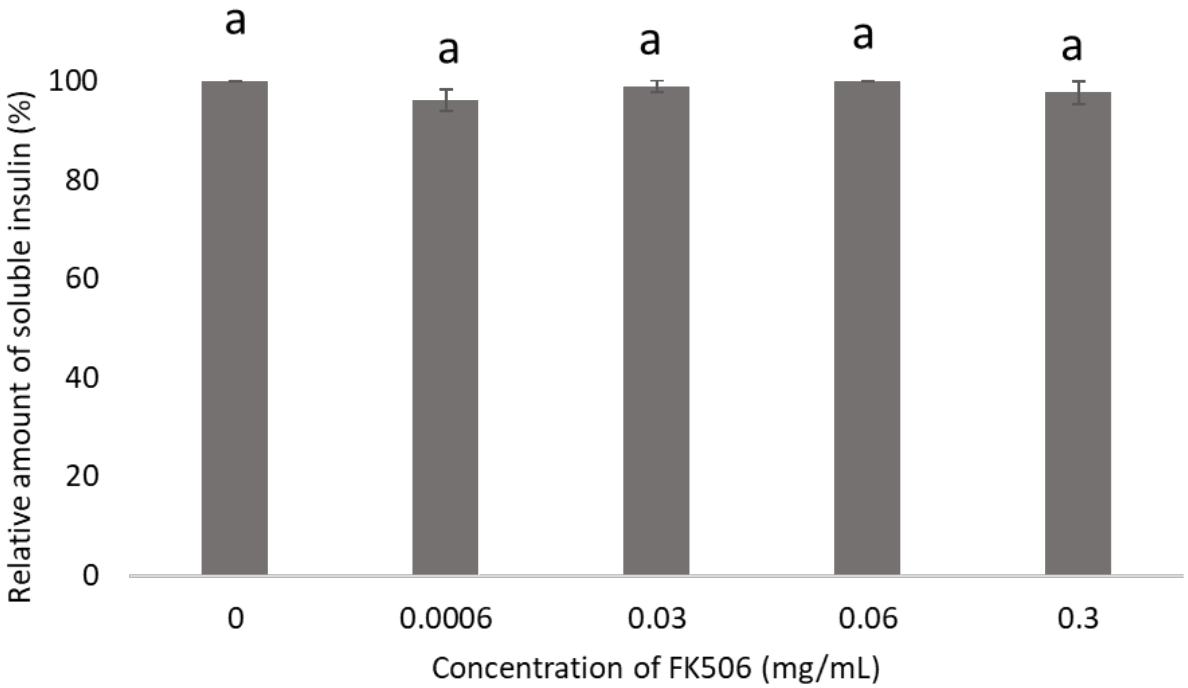
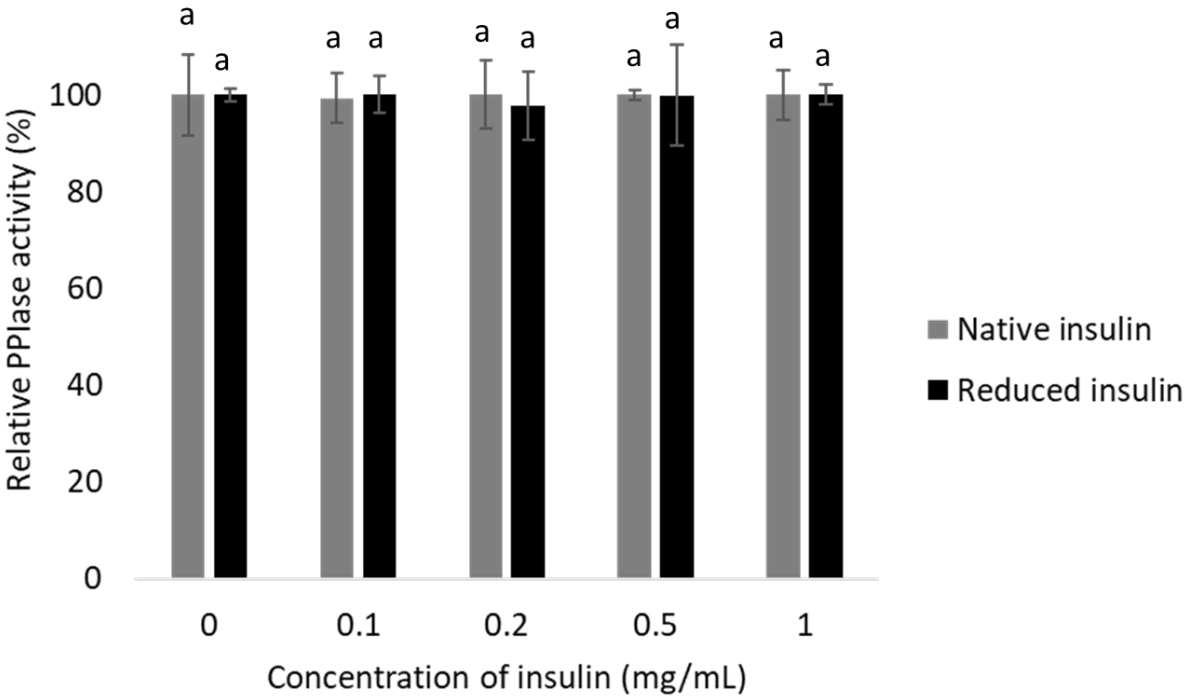


