STABILITY AND ACTIVITY OF LIPID A PHOSPHOETHANOLAMINE TRANSFERASE FROM *NEISSERIA MENINGITIDIS* IN THE PRESENCE OF SODIUM DEOXYCHOLATE

[STABILITAS DAN AKTIVITAS LIPID A PHOSPHOETHANOLAMINE TRANSFERASE DARI NEISSERIA MENINGITIDIS TERHADAP PENAMBAHAN NATRIUM DEOKSIKOLAT]

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ABSTRACT

Antibiotics are often used to fight infections caused by pathogenic bacteria. However, inappropriate use of antibiotics has caused many pathogenic bacteria to gain antibiotic resistance. Some resistance mechanisms arise through enzymatic modification of the bacterial membrane. Lipid A phosphoethanolamine transferase (EptA) modifies bacterial membranes through the addition of phosphoethanolamine to the lipid A moiety of lipopolysaccharide (LPS) or lipooligosaccharide (LOS). Inhibition of EptA would therefore restore the antibiotic susceptibility of the bacterium. In order to develop inhibitors against EptA, *in vitro* studies are required. Biochemical studies of membrane proteins such as EptA, must be carried out in buffers containing detergent to ensure the solubility and stability of the membrane protein. EptA has been found to be most stable in buffers containing the detergent, n-dodecyl β-D-maltoside (DDM). However, due to the fact that LOS is also required for biochemical studies of EptA, the detergent, sodium deoxycholate (DOC) is also required as it prevents the aggregation of LOS. In this study, the stability and activity of EptA in the presence of DOC were investigated using thin layer chromatography (TLC) and circular dichroism (CD) spectroscopy. Additionally, preliminary studies on the interaction of EptA and LOS *in vitro* was carried out using native agarose gel electrophoresis (NAGE). This study shows that EptA remains active in the presence of ≤ 2 mM DOC with the highest activity observed in buffers containing only 1 mM DOC. CD analysis showed that the overall secondary structure of EptA in buffer containing various concentrations of DDM and DOC was maintained. Additionally, through NAGE analysis the interaction between EptA and LOS was successfully observed. Therefore, further *in vitro* studies incorporating both substrates with supplementation of up to 1 mM DOC could be carried out with the long-term goal of studying inhibitors against EptA.

Keywords: circular dichroism; lipid A phosphoethanolamine transferase; lipooligosaccharide, n-dodecyl β-D-maltoside; sodium deoxycholate

ABSTRAK

Antibiotik sering digunakan untuk melawan infeksi yang disebabkan oleh bakteri patogen. Namun, penggunaan antibiotik yang tidak tepat telah menyebabkan banyak bakteri patogen mendapatkan resistensi antibiotik. Beberapa mekanisme resistensi muncul melalui modifikasi enzimatik dari membran bakteri. Lipid A phosphoethanolamine transferase (EptA) mengubah membran bakteri dengan menambahkan fosfoetanolamina ke bagian lipid A dari lipopolisakarida (LPS) atau lipooligosakarida (LOS). Penghambatan EptA akan mengembalikan kerentanan antibiotik dari bakteri tersebut. Untuk mengembangkan inhibitor terhadap EptA, studi in vitro diperlukan. Studi biokimia protein membran seperti EptA, harus dilakukan dalam larutan penyangga yang mengandung deterjen untuk memastikan kelarutan dan stabilitas protein membran. EptA ditemukan paling stabil dalam larutan penyangga yang mengandung deterjen, n-

dodecyl β-D-maltosida (DDM). Namun, karena LOS juga diperlukan untuk studi biokimia EptA, deterjen, sodium deoksikolat (DOC) juga diperlukan karena mencegah agregasi LOS. Dalam penelitian ini, stabilitas dan aktivitas EptA dalam keberadaan DOC diselidiki menggunakan kromatografi lapis tipis (TLC) dan spektroskopi dikroisme sirkular (CD). Selain itu, studi awal tentang interaksi EptA dan LOS in vitro dilakukan menggunakan elektroforesis gel agarosa asli (NAGE). Studi ini menunjukkan bahwa EptA tetap aktif dalam keberadaan ≤ 2 mM DOC dengan aktivitas tertinggi diamati dalam larutan penyangga yang hanya mengandung 1 mM DOC. Analisis CD menunjukkan bahwa struktur sekunder keseluruhan EptA dalam larutan penyangga yang mengandung berbagai konsentrasi DDM dan DOC tetap terjaga. Selain itu, melalui analisis NAGE, interaksi antara EptA dan LOS berhasil diamati. Oleh karena itu, studi in vitro lebih lanjut yang memasukkan kedua substrat dengan suplementasi hingga 1 mM DOC bisa dilakukan dengan tujuan jangka panjang untuk mempelajari inhibitor terhadap EptA.

