Expression and characterization of family 40 Carbohydrate Binding Module (CBM) from *Vibrio cholerae* Non-O1 sialidase

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Abstract. Carbohydrate Binding Module (CBM) is a non-catalytic protein domain found in carbohydrate-active enzyme (glycoside hydrolase) and its role is to bring carbohydrates in close proximity to the enzyme catalytic site for complete hydrolysis. The removal of this CBM from most protein domains often leads to reduced enzyme activity and efficiency. In this study, a gene encoding for family 40 CBM from *Vibrio cholerae* Non-O1 sialidase was cloned and successfully expressed in *E. coli* BL21 (DE3) strain. The CBM40 encoded 195 amino acids with 585 bp of nucleotide sequence. The protein was successfully expressed at 18°C when induced with 1 mM IPTG. Maximum expression was achieved at 20 hours after post-induction time. For purification of the protein, an anionic denaturing detergent method was used containing 1% SDS and 0.1% sarkosyl with gradient affinity elution at 50 mM imidazole concentrations. SDS-PAGE analysis of the purified CBM40 protein displayed a protein band with a molecular mass of 21 kDa. Protein characterization showed optimum stability in 100 mM citrate buffer pH 5.5, with the highest T_m value of 40 °C. The protein was stable between pH 5.5–6.2 and able to retain its activity at 27–56°C. The addition of Mn^{2+} and Mg^{2+} increased the protein melting temperature to 56°C. Meanwhile, the addition of reagents, such as 1% SDS and 1 M urea increased the protein melting temperature (T_m) to approximately 55°C. Protein stability can be influenced by many factors, including different buffers, pHs, temperatures, ionic strengths, and chemical reagents used in a study. The optimum characterization conditions established would further lead to the discovery of CBM40 protein true potential in enhancing substrate binding affinity and protein-carbohydrate recognition, which underpins its broad applications in biotechnology and protein engineering fields.

Keywords: Carbohydrate Binding Module, sialidase, protein characterization, protein expression, *Vibrio cholerae* Non-O1

INTRODUCTION

Bacterial sialidases have been considered as a core enzyme for bacterial infection and reported to execute the virulence mechanism by acting on the glycosidic linkages of the terminal sialic acid residues in glycoconjugates and synthetic substrates (Woo *et al.*, 2011). The initial step for

enzymatic activity is the degradation of sialic acids from various glycoconjugates. Sialidases are multimodular enzymes, consisting of ancillary modules secured at the catalytic domain of the proteins. These prominent modules have been identified as Carbohydrate Binding Modules

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(CBMs) (Lis & Sharon, 1998). CBMs are small monovalent domains that can be found between catalytic modules at the end of N- or C-termini within proteins and are generally expressible in recombinant systems, thus provide an interesting alternative to lectins (Guillén *et al.*, 2010; Duan *et al.*, 2016).

CBMs are organized into 86 families based on the similarity in their amino acid sequences (<http://www.cazy.org>). Five CBM families have been recognized as mammalian glycan-binding domains, namely CBM32, CBM40, CBM47, CBM51, and CBM57 (Ribeiro *et al.*, 2016). Within these, the members of CBM40 are known to bind to the N-acetylneuraminic acid (Neu5Ac) (Figure 1), which is located at the N-terminus of GH33 (glycoside hydrolase 33) sialidase from *V. cholerae* (Moustafa *et al.*, 2004; Connaris *et al.*, 2014). One typical sialidase producer is *V. cholerae* which is a causative agent of cholera and found abundantly in marine and freshwater environments (Mandal & Mandal, 2014). Among over 200 serotypes of *Vibrio cholerae* species, two strains, which are O1 and O139 serotypes produce cholera toxin (CT) and cause cholera disease, while the non-O1/non-O139 strains are restricted to produce CT but may cause sporadic diarrhoea and extraintestinal infections (Finkelstein, 1996; Teh *et al.*, 2012; Tang *et al.*, 2018). In the present study, the researchers report the construction and expression of CBM40 of *Vibrio cholerae* Non-O1 sialidase, its purification, and characterization. For carbohydrate active domain, such as CBM40, protein characterization profile is a really important aspect to be explored. Generally, most proteins are sensitive to changes in temperature and pH as they tend to lose their active conformation structure when these conditions are not optimal. Therefore, protein characterization was carried out to screen the optimal condition for temperature, pH, metal ions, and chemical reagents for CBM40 proteins function and stability. Currently, CBMs application has been established in various biotechnology fields and their use as molecular probes, affinity support for protein immobilization and assistance in vitro glyco-catalysed reactions leading to enhanced yields. This is due to the fact that CBMs are independent folding units and therefore can be engineered to bind and detect varieties of carbohydrates besides being stable to function

efficiently in chimeric proteins. Therefore, CBMs are excellent model systems to study protein-carbohydrate recognition mechanism and various ligand specificities for many biotechnological applications.