FIGURE 4. Mechanism of prevention of insulin aggregation by Pk-FKBP35. (A) DTT-induced insulin aggregation, and (B) Prevention of insulin aggregation by Pk-FKBP35



Same letters above the bars indicate no significant difference (P > 0.05)

FIGURE 5. Relative amount of soluble insulin (%) in the presence of different concentrations of FK506



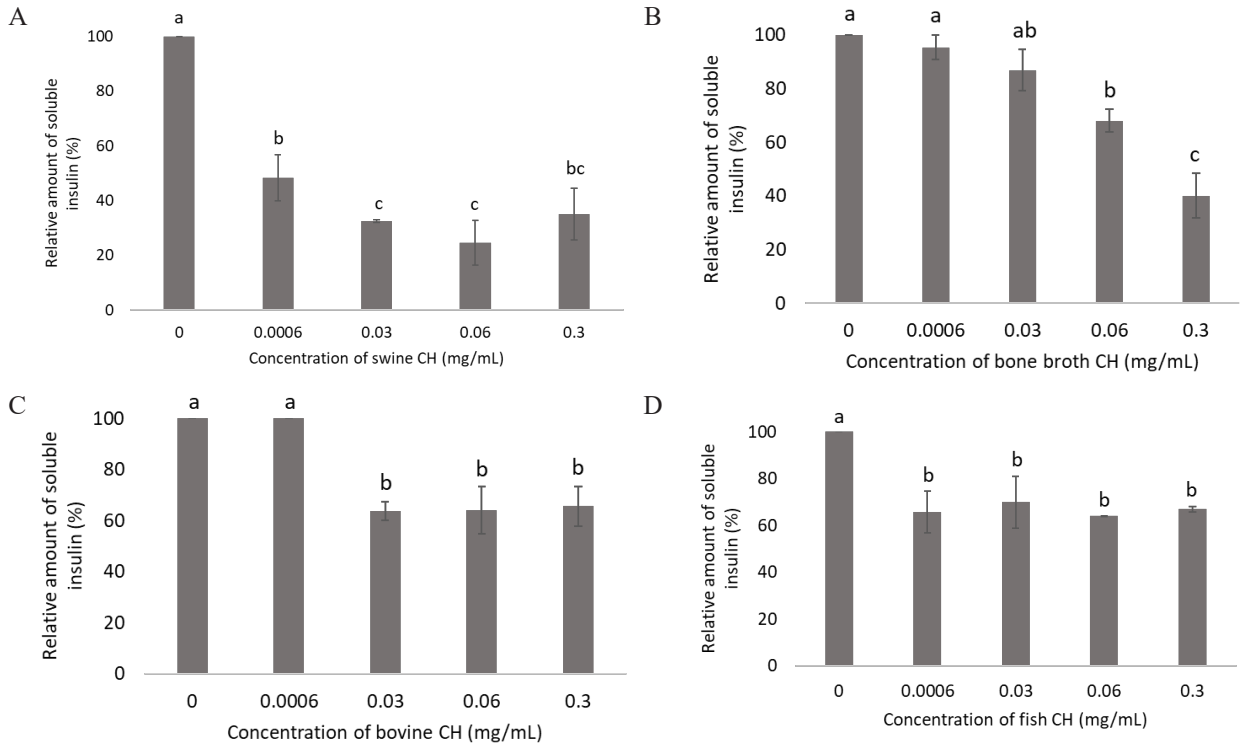
Same letters above the bars in the same group of native or reduced insulin indicate no significant difference (P > 0.05)

FIGURE 6. Relative PPIase activity (%) with the presence of different concentrations of native and DTT-induced insulin (mg/mL)

possibility that the dimeric structure of TPRD plays an important role in the aggregation prevention activity of this protein. The dimeric form of TPRD provides more hydrophobic surfaces for facilitating binding to the intermediate state of protein substrate and further prevents them from forming aggregation. The importance of the dimeric form of FKBP for chaperone function was also earlier reported for FKBP22 of *Shewanella* sp. SIB1, which lost its chaperone function when this protein failed to form a dimeric structure (Budiman et al. 2012, 2009).