Kata kunci: *dikroisme sirkular; lipid A fosfoetanolamina transferase; lipooligosakarida, n-dodecyl β-D-maltosida; natrium deoksikolat*

INTRODUCTION

The human body is complex and is comprised of various types of human cells and normal microbes including fungal and bacterial cells. Many microbes outside the human's normal flora are able to enter the human body and cause infectious diseases (Alberts *et al.,* 2002). Antibiotics are the common therapeutic developed to fight infections caused by bacteria. These antibiotics function by inducing cell death (bactericidal drugs) or inhibiting cell growth and multiplication (bacteriostatic drugs) (Kohanski *et al.*, 2010). However, extensive misuse and overuse of antibiotics have caused antimicrobial resistance, which makes infections significantly more difficult to treat (Murray *et al.,* 2022). There are many ways that infectious agents such as bacteria gain antimicrobial resistance, enabling them to withstand drug exposures that would typically inhibit cell growth or induce cell death (O'Neill, 2024). One example of a resistance mechanism is modification of the antibiotic's target site in the bacterial cell. This results in decreased affinity for the antibiotic drugs, rendering the drugs unable to target the bacterial membrane. Some Gram-negative pathogens have evolved this kind of resistance mechanism by synthesizing enzymes that act by modifying their membrane structure and biochemical features which allows them to evade the action of antibiotics directed at the membrane. One example is the modification of the lipid A moiety of the lipopolysaccharides (LPS)/ lipo-oligosaccharides (LOS) structure (Needham & Trent, 2013). This becomes a major issue as the bacterial membrane is antigenic, which means it has a unique arrangement in contrast to the host membranes and many antibiotics work primarily by targeting the bacterial membrane (Epand *et al.*, 2016).

EptA uses phosphatidyl-ethanolamine (PE) to modify the lipid A portion of LPS/LOS through the addition of phosphoethanolamine (pEtN). This

modification leads to resistance against cationic antimicrobial peptides (CAMPs) such as polymyxin in various Gram-negative bacteria. Because polymyxin is increasingly used as a last-resort antibiotic against multi drug resistant (MDR) Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, and *Enterobacteriaceae*, this developed resistance mechanism in the bacterial cell membrane is a major global health concern (Falagas *et al.*, 2005 & WHO, 2018). Research on pathogenic *Neisseria* indicates that without modification from EptA, their susceptibility to polymyxin increases significantly, in addition to making the bacteria more vulnerable to human serum and the host immune system's natural CAMPs, or defensins, such as LL-37 and protegrin-1. These findings suggest that drugs targeting EptA could enhance the effectiveness of polymyxin as well as aid in clearing infections by the host immune system. (Tzeng *et al.*, 2005 & Lewis et *al.*, 2009).

EptA is an integral membrane protein (IMP) and buffers containing detergent are needed to solubilize and purify EptA for *in vitro* studies. Detergents act by creating a membrane mimicking environment thus allowing EptA to maintain a folded and active state (Jeffery, 2016). One of the most common detergents used in the biochemical studies of membrane proteins is n-dodecyl-β-D-maltoside (DDM) (Anandan & Vrielink, 2016). DDM is considered relatively non-denaturing and, as a consequence, does not affect the overall protein structure.

To study the structure and catalytic mechanism of EptA, particularly in the presence of its substrates (PE and LOS), another surfactant namely sodium deoxycholate (DOC), is needed in order to prevent aggregation of LPS/LOS (Komuro & Galanos, 1988 & Hardy *et al.*, 2016). DOC is an ionic detergent which tends to denature protein thus, in this research the effect of DOC on the activity and structural stability of EptA was investigated. Additionally, the interaction of EptA and LOS in the absence of PE was investigated by NAGE analysis.

MATERIALS AND METHODS

Materials and Tools

The materials used in this research are: Lysogeny Broth (LB) media, bacteriological agar, Terrific Broth (TB) media, Ampicillin, Chloramphenicol, isopropyl β-D-1-thiogalactopyranoside (IPTG), sodium phosphate buffer, NaCl, imidazole, n-dodecyl-β-D-maltoside (DDM) (Anatrace), HEPES buffer, sodium deoxycholate, 1-acyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] dodecanoyl}-sn-glycero-3 phosphoethanolamine (NBD-PE) (Avanti Lipids), silica gel 60 F 254 Thin Layer Chromatography (TLC) plate (Merck), ethyl acetate, methanol, agarose gel

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loading buffer (0.025 % (w/v) bromophenol blue, 30 % (v/v) glycerol, 1x TAE buffer), agarose, imidazole, $ZnSO_4$, and Ethylenediaminetetraacetic acid (EDTA).