Figure 1. Structure of the 9-carbon monosaccharide, sialic acid or N-acetylneuraminic acids (Neu5Ac). This figure was adapted from Moustafa *et al.* (2004).

MATERIALS AND METHODS

Cloning of CBM40 domain and protein expression

A gene encoding for the CBM family 40 domain from *Vibrio cholerae* Non-O1 was amplified by the polymerase chain reaction (PCR). The primers used were CBM (F): 5'- GGA TCC ATG CAC TTT TTG ACT ATA ACG C-3', and CBM (R): 5'- GAA TTC GTC GCC TTG AAT TTC AAA C-3' (*Bam*HI and *Eco*RI restriction sites, as underlined). PCR amplification parameters were performed based on the protocol done by Alias and Mamat (2018). The PCR fragments were purified using QIAquick PCR purification kit (Qiagen), followed by digestion with *Bam*HI and *Eco*RI before being ligated into digested pET-22b(+) vector. The plasmid was transformed into *Escherichia coli* BL21 (DE3) expression strain and the confirmed colonies were verified by colony PCR. In addition, plasmids were sent for sequencing analysis at 1st Base Laboratory Sdn. Bhd., Malaysia in order to identify positive transformant.

The expression of the CBM family 40 protein was carried out by inoculating the recombinant CBM40 clone into Luria Broth (LB) supplemented with 100 µg/ml ampicillin. The bacteria culture was further incubated at 37 °C until the OD (A_{600}) reached 0.6. Then, the bacterial culture was set to a heat shock stage at 42°C for 20 minutes before inducing with 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) followed by overnight incubation at 18°C. The culture was harvested by centrifugation at 12,000

rpm for 20 minutes. The cell pellet was kept in sterile tubes and stored at -80°C until further use.

Recombinant protein purification

The cell pellet was resuspended and lysed in an anionic denaturing buffer, PCL buffer (2.6 mM KCl, 1.4 mM KH_2PO_4 , 8 mM Na_2HPO_4 , 1 % SDS, 0.1 % sarkosyl, and 286 mM NaCl at pH 7.4) containing protease inhibitors. The cell suspension was sonicated on ice prior to lysis and incubated (on ice) for 30 minutes. The chilled lysates were centrifuged at 40,000 g for 30 minutes at 4°C to remove cell debris. The supernatant was filtered through a Sartorius Minisart® Syringe Filter pore size 0.22 μm before being loaded into an affinity column, HisTrap FF (GE Healthcare) connected to AKTA protein purification system (GE Healthcare). The column was equilibrated with PCW buffer (2.6 mM KCl, 1.4 mM KH_2PO_4 , 8 mM Na_2HPO_4 , 0.1% sarkosyl, and 286 mM NaCl at pH 7.4) until the flow-through absorption returned to the baseline level. Bound protein was eluted with PCE buffer (2.6 mM KCl, 1.4 mM KH_2PO_4 , 8 mM Na_2HPO_4 , 0.1% sarkosyl, 286 mM NaCl at pH 7.4) with increased concentrations of imidazole from 10–100 mM. Protein fractions were taken at various points and loaded on 12 % SDS-PAGE. The purified protein fractions were concentrated using 10 kDa cut-off concentrator tubes to a certain volume before undergoing an overnight dialysis in phosphate buffer that contained 20 mM NaCl. All the purified CBM40 proteins were kept at -80°C until further use.

