

## **PROTEIN Cj0391c INTERACTION WITH LIPID BILAYER MEMBRANE IDENTIFICATION USING SMALL ANGLE X-RAY SCATTERING (SAXS), WAXS AND DYNAMIC LIGHT SCATTERING (DLS)**

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### **ABSTRACT**

*Compylobacter jejuni* merupakan salah satu bakteri utama penyebab gastroenteritis akut di seluruh dunia dengan tingkat morbiditas dan mortalitas yang tinggi, terutama di negara-negara berkembang. Oleh karena itu sangat penting untuk mulai mengidentifikasi faktor virulensi dari *C. jejuni*, salah satunya adalah dengan mengidentifikasi protein yang dikeluarkan dari patogen ini. Cj0391c merupakan salah satu protein yang telah mulai dipelajari dan dapat teridentifikasi sebagai  $\alpha$ -Helical pore forming protein (PFP) dan menyebabkan apoptosis pada sel makrofag ayam. Karena diduga bahwa mekanisme apoptosis mungkin mirip dengan mekanisme cytotoxicity dari  $\alpha$ -Helical yang lain maka dalam penelitian ini protein Cj0391c ini kemudian diekspresikan, dimurnikan dan dianalisis untuk melihat interaksinya dengan membran lipid bilayer. Tujuan penelitian utama penelitian ini adalah untuk menentukan apakah Cj0391c bisa menembus dan mengganggu membran sebagai  $\alpha$ -PFP atau tidak. Potensial interaksi protein dan membran dievaluasi menggunakan Small Angle X-Ray Scattering (SAXS), Wide Angle X-Ray Scattering (WAXS), dan Dynamic Light Scattering (DLS). Meskipun hasil tidak menunjukkan indikasi penyisipan protein ke dalam membran, namun penelitian ini menjadi rujukan metode ekspresi dan pemurnian yang optimal untuk Cj0391c dan gambaran bentuk struktur Cj0391c berdasarkan hasil analisis SAXS.

**Keywords:** *Campylobacter jejuni*, Cj0391c, pore forming protein, apoptosis,  $\alpha$ -PFP, lipid bilayer membrane, SAXS, WAXS, DLS.

### **INTRODUCTION**

*Compylobacter jejuni* was identified as one of majority bacterial causes of acute gastroenteritis around the world, having high morbidity and mortality rates, especially in developing countries (Walker, 2005). In such countries, diarrheal illnesses caused by *Campylobacter* species primarily affect children, while in western countries, the incidence of such infection peaks during infancy and again during early adulthood.

Most of the infections come from the consumption and handling of infected poultry, and the broiler chicken is the main potential reservoir of *Campylobacter* strains that are pathogenic to humans; meat from this source, contaminated with *C. jejuni* is reported to be related to about 40% of the cases of human

campylobacteriosis (EFSA, 2010). Horizontal transmission from the environment is the main route of broiler chickens infections (Jacobs-Reilsma, et al., 1995).

Given the ineffectiveness of other control programs for these pathogens, and the high worldwide impact, the successful development of an anti-Campylobacteriosis vaccine for chickens would be an effective way to reduce the number of cases (Bhavsar & Kapadnis, 2007). Although studies of developing anti-campylobacter vaccine have been conducted for a decade, the effective vaccine of Campylobacter is not found yet. The identification of virulence mechanisms associated with *C. jejuni* is an important area of study, as this may be potential targets for a future vaccine. Some of the proteins secreted by *C. jejuni* could be potential virulence factors to be targeted by a vaccine or treatment for Campylobacter (Anderluh & Lakey, 2010).

An example of a secreted protein of *C. jejuni* that has been studied recently is Cj0391c. The potential involvement of this protein was identified by Mu (2014), it was found that inoculation of the recombinant protein of Cj0391c in chicken macrophage cell resulted in apoptosis of these macrophage cells. It is suggested that the importance of this effect may be that this prevents chicken macrophage cells from being able to phagocytose *C. jejuni* and present antigen to T cells. Bioinformatics analyses from the same study found that this protein could be an e-g helical pore forming protein (PFP) because the C-terminus of Cj0391c aligned well with dermcidin, which is an A-helical toxin. Thus, based on this study it was thought that the mechanism of apoptosis might be similar to the cytotoxicity mechanisms of other n-helical toxins (Bischofberger et al., 2012). For this to be so, it is required that the protein first be able to enter the membrane before being able to disrupt it through the formation of pores that lead to apoptosis. The major aim of this study is to determine whether or not Cj0391c could penetrate and disrupt the membrane as an o-PFT, thus testing the hypothesis that this could be the mechanism by which Cj0391c triggers apoptosis. The potential interaction of protein and membrane was evaluated using Small Angle X-Ray Scattering (SAXS) and Dynamic light Scattering (DLS) (Feigin & Svergun, 1987). Although the results did not show that this occurred, insight was obtained into optimal expression of Cj0391c and the likely shape of this protein.