Escherichia coli BL21(DE3) pLysS competent cells were used for recombinant expression of EptA from the plasmid pCMK526 which carries the gene encoding EptA containing a hexahistidine tag at the C-terminus of the protein. The plasmid was kindly provided by Prof. Charlene M. Kahler and Dr. Anandhi Anandan. Unmodified LOS from *Neisseria gonorrhoeae* FA1090 ΔEptA (Neisserial LOS) was also kindly provided by Prof. C.M. Kahler.

The tools utilized in this research are: Ultra Yield 2.5 L flasks (Thomson Instrument), shaker incubator, high-pressure homogeniser (Emulsiflex C5, Avestin), refrigerated centrifuge, ultracentrifuge (Sorvall™ WX+, ThermoFisher), glass/Teflon tissue homogeniser, tube rotator mixer, Filtropur S 0.45 syringe filter (Sarstedt), HisTrap FF column (Cytiva), Superdex 200 10/30 column (Cytiva), Äkta™ pure FPLC system (Cytiva), protein electrophoresis set (Hoefer Inc.), 100 kDa MWCO centrifugal filter unit (Merck-Millipore), NanoDrop lite spectrophotometer (Thermo Scientific), CD spectrophotometer (Jasco J 720), and Chemidoc MP imaging system (Bio-Rad).

Methods

EptA Expression and Purification

Chemically competent *E. coli* BL21(DE3) pLysS cells were transformed with the plasmid pCMK526 (Piek *et al.*, 2014) using a heat shock method (Inoue *et al.*, 1990). The transformed bacteria culture was plated on LB agar plates containing antibiotics (34 μg/mL chloramphenicol and 50 μg/mL ampicillin) and incubated at 37°C overnight. A pre-culture was prepared consisting of Lysogeny Broth (LB) media containing the appropriate antibiotics and inoculated with a single transformed bacterial colony from the plate culture. The pre-culture was incubated overnight at 37°C with 180 rpm shaking. The overnight pre-culture was used to inoculate 3 litres of Terrific Broth (TB) medium containing the appropriate antibiotics to an optical density at 600 nm (OD_{600}) of 0.1. The cultures were incubated at 37°C with 200 rpm shaking until the OD_{600} reached 1.0 – 1.2. The bacterial protein expression was induced by addition of IPTG to a final concentration of 0.4 mM. The bacterial cultures were further incubated at 25°C with 180 rpm shaking for 20 hours. The *E. coli* cells were harvested by centrifugation at 4000 x g for 45 minutes at 4°C. The harvested cells were lysed by a high-pressure homogeniser (Emulsiflex C5, Avestin) at a maximum pressure of 15,000 psi. The lysate was then clarified by centrifugation at 4000 xg for 45 minutes at 4°C. Once clarified the

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lysate was further centrifuged at 100,000 x g for 1 hour at 4°C using an ultracentrifuge (Sorvall™ WX+, ThermoFisher) to isolate the bacterial membrane fraction.

The membranes were solubilized in solubilization buffer consisting of 50 mM sodium phosphate pH 7.5, 300 mM NaCl, 10 mM imidazole and 1% DDM overnight at 6°C with gentle shaking. The mixture was centrifuged at 100,000 x g for 1 hour at 4°C to remove insolubilized membranes, and the supernatant fraction was passed through a 0.45 µm filter disc. Recombinantly expressed EptA solubilized in DDM was purified by Ni^{2+} NTA affinity and size exclusion chromatography (SEC) using an Äkta™ Pure FPLC system following the method outlined by Anandan *et al.* (2017). The final concentration of purified protein was determined by measuring the absorbance at 280 nm using the calculated molar extinction coefficient for EptA (73,980 $M^{-1}cm^{-1}$; calculated based on sequence using ProtParam). The purified protein was concentrated to 20 mg/mL using a 100 kDa molecular weight cut off centrifugal filter unit (Merck-Millipore). To monitor the purification process, a sample from each purification step was analysed with SDS PAGE.

