

## OPTIMAL PROTEIN Cj0391c EXPRESSION AND PURIFICATION METHODS FOR THE PROTEIN IDENTIFICATION

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### ABSTRAK

*Identifikasi mekanisme virulensi yang terkait dengan Campylobacter jejuni dalam bidang yang penting karena merupakan target potensial untuk vaksin masa depan. Contoh protein yang disekresikan oleh C. jejuni yang telah dipelajari baru-baru ini adalah Cj0391c. Analisis bioinformatika menemukan bahwa protein ini bisa menjadi helical pore forming protein (PFP). Untuk mengetahui lebih lanjut mekanisme virulensi dari protein ini maka perlu dilakukan identifikasi lebih mendalam tentang protein. Oleh karena itu penelitian ini bertujuan untuk mengidentifikasi metode ekspresi dan purifikasi yang optimal untuk menghasilkan protein Cj0391c yang murni dan cukup, yaitu 1 mg/ml Cj0391c murni. Pemurnian dilakukan dengan Immobilized metal Affinity Chromatography (IMAC) kolom yang digunakan untuk memulihkan protein rekombinan. SDS-PAGE dan Western blotting digunakan sebagai metode untuk menentukan kondisi optimum untuk elusi protein rekombinan Cj0391c. Hasil dari ekspresi dan pemurnian pada percobaan ini menyimpulkan bahwa konsentrasi optimum Imidazole dalam buffer pencuci untuk menghasilkan konsentrasi tinggi protein Cj0391c murni adalah kombinasi dari langkah awal 20 mM buffer pencuci Imidazole diikuti dengan dua kali mencuci menggunakan 40 mM Imidazole. Konsentrasi akhir 1 mg/mL Cj0391c kemudian siap untuk digunakan untuk Small-angle X-ray scattering (SAXS) dan Dynamic light scattering (DLS).*

*Key words: Campylobacter jejuni, protein Cj0391c, IMAC, SDS-PAGE, Western blotting.*

### INTRODUCTION

Campylobacteriosis, one of the main frequent forms of acute bacterial gastroenteritis in human which has become a serious worldwide problem of public health, is mostly reported to be initiated by two species *Campylobacter jejuni* and *Campylobacter coli* which are zoonotic (Coker *et al.*, 2002). These bacterial are commensals of a range of domestic, farmed and also wild animals. Consumption of undercooked chicken, offal, untreated dairy product and water that is untreated (Kapperud *et al.*, 1992) are some of the human infection risk factors of these pathogens.

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

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area of study, as these 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*. Many studies have been conducted to identify the secreted protein that may give *C. jejuni* the ability to interact with and evade the immune system of chicken (Bhavsar and Kapadnis, 2007). Hypothetical protein Cj1631c and Cj0391c together with 26 other genes were found expressed highly in biofilm-grown cells that involved with biofilm-grown, response of stress, complex and adhesion of the flagellar motility (Kalmokoff *et al.*, 2006).

Testing of cell proliferation and apoptosis induction was done by test of Cj0391c recombinant protein against chicken macrophage cells in a recent study by Mu (2014). This study showed that recombinant Cj0391c protein that was inoculated with chicken macrophage cells induced apoptosis for these cells, which provides a clear mechanism by which this protein could have a significant function in decreasing the immune response of the chicken. Cj0391c also was modelled computationally, with molecular simulations performed under different conditions. The result suggested the possibility that protein Cj0391c could be an alpha-helical pore-forming toxin ( $\alpha$ -PFP), on the basis of its similarity to another alpha-helical pore-forming toxin, dermcidin.

Pore-forming proteins (PFPs) are a kind of protein produced by many organisms secreted as water soluble proteins, but which can be transformed to be a membrane protein or translocate across biological membranes once their targets have been leached. The purpose of producing PFPs varies between organisms. In bacteria they are produced either to kill other bacteria, or have an effect on their hosts of promoting colonization and spread, in the case of pathogenic bacteria (Lakey *et al.*, 1994). The major aim of this is to identify the optimal expression and purification methods for Cj0391c so they can be used for the protein Cj0391c shape and size identification.