The aims of this project are to identify how the Cj0391c protein interacts with the lipid bilayer membrane and also to characterize its shape and size.

Methods used to accomplish this aim include Small Angle X-Ray Scattering (SAXS), Wide Angel X-Ray Scattering (WAXS) and Dynamic Light Scattering (DLS). The results of this experiment will help for greater understanding of the pathogenicity of *C. jejuni* specifically on the mechanisms of pathogenic and also the virulence factors involved. The ultimate goal of this improved understanding is the plan and production of vaccines aimed at reducing the incidence of campylobacter infection by immunizing poultry against *C. jejuni*.

## **METHODS**

### **Materials and Methods**

#### ***Small Angle X-Ray Scattering (SAXS)***

The maximum of 100  $\mu$ l of PBS was added to the capillary tube filling it to 1 cm from the top of the tube. The tube was then sealed using glue that had previously been prepared. The same procedure was done for protein Cj0391c sample. These samples were then ready to be measured by SAXS measurement. SAXS measurements were performed on a Bruker Microcalix instrument, using 50 W Cu K radiation at a wavelength of 1.54  $\text{\AA}$ . A Pilatus 100 k detector was used to detect scattered light. The scattering and transmission measurements were carried out over a 2 hours exposure time.

After the measurement, data analysis including normalization, primary beam masking, and background subtraction was carried out in Fil-2D. Smoothing methods were not used, as normalization did not have a significant effect on the fit.

#### ***Dynamic Light Scattering (DLS)***

DLS was performed on an ALV-5022F spectrometer, using a vertically polarized Helium Neon laser (wavelength 633 nm). The scattering angle was 90°, and measurements were conducted at 20°C. The capillary tube of sample used in this experiment was the same capillary used for SAXS.

#### ***Cj0391c Protein Interaction with Membrane***

Protein Cj0391c was yielded from previous expression and purification experiment, where it was found that Terrific Broth as the optimal medium for Cj0391c expression as high concentration of Cj0391c protein could be expressed using this medium. The lipid bilayer membrane used in this experiment is

Dioleoyl-sn-glycero-3-phosphocholine (DOPC), which has been prepared before and it is naturally occurring phospholipid which is synthesized under cGMP and soluble in most organic solvents. It is known as a synthetic phosphocholine that forms liposomes in aqueous media alone or in combination with other lipids (Tristram-Nagle, Petrache and Nagle, 1998).

The SAXS measurement of lipid membrane thickness was performed according to the same procedure as SAXS measurement for identification of the size and shape of the protein. The differences were in the preparation of the sample before being placed into the capillary tube and the analysis of results. The samples were prepared by mixing the dioleoyl-sft-glycero-3- phosphatidylcoline (DOPC) with PBS in one 1.5 mL eppendorf tube and DOPC with protein Cj0391c) c in another tube. The procedure for mixing these is described as follows. The eppendorf tube was weighed then the DOPC was the loaded into the tube. The tube was then weighed again to get the weight of the HOPC by subtracting this weight from the previous weight. The sample was then loaded into the tube and weighed again, aiming for the same weight of the sample as the DOPC, to give a 1:1 ratio of DOPC: sample. The tube was then centrifuged in 4000rpm for 5 minutes to get the well mixed of DOPC and the sample. The sample preparation procedure for both DOPC and protein Cj0391c followed the same procedure. The mix of DOPC+PBS and DOPC+Cj0391c were then loaded to the capillary tube. The capillary tube was then sealed with glue. The analysis also differs from the SAXS analysis before, where 2 parameters were yielded by this measurement, SAXS and WAXS analysis.

## **DISCUSSION**

### *Protein CJ0391C Interaction with Lipid Bilayer Membrane*

The SAXS measurement and analysis resulted in a graph that compares the distances between the lipid membrane of DOPC-PBS, DOPC-protein Cj0391c in room temperature (20°C), and DOPC-protein Cj0391c in 37°C as can be seen in Figure 1.