Effects of temperature and pH on the stability of the purified CBM40

The thermal stability of the recombinant CBM40 protein was monitored using a thermal shift assay CFX Connect™ Real-Time PCR System (Biorad) through the midpoint of protein melting temperature (T_m). About 2.5 mg/ml of the purified CBM40 enzyme was tested in different buffers (20 mM Tris pH 6.5, 20 mM Tris pH 7.5, 100 mM potassium phosphate pH 6.5, 100 mM potassium phosphate pH 7.5, 100 mM sodium phosphate pH 6.5, 100 mM sodium phosphate pH 7.5, 100 mM sodium citrate pH 5.5, and 100 mM sodium citrate pH 6.2) with 2.5 X SYPRO Orange Dye. The samples were incubated as follows: 25°C for 30 seconds (1 cycle), followed

by an increase of temperature to 100°C for 1 minute (140 cycles) and a finishing cycle at 25°C for 1 minute. The fluorescence was read at a fixed absorbance of 492/610 nm.

Effects of metal ions and chemical reagents on the purified CBM40

The effects of metal ions and chemical reagents are determined using a thermal shift assay CFX Connect™ Real-Time PCR System (Biorad). The reaction mixture consisted of the purified CBM40 (2.5 mg/ml) in 0.2 M sodium citrate buffer, pH 5.5 containing 1 mM of different metal ions (Na^{2+} , Co^{2+} , Zn^{2+} , Ca^{2+} , Mg^{2+} , Fe^{2+} , Mn^{2+} , Cu^{2+} , and K^{+}) and 2.5 X SYPRO Orange Dye. Meanwhile, the effect of surfactants (1% Triton X-100, 1% SDS), organic solvents (5% DMSO, 1 M urea), and metal chelator (1 mM EDTA) was also investigated on the purified CBM40. All tubes were incubated at 25°C for 30 seconds (1 cycle), followed by an increase of temperature from 25°C – 100°C for 1 minute (140 cycles) and a finishing cycle at 25°C for 1 minute. The fluorescence emission was evaluated at a fixed wavelength of 492/610 nm.

RESULTS AND DISCUSSION

Molecular cloning of CBM40 gene

The CBM40 gene of 585 bp, which was isolated from *Vibrio cholerae* Non-O1 sialidase was amplified and cloned in pET-22b(+) vector and transformed in *E. coli* BL21(DE3) strain (Figure 2). The complete ORF sequence consists of 133 adenine (A), 68 thymine (T), 182 guanine (G), and 202 cytosine (C) (Figure 3). Based on the analysis by Compute pI/Mw, the protein isoelectric point (pI) is 4.77 and its molecular weight is 21,074.32 Da. Deduced amino acid sequence analysis showed 98.86% similarity to *Vibrio cholerae* Neuraminidase with Accession No. APF83226 and 2W68_A (Figure 4). The gene was cloned into pET-22b(+) vector and cultivated in *E. coli* BL21 (DE3) host. The pET-22b(+) expression vector encoded a pelB secretion signal peptide under strong bacteriophage T7 promoter (Sokolosky & Szoka, 2013; Kashani *et al.*, 2015). The presence of pelB signal peptide would direct the expression of CBM40 gene in the periplasmic space where

the condition would improve protein folding (Yoon *et al.*, 2010; Malik, 2016).

Figure 2. PCR amplification of CBM40 gene of *Vibrio cholerae* Non-01 sialidase.

Overexpression and purification of CBM40 protein

Expression of the CBM40 protein was carried out in *E. coli* BL21 (DE3) harbouring the pET-22b(+) vector. This vector carried the N-terminal pelB secretion signal peptide, which targeted the unfolded state of the translated protein to the *E. coli* periplasm via the Sec-dependent transport pathway (Yoon *et al.*, 2010). This pelB signal peptide is cleaved upon secretion to the periplasm and the presence of chaperones molecule and disulphide bond isomerases, which are located in the *E. coli* periplasm space help to properly fold the protein, and thus making it an ideal compartment for various therapeutic protein expressions (Sokolosky & Szoka, 2013). In order to express CBM40 protein in soluble form, it was important to go through an additional stage of heat-shock treatment at 42°C for 30 minutes before inducing with 1mM IPTG. After carrying out this additional step, the protein solubility has significantly improved and at the same time, the induction levels were not affected. Crucial stage like heat shock at 42°C could lead to expression of *E. coli* heat-shock proteins, which act as molecular chaperones and assist protein folding

and disassemble protein aggregates. The existence of bacteria chaperones has been widely discovered in *E. coli* insoluble fraction due to the formation of fusion protein aggregation (Carrió & Villaverde, 2005; Guenther *et al.*, 2012). Intracellular chaperones known as heat shock proteins act to assist protein–protein interactions, such as folding, proper protein conformation, and also prevent unwanted protein aggregation. These heat shock proteins help transport proteins across cell membranes and stabilize partially unfolded proteins (Bakthisaran *et al.*, 2015). In addition, deficiency of eukaryotic chaperones molecule and post-translational modification mechanism also assist in the inclusion bodies formation in bacteria (Carrió *et al.*, 2000; Walter & Buchner, 2002).