The construct of the PREST-A/Cj0391c in *E. coli* DH5a was attained from the earlier study. Extraction and sequencing of the plasmid DNA from the cells was carried out, confirming the sequence of the plasmid and that the protein-coding sequence was in the same reading frame as the his-tag. Following this, the plasmid was transfected into the *E. coli* BL21 (DE3) pLysS expression system. Finally, recombinant protein Cj0391c from pREST-A was expressed, purified and

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analysed. It has been determined by the previous study that 5 hours is the optimum expression time after Isopropyl- $\beta$ -D-galactopyranoside (IPTG) induction.

## METHODS

### *Bacterial Strains and Culture Methods*

Throughout this study, pRSET-A (Invitrogen Corporation, USA) was used as a plasmid containing all recombinant constructs within *E.coli*BL21(DE3) (Invitrogen Corporation, USA). The growth media used were LB Agar or LB broth containing ampicillin prepared before. For LB broth orbital shaking at 200 revolutions per min (rpm) was used to maintain oxygenation. Following growth at 37°C, for short term storage *E.coli* were kept at 4°C, or for longer term, at -8°C in a 30% glycerol solution.

### *Protein Expression and Cell Lysate Preparation*

For each induction using IPTG, a seed culture was freshly prepared the day before and incubated overnight. Cells were grown two days prior on LB agar from which a single colony was inoculated into 10mL of LB broth grown overnight from which, 2 mL were used as the seed culture for 200mL of LB broth, all with antibiotics as previously described. They were then grown to an optical density at 600nm of 0.4-0.7. Once this concentration was reached, 1 mM IPTG was added to induce protein expression for the optimally determined time of 5 hours.

50 mL Falcon tubes containing cells were centrifuged at 4700x G for 15 minutes then resuspended in 10-12 mL of protein lysis buffer to harvest cells. These were then placed on a plastic beaker filled with ice to be sonicated at amplitude of 35%, for three 15s pulse cycles with pauses of 30s. Whole cells were also stored at -80°C to be sonicated and purified in the future.

### *Immobilised Metal Affinity Chromatography (IMAC)*

#### *IMAC purification*

After being sonicated, the lysed cells were centrifuged in 50 mL Falcon tube at 4,700 x g for 15 min at 4°C. 0.2  $\mu$ m filter was then used to filter the supernatant before purification. All the soluble cell lysate containing expressed recombinant protein was then mixed with the sepharose in a pre-equilibrated IMAC column by putting some into the column then mixing, taking some, putting back to the supernatant then repeating all steps until the column was clean. The solution was

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then transferred to 50 mL Fallon tube and kept in 4°C horizontally for 1 hour (with manually shaking every 10 mins) to ensure optimal binding of the polyHis-tag to the nickel ions in the resin.

The column was then verticalised and left for 15 min to settle before collecting the crude eluent. 5mL washes of post-sample wash buffers with increasing imidazole concentrations were used to wash the column and the flow-through collected in 5mL fractions. The nickel- bound polyHistidine-tagged protein was then eluted with 5mL of EB containing 140mM imidazole, and the flow through collected in 10 mL fractions. 40 µl aliquots from each purification step were taken for analysis by SDS-PAGE.

#### *Protein Concentration and Buffer Exchange*

Concentration of the IMAC purified protein was carried out by centrifugation to remove non-specific proteins of a smaller molecular weight. EB was replaced with PBS by buffer exchange, following a wash step with 10mL of MQ water, 5 ml was added of elution buffer into to a difiltration cup that had been inserted into the Amicon Ultra- 15. Centrifugation continued for 45 minutes at a temperature of 4°C at 4,000x g. Elution buffer was run through the difiltration cup, leaving approximately 500 µt of solution remained. Following the addition of 10 mL of PBS centrifugation continued for up to 45 minutes, or until only 500 µl of protein solution (in PBS) remained. Two rounds of buffer exchange were carried out per sample. The final protein solution (in PBS) was stored in a 1.5 mL microfuge at -20°C.