EptA Enzymatic Activity Assay

Enzyme activity assays were performed according to the protocol by Anandan *et al.* (2017) with some modifications. To assess the enzymatic hydrolysis of pEtN from NBD-PE, NBD-PE was utilized. Each reaction (50 µL) comprised 0.32 µM EptA and 1.6 µM NBD-PE in various activity assay buffers. The activity test buffers tested were 50 mM HEPES pH 7,0, 100 mM NaCl, with varied concentrations of DDM (0 - 450 µM) and DOC (0 – 12 mM). The mixture was incubated at 25 °C for defined time periods. Every five minutes 1 µL of each mixture were taken and was added to 2 µL methanol to halt the enzymatic reaction. The mixtures were applied to a pre-warmed TLC plate. A mixture of ethyl acetate:methanol:water (7:2:1) was used to develop the TLC plate and the fluorescent signal on the plate was visualized with a Chemidoc MP imaging system (Bio-Rad) using Epi blue light (455-485 nm) and a corresponding filter (530 nm +/- 28 nm). Image Lab software (Bio-Rad) was used to quantitatively analyse the fluorescent bands to determine the relative amounts of the substrate (NBD-PE) and product (NBD-diacylglycerol/NBD-DAG) at each time point of the enzymatic reaction.

Analysis of EptA Secondary Structure

Pure EptA at 20 mg/mL was diluted to a final concentration at 0.1 mg/mL in various CD test buffers to a final volume of 1 mL. The CD test buffers were 20 mM sodium phosphate pH 7.0 with varied concentrations of DDM (0 - 450 µM) with and without 1 mM DOC. For each mixture, the exact protein

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concentration in the CD test buffer was determined by measuring the absorbance at 280 nm. The purified protein was placed in a quartz cuvette with a 1 mm path length, and the far-UV spectra were measured using a CD spectrophotometer (Jasco J 720) at 20 °C. Data was collected every 1 nm with 1 nm bandwidth in the wavelength range of 190 – 260 nm, using an integration time of 5 seconds per step. The spectra underwent buffer-baseline correction, and the measurement was conducted in triplicate.

Analyses of the CD spectra was performed using BeStSel. (Micsonai *et al.*, 2022). BeStSel is a free webserver analysis tool, which uses empirical algorithms to determine a protein's secondary structure from CD spectroscopic data in comparison to available reference datasets. Eight secondary structural components of protein based on the Dictionary of Secondary Structure of Proteins (DSSP) (Cooley *et al.*, 2010; Kabsch & Sander, 1983) are defined in BeStSel, which made this method higher in accuracy compared to other methods currently available such as SELCON3 which distinguishes six secondary structural components of protein (Sreerama *et al*., 1999; Micsonai *et al*., 2015). The basis of secondary structural components utilised by SELCON3 were also used by CONTIN (Provencher & Gloeckner, 1981) and CDSSTR (Sreerama & Woody, 2000).

Interaction of EptA and LOS *in vitro*

The interaction of EptA and LOS *in vitro* was investigated by NAGE according to Hardy *et al.*, (2016) with modifications. Mixtures containing varied concentrations of purified EptA (1.6 µM – 40 µM) and varied concentrations of LOS (20.8 µM – 520 µM) dissolved in activity assay buffer (50 mM HEPES pH 7.0, 100 mM NaCl, 1 mM DOC) were mixed and incubated at 37 °C for 1 hour. Samples (36 µL) were then mixed with 4 µL 10X gel loading buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) and loaded onto an 0.8 % (w/v) agarose gel. Electrophoresis was performed at 80 V for 1 hour or until the tracking dye reached the bottom of the gel. The gel was then rinsed twice in ddH_2O, soaked in 0.2 M imidazole with gentle rocking for 20 mins, rinsed twice in ddH_2O, soaked in 0.2 M $ZnSO_4$ with gentle agitation until transparent bands were developed, then finally rinsed with ddH_2O several times. The gel was imaged using a Chemidoc MP imaging system (Bio-Rad) with a colorimetric protocol. Following the imaging, the gel was soaked in 100 mM EDTA for 15 mins with gentle agitation, then soaked in ddH_2O with gentle agitation for 15 minutes three times, stained with Coomassie blue for at least 120 minutes and destained in destaining solution overnight. The gel was rinsed with ddH_2O several times. The gel was imaged using a Chemidoc MP imaging system (Bio-Rad) with a colorimetric protocol.

RESULTS AND DISCUSSION

EptA Expression and Purification

A single colony of transformed competent cell was used to inoculate 500 mL LB pre-culture with the appropriate antibiotic. After overnight incubation the pre-culture OD_{600} was measured to be 1.72. The pre-culture was then used to inoculate six flasks each containing 500 mL TB as the main culture with the OD_{600} adjusted to 0.1 at the start of the incubation. After four hours of incubation, the OD_{600} was measured to be 1.5. Due to the presence of the T7 lac operon in the plasmid vector, overexpression of EptA is induced by addition of IPTG to the main cultures. From 3 L culture 72 gr cell pellet was harvested.