In order to investigate the effect of different post-induction time on the yield of recombinant CBM40, five incubation times (4, 8, 12, 16, and 20 hours) were evaluated. According to our results, the highest protein expression was observed at 20 hours after the post-induction time (Figure 5). Despite using lower post-induction temperature of 18°C to slow down protein folding and fusion protein oligomerization, some of the proteins were still expressed as insoluble form in the pellet. According to Hoell *et al.* (2005), incompatible growth temperature can cause protein misfolding, which eventually promotes inclusion bodies formation. Previous studies have suggested to use low expression temperature to promote the formation of correctly folded protein and soft inclusion bodies where non-denaturing solvents can be used as solubilizing agents (Jevševar *et al.*, 2005; Peternel *et al.*, 2008; Alias *et al.*, 2009). Besides that, protein expression at a high translational rate often resulted from unsuitable post-induction temperature, higher concentration of inducer, and strong promoter system, which also lead to protein aggregations and inclusion bodies formation (Costa *et al.*, 2014). Inclusion bodies are highly dynamic in nature but they can be reversibly converted to their native form by proper strategies during protein recovery (Singh *et al.*, 2015).

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1 GGC GCC ATG GCC CTG TTC GAC TAC AAC GCC ACC GGC GAC ACC GAG TTC GAC AGC CCC GCC 60
1  G  A  M  A  L  F  D  Y  N  A  T  G  D  T  E  F  D  S  P  A  20

61 AAG CAG GGC TGG ATG CAG GAC AAC ACC AAC AAC GGC AGC GGC GTT TTA ACC AAT GCA GAT 120
21  K  Q  G  W  M  Q  D  N  T  N  N  G  S  G  V  L  T  N  A  D  40

121 GGA ATG CCC GCT TGG TTG GTG CAA GGT ATT GGA GGG AGA GCT CAA TGG ACA TAT TCT CTC 180
41  G  M  P  A  W  L  V  Q  G  I  G  G  R  A  Q  W  T  Y  S  L  60

181 TCT ACT AAT CAA CAT GCC CAA GCA TCA AGT TTC GGT TGG CGA ATG ACG ACA GAA ATG AAA 240
61  S  T  N  Q  H  A  Q  A  S  S  F  G  W  R  M  T  T  E  M  K  80

241 GTG CTC AGT GGT GGA ATG ATC ACA AAC TAC TAC GCC AAC GGC ACT CAG CGT GTC TTA CCC 300
81  V  L  S  G  G  M  I  T  N  Y  Y  A  N  G  T  Q  R  V  L  P  100

301 ATC ATT TCA TTA GAT AGC AGT GGT AAC TTA GTT GTT GAG TTT GAA GGG CAA ACT GGA CGC 360
101 I  I  S  L  D  S  S  G  N  L  V  V  E  F  E  G  Q  T  G  R  120

361 ACC GTT TTG GCA ACC GGC ACA GCA GCA ACG GAA TAT CAT AAA TTT GAA TTG GTA TTC CTT 420
121 T  V  L  A  T  G  T  A  A  T  E  Y  H  K  F  E  L  V  F  L  140

421 CCT GGA AGT AAC CCA TCC GCT AGC TTT TAC TTC GAT GGC AAA CTC ATT CGT GAC AAC ATC 480
141 P  G  S  N  P  S  A  S  F  Y  F  D  G  K  L  I  R  D  N  I  160

481 CAG CCG ACT GCA TCA AAA CAA AAT ATG ATC GTA TGG GGG AAT GGC TCA TCA AAT ACG GAT 540
161 Q  P  T  A  S  K  Q  N  M  I  V  W  G  N  G  S  S  N  T  D  180

541 GGT GTC GCC GCT TAT CGT GAT ATT AAG TTT GAA ATT CAA GGC GAC 585
181 G  V  A  A  Y  R  D  I  K  F  E  I  Q  G  D  195

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Figure 3. The gene sequences of CBM40 of *Vibrio cholerae* Non-O1 sialidase. The translated amino acid sequence is shown in one-letter codes below the nucleotide sequence.