#### *Bradford Assay and Spectrophotometer Measurement*

The Bradford method (Bradford, 1976) and Spectrophotometer Measurement were used to determine the concentration of protein. Protein standards were prepared using BSA containing between 0- 20 µg/100 µL of 0.15 M NaCl. 90 µt of 0.15 M NaCl was added to 10µl of sample to make 100 µL aliquots. 900 µL of Bradford reagent was added to the sample and each of the standards. Following mixing using a pipette, these were left for five minutes at room temperature. 200 µL from each sample or standard were added in duplicate to a 96-well microtitre plate. An iMark microplate absorbance reader read the absorbance at 600 nm. The concentration of sample protein was estimated by plotting the sample absorbance reading against a standard curve of the standards (µg) versus absorbance.

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Another method used to estimate the concentration of protein Cj0391c protein was Spectrophotometer absorbance at 280nm. In order to test the accuracy of the spectrophotometer value, amounts of the protein based on the calculated concentrations were run on a gel. The bands were compared to marker bands with a known amount of the protein. In this experiment, 1 µg and 5 µg of the protein were calculated based on the spectrophotometer value. These amounts were then loaded to the gel, and the bands were compared to the 1 µg marker band.

#### *SDS-PAGE with Coomassie Blue Stain*

1D SDS-PAGE with a discontinuous buffer system was used to separate proteins of different sizes. Wells were formed by inserting a 12-comb cast between the glass slides after setting the 12.5% resolving gel with a 4.5% stacking gel layered over the top. Assembly according to the manufacturer's (BioRad Laboratories, USA) instructions of the protein gel system was carried out and this was placed inside a gel tank with SDS-PAGE running buffer. Protein samples in 5x SDS sample loading buffer were heated to 100°C for five minutes, and loaded along with the Precision Plus Protein Standard for comparison. For gels contained the Coomassie stain, Unstained Precision PlusProtein standard was used instead. Wherever the gel was intended to be subject to immuno-blotting, a pre-stained standard, the Kaleidoscope protein standard was used.

After thirty minutes of electrophoresis at 60V and fifty minutes of electrophoresis at 180a, the glass plates were separated and the stacking gel removed. The resolving gel was covered by Coomassie gel stain in a square plastic container and left on a shaker for an hour or two, until bands could be visualized. The gel was then destained using destaining solution for three hours and this solution was stopped using distilled water.

#### *Immunotransfer and Immunoblotting*

The iBlot system was used for Western blotting to transfer the protein onto a nitrocellulose membrane from the gel. This was done according to the protocol provided by the manufacturer. Following the removal of any visible air bubbles from the gel and filter paper, the cassette was closed for seven minutes to carry out the transfer. Following this, the nitrocellulose membrane was retained for the immunoblotting.

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All incubation or wash steps of the immunoblotting process were carried out on a rotating shaker. The first step was to block the nitrocellulose mean for an hour in blocking buffer. Two wash steps of 15mL TBS for five minutes each were then carried out to remove the buffer. Following this, the membrane was incubated in the primary antibody and either left at room temperature for two hours, or refrigerated overnight at 4°C. Following another double wash step with TBS for five minutes each time, the secondary antibody, conjugated to horse radish peroxidase (HRP) was added, and incubated under the same set of conditions as the first antibody. A triple wash with 15mL TBS for five minutes was carried out before development of the membrane with 50 mL chloronaphtol substrate solution for up to thirty minutes in darkness, until the bands could be visualized. Distilled water was used to stop the reaction while bands could be seen, and the background was still light.

## **RESULTS AND DISCUSSION**

### *Protein Expression and Purification*

The aim of this phase of the study was to produce 1 mg/ml pure Cj0391c. It was indicated from data obtained in the previous pilot study that the optimum condition to express Cj0391c protein expression is to incubate in 1 mM IPTG at 37°C for five hours and the best 40mM imidazole is ideal for elution of the target protein. The expression and purification procedure had been modified four times in order to achieve pure protein at a sufficient concentration.

The very first run followed the procedure used (or previous research, which found that the pure protein could be achieved by a single wash step containing 20 mM imidazole (Mu, 2014). The function of imidazole in buffer is to elute tagged proteins bound to the Nickel ion attached to the surface of beads in the IMAC column. Imidazole is the side chain of histidine amino acid to which Nickel ions bind, so that added imidazole is in the column, competes against the 6-His-tag for nickel binding sites. This leads to the tagged protein being eluted from the column. Imidazole also reduces the level of nonspecific binding of the protein to the column (Chiang, Chen, & Chang, 2008).