EptA is a membrane protein, therefore the bacterial cell must be lysed in order to isolate the protein. Cell lysis using high pressure homogenization was chosen as the lysis method because the high efficiency of this method (Islam *et al.*, 2017). The only drawback of this method is the generated heat which could disrupt the protein. However, this problem could be easily mitigated by ensuring the system is cooled through the utilization of an ice bath.

Following the cell lysis, two step centrifugation was carried out. In the first centrifugation step, cell debris were pelleted while the protein could be collected from in the supernatant. The second centrifugation was carried out using an ultracentrifuge to pellet the membrane component of the cells, into which EptA is embedded. Prior to the purification process, EptA is solubilized from the membrane by using DDM detergent.

EptA can be purified from other proteins in the mixture by immobilized metal ion affinity chromatography (IMAC) using a Ni^{2+} NTA column due to the presence of the hexa-histidine tag at the C-terminal end of the protein. Size exclusion chromatography (SEC) was employed to obtain highly pure EptA as well as to perform buffer exchange into EptA storage buffer. The storage buffer has been previously optimized to ensure the solubility and stability of EptA (Anandan, 2018). After purification approximately 12 mg of protein was obtained from 72 gr cell pellet. The protein was confirmed to be > 95% pure based on SDS PAGE analysis as shown in Figure 1, and therefore was considered suitable for further analysis.

EptA Enzymatic Activity Assay

Anandan *et al.*, 2017 developed an enzymatic assay which demonstrates the cleavage of the pEtN moiety, by EptA, from a fluorescently labelled substrate, NBD-PE. This reaction leads to the release of NBD-DAG as a side-product (Anandan *et al.*, 2017). To separate NBD-PE and NBD-DAG in the reaction mixture, thin layer chromatography can be used. The corresponding fluorescent intensities can be measured and quantitated. With this method,

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EptA's activity in the presence of 1 mM – 12 mM DOC can be assayed.

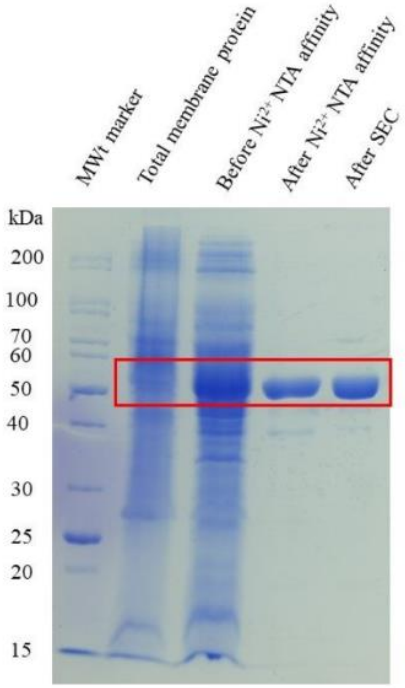

Figure 1. SDS PAGE analysis of EptA through various steps in the purification procedure. The band corresponding to EptA is marked by the red box.

Furthermore, this assay aims to identify the suitable DOC concentration for an *in vitro* assay of EptA in the presence of LOS. As a detergent, DOC is necessary to prevent LOS aggregation for *in vitro* assays. However too much DOC might interfere with EptA's activity or even cause denaturation of the protein. Purified EptA is stored in buffer containing 50 mM HEPES pH 7.0, 150 mM NaCl and 450 µM DDM. The buffer composition has been previously optimized by Anandan (2018) to maintain EptA's stability. Therefore, 1 mM – 12 mM DOC was added to EptA's storage buffer in the initial assay. For the activity assay, measurements were taken for a total of 30 minutes at 25 °C; samples were taken every 5 minutes, and the reaction was halted by the addition of methanol, then applied to the TLC plate for analysis. The results are presented in Figure 2.