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CBM40      -----GAMALFDYNATGDTEFDSPAKQGWMQDNTNNGSGVLTNA 39
2W68A      -----AAMALFDYNATGDTEFDSPAKQGWMQDNTNNGSGVLTNA 39
APF83226   MRFKNVKKTALMLAMFGMATSSNAALFDYNATGDTEFDSPAKQGWMQDNTNNGSGVLTNA 60
          . *****

CBM40      DGMPAWLVQGIGGRAQWTYSLSTNQHAQASSFGWRMTTEMKVLSSGGMITNYYANGTQRVL 99
2W68A      DGMPAWLVQGIGGRAQWTYSLSTNQHAQASSFGWRMTTEMKVLSSGGMITNYYANGTQRVL 99
APF83226   DGMPAWLVQGIGGRAQWTYSLSTNQHAQASSFGWRMTTEMKVLSSGGMITNYYANGTQRVL 120
          *****

CBM40      PIISLDSSGNLVVEFEGQTGRTVLATGTAATEYHKFELVFLPGSNPSASFYFDGKLIRDN 159
2W68A      PIISLDSSGNLVVEFEGQTGRTVLATGTAATEYHKFELVFLPGSNPSASFYFDGKLIRDN 159
APF83226   PIISLDSSGNLVVEFEGQTGRTVLATGTAATEYHKFELVFLPGSNPSASFYFDGKLIRDN 180
          *****

CBM40      IQPTASKQNMIVWGNSSNTDGVAAYRDIKFIEIQGD----- 195
2W68A      IQPTASKQNMIVWGNSSNTDGVAAYRDIKFIEIQGD----- 195
APF83226   IQPTASKQNMIVWGNSSNTDGVAAYRDIKFIEIQGDVIFRGPDRIPSIVASSVTPGVVTA 240
          *****

CBM40      ----- 195
2W68A      ----- 195
APF83226   FAEKRVGGRRSRCSE 255

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Figure 4. The alignment of the amino acid sequence of the recombinant CBM40 gene of the *Vibrio cholerae* Neuraminidases (Accession No. APF83226 and 2W68_A). The alignment was performed using the Clustal Omega program.

Figure 5. Expression of the recombinant CBM40 protein at different time interval from 4 hours until 20 hours after post induction time. All samples were sonicated and separated by centrifugation. S: Supernatant; P: Pellet.

In order to recover inactive CBM40 protein from the pellet, four main strategies were carried out as stated by Singhvi *et al.* (2020), which were inclusion bodies isolation, homogeneous inclusion bodies solubilization, refolding of the solubilized proteins, and refolded proteins purification through chromatographic approaches (Singhvi *et al.*, 2020). In order to solubilize the pellet, sonication was performed on ice in an anionic denaturing buffer containing 1% w/v SDS and 0.1% sarkosyl. Shlager *et al.* (2012) stated that adding both 0.1% sarkosyl and 1% SDS in the lysis buffer helped to break the protein solubility barrier. Another study by Burgess (2009) mentioned that adding as low as 0.1% to 2% of sarkosyl in the lysis buffer led to the improvement of protein solubility from inclusion bodies. Previous research by Massiah *et al.* (2016) stated that an addition of 10% sarkosyl in the buffer helped recover more than 95% of active protein from the inclusion bodies, thus enhanced protein solubility and efficiently helped in the cell lysis. Besides that, as reported by Jevševar *et al.* (2005), in order to solubilise inclusion bodies, less concentrated organic solvents such as 5% n-propanol, 5% DMSO, 2 M urea, and 0.1% and 0.2% N-lauryl sarcosine (detergent) were used as mild solubilization agents (Jevševar *et al.*, 2005; Upadhyay *et al.*, 2014). These mild solubilization

techniques have been proven to enhance bioactive protein recovery from inclusion bodies (Burgess, 1996; Kudou *et al.*, 2011). Furthermore, as reported by Singh *et al.* (2015), for protein containing multiple cysteine residues, reagents such as β -mercaptoethanol or dithiothreitol (DTT) are normally added during the solubilization stage to decrease the misformation of disulfide bond.