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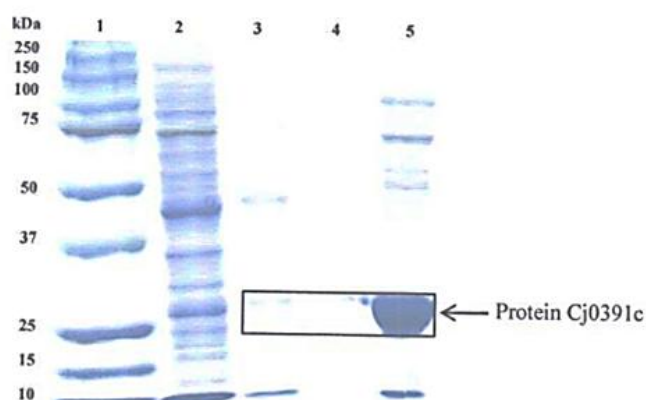


Figure 1. SDS-PAGE Analysis of Post IMAC purification, concentrated, and buffer exchange of Recombinant Protein Cj0391c

Lane 1: Unstained Standard Marker

Lane 2: Flow Through

Lane 3: IMAC-purified bound-protein Cj0391c from wash buffer (20 mM Imidazole)

Lane 4: IMAC-purified bound-protein Cj0391c from the elution buffer (140 mM Imidazole)

Lane 5: concentrated and buffer exchanged protein Cj0391c in PBS

It can be seen from the result in Figure 1 that many contaminants still appear in the concentrated protein (column 5) by using this wash buffer. Imidazole at low concentrations inhibits non-specific binding to the immobilized metal ion by other proteins, which leads to better controlled purification, but at higher concentrations can compete against the 6-His-tagged protein (Bornhorst&Falke, 2000). Therefore, it is necessary to optimize the amount of imidazole for high purity and high yield of the target protein.

IMAC optimization was then tried by adding more wash buffers before the elution buffer (40mM, 60 mM, 80 mM and 100 MM Imidazole).

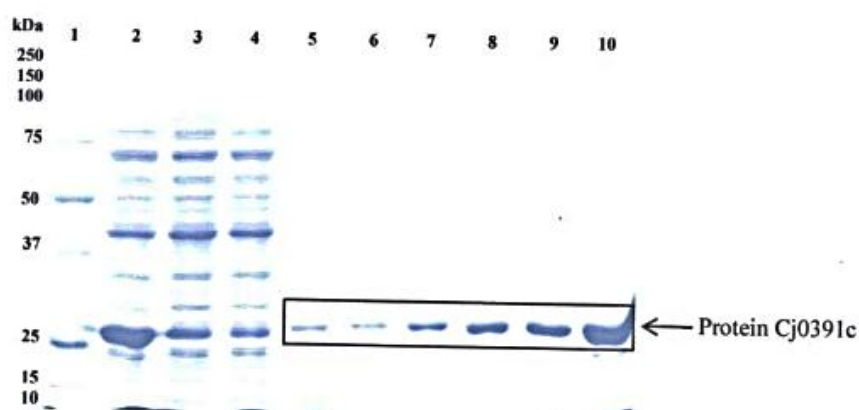


Figure 2. SDS-PAGE Analysis of Post IMAC purification, concentrated, and buffer exchange of Recombinant Protein Cj0391c

Lane 1: Precision PlusProtein<sup>TM</sup>Unstained Standard

Lane 2: Pre Column Sample

Lane 3: Flow Through

Lane 4: IMAC-purified bound-protein Cj0391c from 1st wash buffer (20 mM Imidazole)

Lane 5: IMAC-purified bound-protein Cj0391c from 2<sup>nd</sup> wash buffer (40 mM Imidazole)

Lane 6: IMAC-purified bound-protein Cj0391c from 3rd wash buffer (60 mM Imidazole)

Lane 7: IMAC-purified bound-protein Cj0391c from 4th wash buffer (80 mM Imidazole)

Lane 8: IMAC-purified bound-protein Cj0391c from 5th wash buffer (100 mM Imidazole)

Lane 9: IMAC-purified bound-protein Cj0391c from the elution buffer (140 mM Imidazole)

Lane 10: concentrated and buffer exchanged protein Cj0391c in PBS

The gel in Figure 2 clearly shows that the additional wash buffers had a large effect on purity of the protein as much less contamination in the concentrated protein was found compared to the previous purification. However, the 100 mM wash buffer washed out a large amount of Cj0391c protein itself, almost the same amount as with the eluent of 140 mM Imidazole. This purification resulted in low concentrations of the protein since higher concentrations of Imidazole can reduce the binding of histidine-tagged proteins (Bomhorst&Falke, 2000). Based on this result the 100 mM Imidazole wash buffer was left out of the next purification.