The ability of Pk-FKBP35 to inhibit protein aggregation is notably beneficial for malaria parasites, as it ensures the maintenance of proper protein folding and functionality within the parasite cells. Earlier reports indicated that the ability of *P. falciparum* to prevent its protein aggregation is a vital process for cell viability (Muralidharan et al. 2012). Accordingly, the compounds capable of inhibiting the ability of this parasite to suppress the protein aggregation are expected to have a negative impact on the parasite viability. Figure 7(A)-7(D) shows the relative amount of soluble insulin in the presence of different concentrations of CH from swine, bone broth, and bovine. These were significantly different from the absence of CH ($P<0.05$). This implied that all CH were able to block the capability of Pk-FKBP35 to prevent DTT-induced aggregation of insulin which leads to less soluble insulin in the cocktail's supernatant. Nevertheless, the amount of soluble insulin was different under different sources of CH, which suggested that each type of CH exhibited the capacity to inhibit the chaperone function of Pk-FKBP35 to varying degrees. Swine and bone broth CH illustrate more than 50% reduction in the chaperone activity of Pk-FKBP35 through the prevention of DTT-induced insulin aggregation (Figure 7(A) & 7(B)). On the other side, bovine and fish CH only reduced the chaperone activity by 20% to 30%, respectively (Figure 7(C) & 7(D)).

To note, CH is essentially a mixture of peptide fragments released from collagen upon hydrolysis by protease (presumably trypsin). Richard-Blum (2011) indicated that collagen is mainly composed of glycine, proline, and hydroxyproline. As collagen is rich in proline, the peptide fragments in CH are also expected to be rich in proline contents. This postulation aligns well with the findings of Asai (2020), which highlight the elevated proline content in CH from diverse sources. As Pk-FKBP35 is a member of the PPIase family protein (Goh et al. 2018), the substrate-binding cavity of this protein is therefore exhibiting high specificity towards proline-containing



Different letters above the bars indicate statistically significant difference at $P<0.05$

FIGURE 7. The relative band intensity (%) against the concentration of different collagen hydrolysates (CH) in mg/mL. (A) Swine CH, (B) Bone broth CH, (C) Bovine CH, and (D) Fish CH

proteins or peptides. This is congruent with Budiman et al. (2020), which confirmed the high specificity of Pk-FKBP35 to bind to X-pro containing peptides, where X is preferably to be hydrophobic bulky residues. Accordingly, proline-rich peptides of CH should be therefore able to bind to the substrate-binding cavity of Pk-FKBP35. This substrate-binding cavity is previously reported to be localized at FKBD, where FK506 binds and inhibits PPIase activity of this protein (Budiman et al. 2020; Goh et al. 2018). However, as Figure 4 demonstrated that FKBD has no role in the prevention aggregation activity of this protein, this consequently indicated that binding of CH peptides to the substrate-binding cavity should not interfere with the ability to the aggregation prevention activity. It is therefore proposed that CH peptides might not only bind to the substrate-binding cavity but also to other regions of Pk-FKBP35 that are involved in the binding to the folding intermediate of insulin. The region is presumably TPRD as this was previously reported to be a non-catalytic domain responsible for binding to protein substrate (Goh et al. 2018). This may be due to the myriad range of peptides as a product of hydrolyzed collagen (León-López et al. 2019). Occupancy of TPRD by CH peptides leads this region to be inaccessible by folding intermediate insulin substrates for further protection from the DTT-induced aggregation event. Nevertheless, whether or not the binding event of the CH peptides to TPRD is a specific binding event should be further experimentally confirmed.

CONCLUSIONS

The current study for the first time confirmed the ability of Pk-FKBP35 in preventing DTT-induced insulin aggregation. The prevention ability was found to be independent from the influence of FK506 which implied that it was not associated with the catalytic domain FKBD. Alternatively, the non-catalytic domain of this protein (TPRD) is presumably responsible for the aggregation prevention activity. Further, CH from various sources demonstrated the ability to reduce the capability of Pk-FKBP35 to prevent DTT-induced insulin aggregation which is likely due to occupancy of TPRD by CH peptides which leads to failure of complex formation between Pk-FKBP35 and folding intermediate state of insulin for aggregation prevention. This study demonstrated the potential application of CH for the development of antimalarial specifically targeting Pk-FKBP35, indicating a promising avenue for further exploration. However, it is imperative to assess their efficacy in inhibiting the parasite *in vivo* using cell cultures to validate this antimalarial properties.

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