The lysates were then cooled down to precipitate free SDS, which also contained trace amounts of protein. Centrifugation helped to separate the precipitated SDS from host proteins and overexpressed target protein. Usually, the isolated inclusion bodies contain certain impurities, such as proteins from the host, membrane fragments, and RNA, hence, further purification steps need to be carried out. During refolding of the solubilized proteins, removal of solubilization agents with optimal refolding buffer is very important. Many chromatographic techniques have been widely used for protein refolding, which included size exclusion, ion exchange, and affinity chromatography. Choosing the correct and suitable chromatography methods is very crucial in order to optimize and recover a large quantity of bioactive protein from the inclusion bodies. In this study, affinity column chromatography was performed using HisTrap

FF column. For protein purification, two-step strategy of the affinity chromatography was carried out with the final concentration of 50 mM imidazole. Improved protein purity was observed after elution with gradient imidazole concentrations (10–50 mM). As seen in Figure 6, most of the high molecular weight protein contaminants associated with the protein of interest were successfully removed. The purified CBM40 protein displayed a protein molecular mass of 21 kDa on SDS PAGE (Figure 6).

Figure 6. Purified CBM40 protein showed an approximate size of 21 kDa after purification stages. M: Protein marker; C: Crude enzyme; FT: Flow through; W1-W2: Wash stages; E1-E2: Elution stages.

Temperature and pH stability studies of purified CBM40

Protein characterization is one of the important stages to be analysed especially for a newly developed engineered protein. Protein characterization profile is related to protein separation, purity, and also its biological, chemical, and physical properties. So, when it comes to developing a strategy for protein characterizations, a few factors need to be considered, which include protein purity and its stability. There are several options of biophysical techniques available to measure protein interactions, such as direct fluorescence-based method known as thermal shift assay. In this assay, protein thermal stability can be calculated through the temperature midpoint or melting point (T_m) of the protein samples' curve. Melting temperature (T_m) is defined as the temperature where 50% of the protein molecules are unfolded. According to Niesen *et al.* (2007), protein structure is likely denatured at certain temperatures and throughout this process, the protein midpoint can be measured. Protein with higher T_m value will have greater thermal

resistance since a higher temperature is needed to unfold the protein structure.

The importance of buffers as part of the integral component of protein formulations helped to stabilize protein by specific mechanisms. The addition of buffers not only helped to regulate pH shifts but also managed to increase protein conformation stability either by ligand binding or through excluded solute mechanism (Zbacnik *et al.*, 2017). From Figure 7, the highest stability of the protein was obtained in 100 mM citrate buffer at pH 5.5, with melting temperature of 40°C. The protein exhibited stability between pH 5.5–6.2 and was able to retain its activity from 27–56°C. In contrast to CBM32 isolated from *Thalassomonas* sp. LD5, the protein has optimal enzymatic activity at 30°C and pH at 7.6, as reported by Zhang *et al.* (2019). Therefore, these data suggested that CBM40 had a slightly better thermal stability with 10 °C higher temperature as compared to CBM32. In addition, for optimal pH condition, the results showed that both CBMs had a significant effect on the optimal pH of the enzyme where CBM40 exhibited better protein stability in highly acidic environment in contrast to CBM32, which showed stability in alkaline environment. Based on previous research by Ali *et al.* (2005) on family 22 CBM from thermostable strain of *Clostridium thermocellum* Xyn10C, the contiguous presence of CBM domain to the catalytic module increased the enzyme optimal temperature to 80°C from 60°C when CBM domain was attached. Meanwhile, pH of the recombinant CBM (rCBM-CM) protein was also found to be more stable in acidic pH range (Ali *et al.*, 2005). In addition, according to Zhou and Pang (2018), protein stability will be affected by pH of buffers and the electrostatic interactions between charged amino acids present on the protein. Through thermal shift assay, the consequent changing of protein melting temperature (T_m) in a solution can be applied to measure protein stability at different relative pHs. According to a study by De Lencastre *et al.* (2011), the addition of phosphate and citrate in Green Fluorescent Protein (GFP) expression showed that both citrate and phosphate aided to protect GFP protein from inactivation at elevated temperatures from 70–90°C by promoting protein conformation stability. These salts bind to the native protein state and stabilize it via their

anionic multivalent interactions. Another study by Byrt *et al.* (2012) on the hydrolytic activity of cellulase enzyme found that the existence of CBM next to the enzyme molecule did not reduce the rate of enzyme hydrolysis. In addition, it increased the protein stability towards pH changes, which were between pH 4.0 and 5.5. This was in agreement with a study by Liu and Ding (2016), which also reported that thermostability of cellulases and hemicellulases enzyme against lignocellulosic substrate improved when CBM42 domain was fused to the main enzyme. This