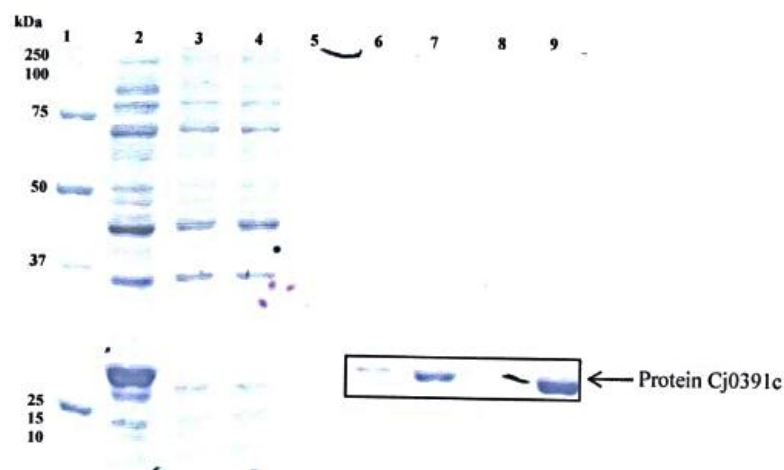


Figure 3. SDS-PAGE Analysis of Post IMAC purification, concentrated, and buffer exchange of Recombinant Protein Cj0391c

Lane 1: Precision PlusProtein™ Unstained Standard

Lane 2: Pre Column Sample

Lane 3: Flow Through

Lane 4: IMAC-purified bound-protein Cj0391c from 1st wash buffer (20 mM Imidazole)

Lane 5: IMAC-purified bound-protein Cj0391c from 2<sup>nd</sup> wash buffer (40 mM Imidazole)

Lane 6: IMAC-purified bound-protein Cj0391c from 3rd wash buffer (60 mM Imidazole)

Lane 7: IMAC-purified bound-protein Cj0391c from 4th wash buffer (80 mM Imidazole)

Lane 8: IMAC-purified bound-protein Cj0391c from the elution buffer (140 mM Imidazole)

Lane 9: concentrated and buffer exchanged protein Cj0391c in PBS

The result of leaving out the 100 mM Imidazole wash step is shown in Figure 3. It can be seen that 80 mM in lane 7 was also too high, washing out the majority of the protein but it had higher amount of protein compared to the eluent seen in lane 8.

The next purification was performed with 20, 40, and 60 mM washes buffers, but the result as described in Figure 4 shows that 60mM imidazole also washed out much of the protein resulting in low concentration in the elution.

Finally, the purification with performed with 20 mM and two sequential 40 mM Imidazole washes, which resulted in high purity and concentration of protein as shown in Figure 5, where not much Cj0391c was lost during washing steps.

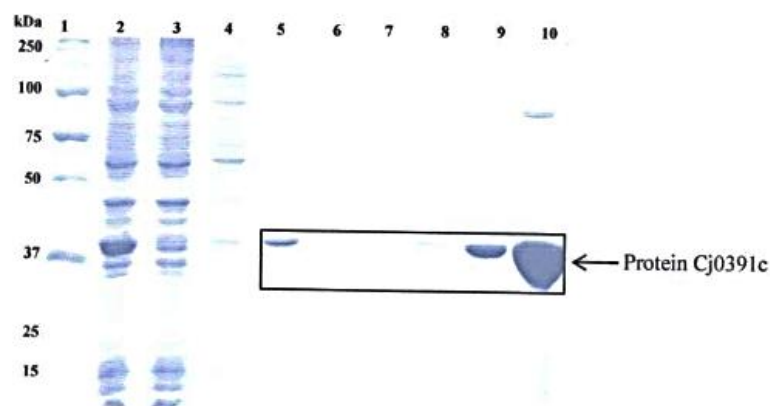


Figure 4. SDS-PAGE Analysis of Post IMAC purification, concentrated, and buffer exchange of Recombinant Protein Cj0391c