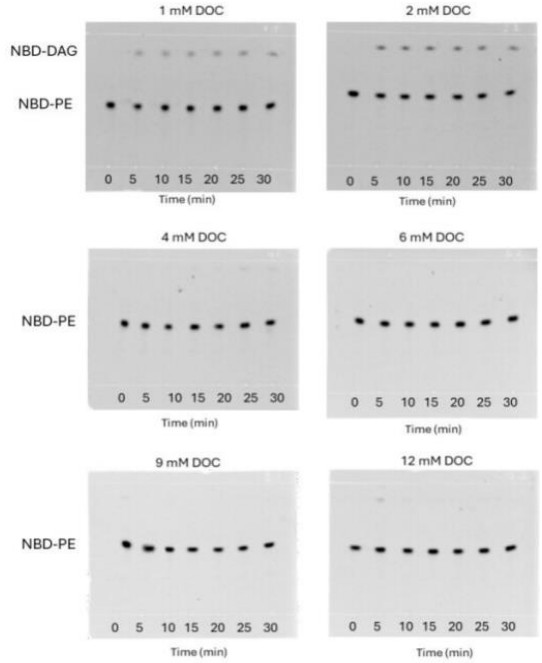

Figure 2. TLC based analysis of the EptA enzymatic activity assay in the presence of various concentrations of DOC. The fluorescently labeled substrate, NBD-PE, was used for visualization on the TLC plates and enzymatic activity assessed by the production of NBD-DAG.

The presence of DOC at high concentrations (> 2 mM) disturbs the activity of EptA as indicated by the absence of an NBD-DAG spot on the TLC plate. In the presence of 4 mM DOC, the reaction was significantly slower, and no EptA activity was observed in the presence of > 6 mM DOC. The critical micelle concentration of DOC is 2-6 mM at 20-25 °C. The EptA storage buffer already contains DDM at 3 times its critical micelle concentration (CMC), general concentration used in solubilization and purification of integral

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membrane proteins (IMP). Therefore, the presence of additional detergent at a concentration above its CMC may lower the likelihood for EptA to encounter its substrate. Hence, to avoid excess detergent in the buffer, 1 mM DOC concentration was chosen for further screening.

To further investigate the most suitable concentration of detergent present in the activity assay buffer for EptA, additional screening was carried out with different concentrations of DDM. Buffers containing 0-450 µM DDM (0-3x CMC) with and without 1 mM DOC were tested. The reaction was also performed for 30 minutes at 25°C and the amount of the reaction product was quantitated by measuring the intensities of the band corresponding to NBD-DAG on the TLC plate (Figure 3). The results indicate the lowest EptA activity in the buffer containing 37.5 µM DDM and 1 mM DOC whereas the highest EptA activity was observed in buffer containing 1 mM DOC in the absence of any DDM.

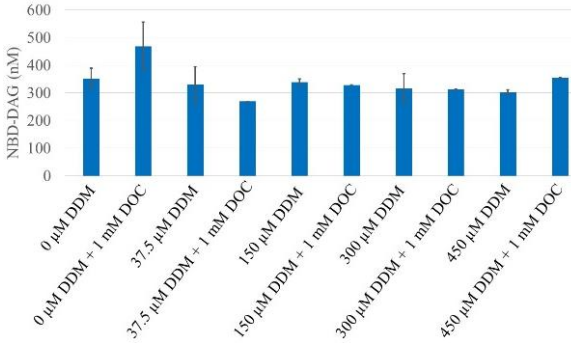

Figure 3. The EptA enzymatic assay as measured through the production of NBD-DAG in the presence of various concentrations of DDM and DOC. Each bar corresponds to the average from duplicate measurements with standard deviations shown.

Analysis of EptA Secondary Structure

The results of the CD analyses are shown in Figure 4 and Table 1. BeStSel was used to estimate the secondary structures present in the protein. The differences in percentage of α-helical, β-strand, turns content, and others (disordered) structures as a function of different buffers were compared.

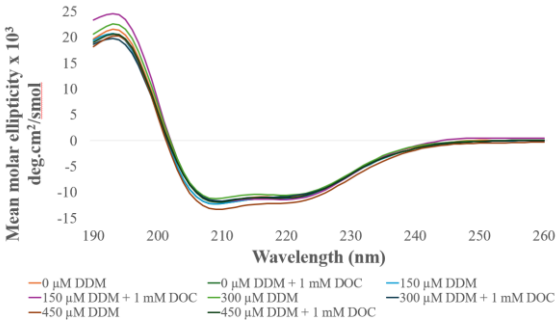

Figure 4. CD Spectra analyses of the predicted binding site residue of EptA in various types of buffers. The CD spectra obtained from seven different buffers were compared to 3x CMC (450 µM)

From the CD spectral analyses, no major differences were observed between each treatment when compared to the control buffer where the DDM concentration in the buffer was at 3x CMC. The abundance of specific secondary structural elements, which is observable through the intensity of CD spectra at certain wavelength, were highly similar.