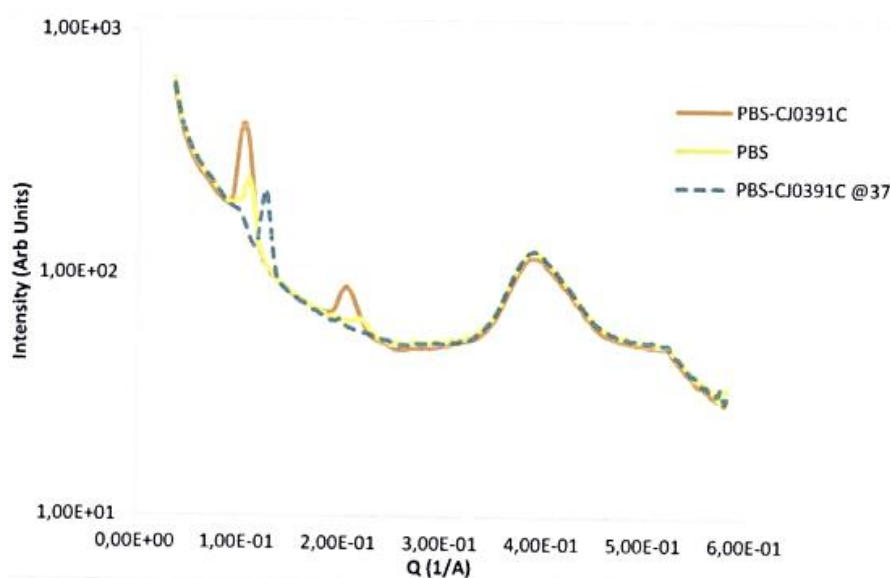


Figure 1. SAXS Result of the Cj0391c in DOPC/PBS analysis under two different temperature conditions (room temperature (20°C) and 37°C).

The graph of SAXS analysis in Figure 1 indicates the repeat spacing of the DOPC (as can be seen in Table 1) as a result of its interaction with PBS and protein Cj0391c which were performed in two different temperature conditions.

Table 1. Repeat spacing of the DOPC from three different samples resulted from SAXS analysis

Samples	Repeat Spacing
DOPC in PBS	57.16
DOPC in PBS with CJ0391 C at 20°C	60.44
DOPC in PBS with CJ0391 C at 37°C	50.07

The result of SAXS analysis for the interaction of protein with DOPC clearly shows that the presence of the protein has an effect of increasing the thickness of the lipid bilayer membrane (Lipfert & Doniach, 2007). The next experiment of increasing the temperature from 20°C to 37°C decreased the repeat spacing by about 20% (from 60.44 Å to 50.07 Å). These results clearly indicate that the presence of Cj0391c and increasing temperature affect the structure of the lipid bilayer membrane.

The interaction of the protein with the DOPC also was analyzed with WAXS, and resulted in a graph that compared the distances between the lipid chains in the bilayers of DOPC-PBS, DOPC-protein Cj0391c in room temperature (20°C), and DOPC-protein Cj0391 c in 37°C as can be seen in Figure 2.

The graph of WAXS analysis in Figure 2 indicates the chain spacing of the DOPC (as can be seen in Table 2) in the presence of PBD and protein Cj0391c under two different temperature conditions.

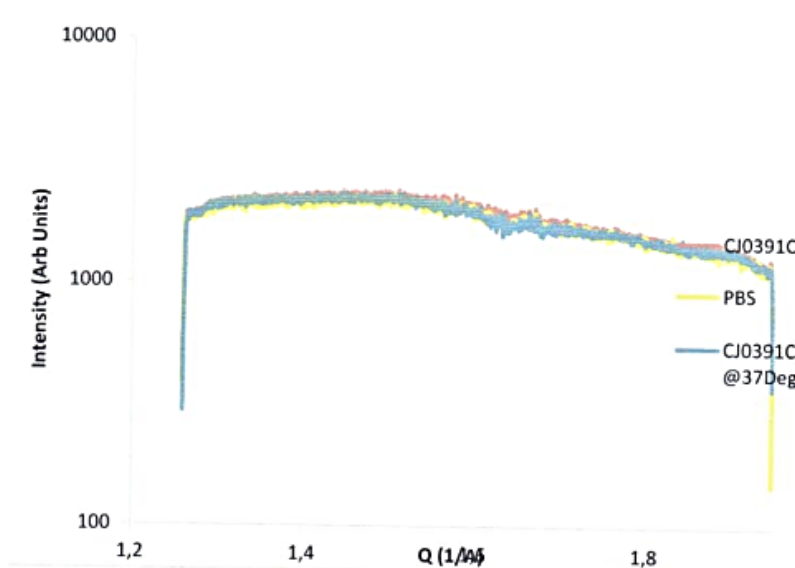


Figure 2. WAXS Results of the Cj0391c in DOPC/PBS analysis at two different temperature conditions (room temperature (20°C) and 37°C).