Lane 1: Precision PlusProtein™ Unstained Standard

Lane 2: Pre Column Sample

Lane 3: Flow Through

Lane 4: IMAC-purified bound-protein Cj0391c from 1st wash buffer (20 mM Imidazole)

Lane 5: IMAC-purified bound-protein Cj0391c from 2<sup>nd</sup> wash buffer (40 mM Imidazole)

Lane 6: IMAC-purified bound-protein Cj0391c from 3rd wash buffer (60 mM Imidazole)

Lane 7: IMAC-purified bound-protein Cj0391c from the elution buffer (140 mM Imidazole)

Lane 8: concentrated and buffer exchanged protein Cj0391c in PBS

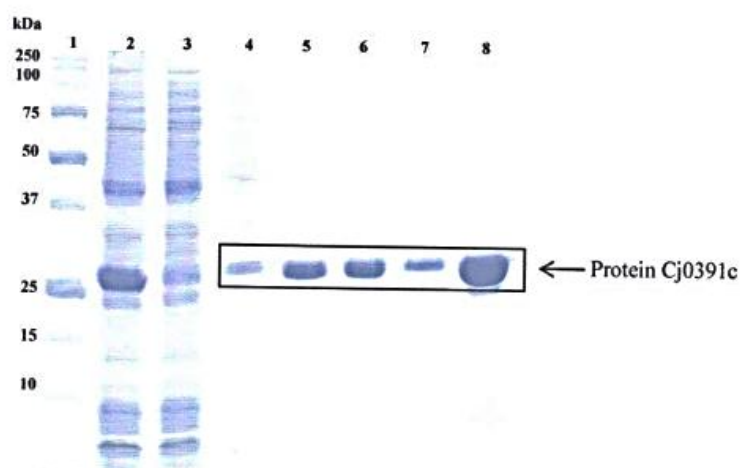


Figure 5. SDS-PAGE Analysis of Post IMAC purification, concentrated, and buffer exchange of Recombinant Protein Cj0391c

Lane 1: Precision PlusProtein™ Unstained Standard

Lane 2: Pre Column Sample

Lane 3: Flow Through

Lane 4: IMAC-purified bound-protein Cj0391c from 1st wash buffer (20 mM Imidazole)

Lane 5: IMAC-purified bound-protein Cj0391c from 2<sup>nd</sup> wash buffer (40 mM Imidazole)

Lane 6: IMAC-purified bound-protein Cj0391c from 3rd wash buffer (40 mM Imidazole)

Lane 7: IMAC-purified bound-protein Cj0391c from the elution buffer (140 mM Imidazole)

Lane 8: concentrated and buffer exchanged protein Cj0391c in PBS

There is still some contamination in the eluent which always appears together with protein Cj0391c, and cannot be washed out completely without losing the Cj0391c, which could be the result of contaminants from *E. coli*. According to Bolanos-Garcia and Davies (2006), in stress conditions like starvation, oxidative damage or heat-shock, *E. coli* responds by shutting down the transcription of genes for protein synthesis, while inducing genes for stress response proteins. They stated that these stress response proteins are the major contaminants during purification using conventional IMAC approaches. Most importantly, this experience showed that the particular contaminant proteins from *E. coli* expressed variably and depending on a variety factors, especially culture conditions, such as temperature, or the specific growth media used. The outcome of these expression and purification experiments is to conclude that 20 mM and two sequential washed of 40 mM Imidazole are the optimum Imidazole concentrations for the production of 1 mg/ml pure Cj0391c protein.

## CONCLUSION

This expression and purification experiment of Cj0391c found that the optimum concentration of Imidazole in the wash buffer to produce a high concentration of pure protein Cj0391c is the combination of an initial step of 20 mM Imidazole wash buffer followed by two times washes using 40 mM Imidazole. These steps then followed by a final step of elution with 140 mM Imidazole eluent buffer.

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