approach thus significantly enhanced the enzyme catalytic activity and thermostability towards 4-methylumbelliferyl acetate substrate. A study by Koseki *et al.* (2010) on chimeric enzyme of *Aspergillus awamori* feruloyl esterase and family 42 CBM managed to retain the enzyme activity up to 40% after incubation at 55°C for 30 minutes as opposed to the enzyme without CBM domain, which had lost activity at the same temperature tested. Therefore, this showed the role of CBM in maintaining the thermal stability of protein.

Figure 7. Protein melting curve analysis for the CBM40 in different buffers. (■) 100 mM sodium citrate pH 5.5, (■) 100 mM sodium citrate pH 6.2, (■) 100 mM potassium phosphate pH 6.5, (■) 100 mM potassium phosphate pH 7.5, (■) 100 mM sodium phosphate pH 6.5, (■) 100 mM sodium phosphate pH 7.5, (■) 20 mM Tris pH 6.5 and (■) 20 mM Tris pH 7.5.

Effect of metal ions and reagents on stability of CBM40

One of the approaches to stabilize protein structure and mechanism is identifying the interaction of metal ions with specific amino acid on a protein (Kardinahl *et al.*, 2000; Villalonga *et al.*, 2004). The effect of metal ions (Na^{2+} , Ca^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Mn^{2+} , Mg^{2+} , Zn^{2+} , and K^{+}) on the stability of CBM40 domain was determined by the thermal shift assay approach (Figure 8). From the temperature curve analysis, Mn^{2+} and Mg^{2+} ions managed to increase protein melting temperature to 56°C as compared to other metal ions including the control (sample without

addition of metal ion). Meanwhile, K^{+} showed the lowest T_m , which was around 40°C (Figure 8). A previous study on human lymphotactin revealed that protein function and its structure could be coordinated by ionic interactions between metal ion and salts (Formanek *et al.*, 2006). The roles of electrostatic interactions in protein folding involved in protein-metal complexes show that metals are utilized by proteins in cells on a molecular level, which affects thermodynamic and kinetic properties of proteins. Effect of metal ions and reagents on protein folding and unfolding depends on types and concentration of metal ions, ionic strengths, and also protein-charged residues.

These factors affect both native protein and denatured states of the protein (Palm-Espling *et al.*, 2012). A study by Zhang *et al.* (2020) on CBM32 and CBM9 from a marine bacterium, *Thalassomonas* sp. LD5 revealed that combination of both domains, i.e. CBM9 and CBM32 to the main protein managed to maintain 5% of its activity up to 80°C, which enhanced thermostability. The addition of Ca^{2+} into the

protein reaction led to 14% increase in the protein activity compared to the reaction without metal ion. These results proved that CBM9 and CBM32 improved the catalytic module activity against metal ion. Nevertheless, when comparing their melting temperature without the addition of metal ion, CBM40 showed a relatively higher melting temperature of 40°C as compared to the chimeric protein (CBM9-CBM32), which was at 30°C.

Figure 8. Effect of metal ions on protein melting temperature analysis. (—) Control, (—) Co^{2+} , (—) Mn^{2+} , (—) K^{+} , (—) Cu^{2+} , (—) Zn^{2+} , (—) Mg^{2+} , (—) Na^{2+} , (—) Fe^{2+} and (—) Ca^{2+} .

Due to the protein stabilization complex, protein stability can be deliberated through the increase in melting temperature. The presence and absence of ligands can lead to differences in melting temperature, which correlate to substrate and protein molecule binding affinity. Various research found that greater than three-tenths of many folded proteins have metal ions bound to specific sites on the proteins in order to obtain the desired function (Lin *et al.*, 2006; Palm-Espling *et al.*, 2012). Metal ions have been proven to play significant roles in protein structural stability (i.e. zinc in zinc-finger domains) and enzyme