Table 2. Chain spacing of the DOPC mm three different samples resulting from SAXS analysis.

Samples	Repeat Spacing
DOPC in PBS	4.36
DOPC in PBS with CJ0391 C at 20°C	4.35
DOPC in PBS with CJ0391 C at 37°C	4.38

In contrast to the repeat spacing, the present of this protein in this experiment did not show an effect on the chain spacing of the lipid bilayer membrane. Increasing the temperature to 37°C also did not have an effect on the chain spacing. This result clearly indicates that in this experiment protein Cj0391c did not insert into the membrane because the penetration of the proteins into the membranes would have affected the membrane by increasing the chain spacing (Gilbert et al., 2014).

TetC, which had been planned to be used as negative control for membrane insertion, had been expressed and purified with a high protein concentration produced. The protein obtained was suitable to be used for this experiment as a control, unfortunately, the experiments of TetC interaction with lipid bilayer membrane could not be undertaken due to time limitations.

As described by Thalhammer et al. (2014), using small-angle x-ray scattering (SAXS) and wide-angle x-ray scattering (WAXS) for characterizing COR 15 protein membrane systems in water or 50% (v/v) glycerol resulted in  $d_{\text{repeat}} = d_{\text{lipid}} + d_{\text{water}}$  (as described in Figure 3). This figure shows a schematic drawing interpretation of what is measured by WAXS. The change in protein interchain distance shown by SAXS analysis can identify the penetration of the protein into the membrane.

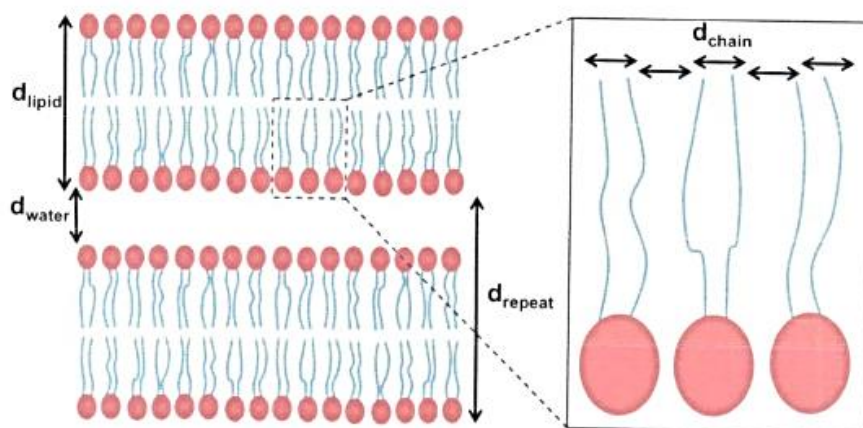


Figure 3. Schematic of a lipid bilayer membrane

The left hand figure shows two neighbouring bilayers. The primary repeat spacing measured using SAXS gives  $d_{\text{repeat}} = d_{\text{lipid}} + d_{\text{water}}$ . The broad peak observed in WAXS gives  $d_{\text{chain}}$  the average distance between lipid chains in two dimensions (represented schematically on the right). As the lipids are in the fluid phase, the chains are mobile and not regularly spaced, resulting in a broad peak. Subtle changes in packing leads to changes in the position of the peak maximum, as well as the shape of the peak (Figure and legend from Thalhammer et al., 2014).

## CONCLUSION

Based on the SAXS and DNS analysis in this study, it can be concluded that regarding the interaction of the protein with membrane, it was found that the presence of Cj0391c in 20°C increased the lipid bilayer thickness, while increasing the temperature by 17°C decreased the repeat spacing. In contrast, the presence of Cj0391c did not have a significant effect on the membrane chain spacing, which indicates that there is no evidence of insertion of the protein into the membrane based on this project. Based on these findings, a variety of research directions could potentially be pursued to better understand the role of protein Cj0391c as a virulence factor. One suggestion is comparing the structure of the protein based on

SAXS and DLS analysis with the structure of Bioinformatics prediction has been studied before.

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