Despite the overall similarity of the CD spectra, further quantitative analysis must be carried out to help identify any differences in the secondary structure. Detailed comparisons are shown in Table 1. The secondary structure content prediction using BeStSel has shown a good fit as indicated by normal root mean squared

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deviation (NRMSD) of 0.05 or less for all data (Miles *et al.*, 2021). NRMSD is a correspondence value between the experimental data and the back-calculated spectrum produced from the derived secondary structures. Thus, low (< 0.1) NRMSD value suggests that the best solution for deconvolution data analysis have resulted in an accurate reflection of the secondary structure. In agreement with the CD spectral data, the overall secondary structure content of EptA in various buffers are very similar.The alpha helical content of the protein on average differs by approximately 1% between different buffer compositions, with the highest difference (2-3%) observed when the protein buffer contains 150 µM DDM (equal to 1x CMC) and 1 mM DOC. The differences in the β-strand content of the protein are very subtle as well, which are on average less than 5% except for the content of antiparallel β-strands in control buffer containing 450 µM DDM (3x CMC) which differed by approximately 9% from 0 µM DDM + 1 µM DOC, 150 µM DDM + 1 µM DOC, and 300 µM DDM. Lastly, the content of other (disordered) structures also differed by 7% – 13% in buffer containing 150 µM DDM (1x CMC) + 1 mM DOC when compared to all other samples. However, these differences are still within the acceptable range because up to 10% differences were also observed between calculated secondary structure content from CD data and the crystal structures of caletexin, antithrombin, Bj-xtrIT and hemerythrin (Miles *et al.*, 2021).

Table 1. Analysis of the secondary structure elements of EptA in eight buffers.

Buffer	Estimated Secondary Structure Content (%)						NRMSD
	Regular α helix	Distorted α helix	Anti-parallel β strand	Parallel β strand	Turns	Others	
0 µM DDM	15.3	11.0	16.6	7.5	11.2	38.4	0.05
0 µM DDM + 1 µM DOC	14.3	10.5	19.4	6.3	11.8	37.7	0.01
150 µM DDM	14.2	11.3	15.4	6.6	11.6	40.9	0.05
150 µM DDM + 1 µM DOC	17.1	10.3	19.2	4.0	11.6	30.8	0.01
300 µM DDM	15.5	10.3	19.5	4.6	11.8	38.3	0.01
300 µM DDM + 1 µM DOC	14.8	10.2	16.2	7.9	11.8	39.1	0.01
450 µM DDM	14.8	11.8	10.5	7.0	12.1	43.7	0.01
450 µM DDM + 1 µM DOC	14.8	11.1	16.2	7.1	11.7	39.0	0.005

Interaction of EptA and LOS *in vitro*

It has been proposed that EptA follows a ping-pong mechanism (Anandan *et al.*, 2017). EptA has two substrates: PE as the donor substrate and LOS as the receiver substrate. It has been proposed that pEtN is transferred from PE to Thr280 on the enzyme as the first reaction in the ping-pong mechanism producing a bound enzyme-pEtN intermediate and resulting in the release of

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DAG. The TLC based activity assay has shown that EptA is able to perform this first part of enzymatic reaction in the absence of the acceptor substrate. However, the enzyme's *in vitro* interaction with the acceptor substrate in the absence of the donor substrate has not yet been tested.

Rodríguez and Hardy (2015) have developed a simple technique to detect and analyse the interaction of protein and LPS using NAGE. Visualization of LPS in the agarose gel was carried out by equilibrating the gel in an imidazole solution followed by immersion in a $ZnSO_4$ solution. The overall negative charge of the LPS molecules electrostatically attracts zinc ions to diffuse into the agarose matrix. The zinc ions and the LPS will form a complex resulting in a transparent zone in the gel (observed as a dark zone in the image due to the fact that the gel was imaged on a dark background), while the zinc ions around the LPS will react with imidazole resulting in an imidazolate precipitate which stains the background. After the image of the gel was taken, the gel was immersed in an EDTA solution to dissolve the imidazolate precipitate, rinsed with ddH_2O to remove the EDTA, then stained with Coomasie blue to visualize the protein. The protein bands were then compared with the LPS bands. Through employing this method, the interaction of EptA and LOS was analysed. In essence, LOS is LPS without O-antigen. O-antigen is commonly found in enteric bacteria. Bacteria that reside on the respiratory and genital mucosal surfaces, such as pathogenic *Neisseria*, lack the O-antigen (Preston *et al.*, 1996). The result of EptA and LOS interaction analysed by NAGE was shown in Figure 5.