metabolisms by the mechanism of metal-bound proteins, which folds in cellular environments where their related metal ions are present free in solution or bound to delivery proteins (i.e. zinc in carbonic anhydrase) (Palm-Espling *et al.*, 2012). Based on a study carried out by Sissi and Palumbo (2009) on the effects of divalent metal ion (Mg^{2+}), this cofactor helped in improving topoisomerases DNA cleavage through supporting the catalytic events. On the contrary, metal ions destabilizing effect of Na^{+} , Ca^{2+} , and Mg^{2+} on recombinant pyrolysin protein was been observed due to the disruption of protein surface electrostatic

interactions. Notably, the protein's melting curve was shifted to a lower temperature, displaying destabilizing effects on the tested divalent cations. This destabilizing effect may also be compensated by other biotic and abiotic factors (Zeng *et al.*, 2014). Another study by Villalonga *et al.* (2004) on trypsin also found that Mg^{2+} , Zn^{2+} , and Mn^{2+} metal ions helped to increase trypsin thermal stability up to four-fold after the addition of the respective metal ions. Therefore, correlated from that study, the addition of Mn^{2+} and Mg^{2+} ions also showed a significant impact on CBM40 protein thermal stability (up to 56°C) as compared to other metal ions tested.

In addition, stability effect of reagents on the purified CBM40 protein was tested using five different reagents, namely 1 M urea, 1 mM EDTA, 1% SDS, 5% DMSO, and 1% Triton X-100 in 100 mM sodium citrate buffer pH 5.5. From the observation, three reagents (1% Triton

X-100, 1% SDS, and 1 M urea) managed to slightly increase the melting temperature of the recombinant CBM40 protein (Figure 9). The highest melting temperature was found after the addition of 1% SDS, which was at 55°C, followed by 1 M urea and 1% Triton-X at 54°C and 52°C, respectively. From this study, the addition of polar aprotic solvent-like DMSO and the chelating agent EDTA showed a lower melting temperature which was at 38°C and 46°C, respectively as compared to the control when reacted with the CBM40 protein. A previous study by Lu *et al.* (2014) reported that recombinant gene CBM1 and *Aspergillus nidulans* endo- β -1,4-mannanase (Man5XZ3) managed to retain higher enzymatic activity (more than 80%) in the presence of chaotropic agent (4 M urea), 60% acetone, and two surfactants (1% SDS and 1% Triton X-100) as compared to the protein alone without the CBM1 domain.

Figure 9. Effect of different chemical reagents on CBM40 protein stability. (—) Control, (—) 1% SDS, (—) 5% DMSO, (—) 1% Triton X-100, (—) 1 mM EDTA, (—) 1 M urea.

Based on a previous study by Pereira *et al.* (2017), the addition of anionic detergent, such as sodium dodecyl sulphate (SDS) at low concentrations of 1–2 mM would enhance the enzyme activity, while could cause inhibitory effect at 20 mM

concentration. Higher concentration of SDS would interfere in the hydrophobic regions of a protein, thus modifying protein three-dimensional structure and causing enzyme denaturation (Bhuyan, 2010). A study conducted

by Szymczyk *et al.* (2018) on different non-ionic detergents (Tween-20, Tween-40, Tween-60, Tween-80, Tween-100, Triton X-100, and polyethylene glycol (PEG)) had proven to reduce the fluidic systems' surface tension, which led to changes in liquid properties. Even though Triton X-100 is a non-ionic mild detergent that usually leads to slow cell lysis, this reagent has the tendency to denature proteins and break up protein complexes (Islam *et al.*, 2017). Based on this study, the CBM40 melting temperature was distinguished by only 1°C when SDS and urea were added to the protein mixture. Some of the advantages of using SDS in protein purification over urea are that SDS can be applied at comparably low concentrations and does not tend to crystallize in valves and pumps of chromatography devices, such as FPLC (Schlager *et al.*, 2012).

CONCLUSION

In this study, the recombinant CBM40 was successfully expressed in *Escherichia coli* BL21(DE3) strain despite having inclusion bodies formation. Our study indicated that the yield of recombinant CBM40 was markedly increased when produced under optimized protocol. The purified CBM40 showed an increase in thermal stability to withstand acidic environment and was able to retain its activity at a short period of time by increasing the temperature up to 50°C. The addition of metal ions managed to increase protein melting temperature, meanwhile, the addition of lower concentrations of ionic, non-ionic, and chaotropic detergents showed a significant increase in protein stability. This discovery provides new insights on the development of stable recombinant family 40 CBM, which can be further developed as a potential biologic molecule for diagnostic uses in the biotechnology and biomedical fields.

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