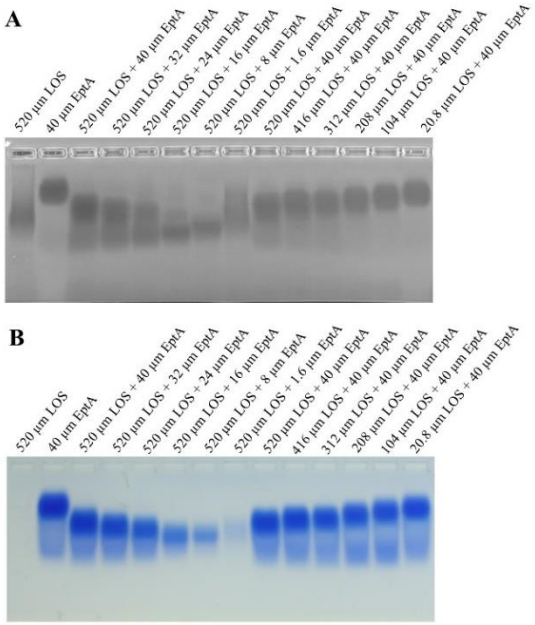

Figure 5. NAGE analysis of EptA and LOS. The gel was stained with (A) imidazole and zinc, (B) coomasie blue.

Using this method, changes in the electrophoretic movement of mixtures of EptA and LOS in various ratios were observed. EptA by itself as well as LOS by itself were used as negative controls. Figure 5A shows the EptA band as a transparent zone in the gel similar in position to the LOS band. This result differs from that observed by Rodríguez and Hardy (2015). EptA is a metallo-enzyme which contains a zinc ion bound to the protein. Therefore, similar to the LOS-zinc complex which appears as a transparent zone, EptA is observed as transparent zone on the gel as well. In

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contrast, Rodríguez and Hardy used lysozyme (contains no metal) in their experiment, hence they observed an opaque zone.

Similar to Rodríguez and Hardy's observation of the LPS sample, the LOS band is also observed as a relatively broad band due to the high polydisperse molecular mass of LOS (Figure 5A, first lane). LOS is highly polydisperse due to the phase variation mechanism which influence the expression and/or function of various glycosyltransferases involved in the LOS biosynthesis (Bartley & Kahler, 2014).

As shown in Figure 5, the presence of EptA changes the electrophoretic movement of the LOS. When the concentration of LOS was high (520 µM) and the concentration of EptA was high as well (40 µM), the EptA band moved further from the well and the pattern of the clear zone became similar to the pattern of EptA band. As the EptA concentration became lower while the LOS concentration remained constant, the clear zone became more compact. On the other hand, when the EptA concentration was constantly high while the LOS concentration became lower, the pattern of the band became more similar to the band corresponding to EptA. Therefore, it can be concluded that *in vitro* EptA is able to interact with the second (acceptor) substrate even in the absence of the first (donor) substrate.

The NAGE analysis also indicates that 1 molecule of EptA could interact with up to 65 molecules of LOS. This is shown in Figure 5, where, compared to the lane containing only 520 µM LOS, the band of the lane containing a mixture of 520 µM LOS and 8 µM EptA (65 LOS:1 EptA) was observed as a compact zone (Figure 5A). After Coomasie staining (Figure 5B), the corresponding zone was stained blue which suggest that EptA was detected on the same zone as the transparent zone corresponding to LOS. Meanwhile the transparent zone on the lane containing a mixture of 520 µM LOS and 1.6 µM EptA (325 LOS:1 EptA) was observed as a broad zone similar to the zone of the lane containing only LOS.

CONCLUSION

Highly pure EptA in sufficient amount was successfully obtained through recombinant expression using *E. coli* BL21(DE3) pLysS as the host cell, followed by Ni^{2+} NTA affinity and size exclusion chromatography. The enzymatic activity of EptA was significantly disturbed in the presence of > 2mM DOC. The enzymatic activity of EptA in various DDM concentration (0 – 450 µM) with and without 1 mM DOC was maintained, with highest enzymatic activity observed when only 1 mM DOC was present in the buffer. Supporting the enzymatic activity assay, the secondary structure of EptA in the same buffer utilized in the activity assay was also maintained. Lastly, EptA interaction with its acceptor

substrate (LOS) in the absence of its donor substrate (PE) was observed *in vitro*. Therefore, further *in vitro* studies incorporating both PE and LOS with supplementation of up to 1 mM DOC to prevent LOS aggregation could be carried out to enable a more detailed study of the EptA enzymatic mechanism, and with the long-term final goal of developing inhibitors against EptA.

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