J. Trop. Agric. and Fd. Sc. 39(2)(2011): 179–189

Cloning, expression and purification of a partial outer membrane protein (Omp) from *Candidatus* Liberibacter asiaticus causing greening disease of citrus

[Pengklonan, ekspresi dan penulenan protein rekombinan membran luaran separa (Omp) *Candidatus* Liberibacter asiaticus yang menyebabkan penyakit *greening* tanaman limau]

H.Y. Lau*, H. Habibuddin** and C.S. Tan*

Keywords: Citrus greening disease, outer membrane protein (Omp), *Candidatus* Liberibacter asiaticus, protein expression and purification

Abstract

Citrus greening is one of the most serious diseases affecting citrus cultivation in many countries including Malaysia. The disease is caused by a non-cultured, phloem-restricted bacterium-like organism, *Candidatus* Liberibacter asiaticus. The low concentration of the organism in infected plants makes it difficult to obtain sufficient amounts of the pathogen to be used as antigen in the production of its antibodies. This study was carried out to produce recombinant protein from the partial outer membrane protein (Omp) that could be used as the antigen during polyclonal antibody production. The gene encoding the Omp of the organism was amplified using total DNA extracted from disease infected citrus midribs. The amplified PCR fragment of length 877 bp was purified, restriction enzyme digested and ligated into a bacterial expression plasmid vector, pRSET B. After the construct was verified by restriction enzyme and DNA sequencing, the bacterial expression was carried out using *E. coli* JM109 (DE3) and the expression of recombinant Omp was induced with isopropyl thiogalactoside (IPTG). The expected 30 kDa expressed protein was estimated by migration in 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The recombinant Omp was purified by nickel-chelating resin under denaturing conditions. This protein will be used in our subsequent studies to obtain antibodies against it.

Introduction

Citrus greening disease caused by *Candidatus* Liberibacter asiaticus is one of the most destructive diseases affecting citrus cultivation in Malaysia and other Asian countries (Ohtsu 1998; Bové 2006; Hajivand et al. 2011). Most of the known citrus varieties cultivated in Malaysia are susceptible to the disease. Pomelo, which was long considered to be free from this disease, has been found to be naturally infected with the disease, first in the East and then in Peninsular Malaysia in 1995 (Teo 1995).

*Biotechnology Research Centre, MARDI Headquarters, Serdang, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia
**Rice and Industrial Crops Research Centre, MARDI Headquarters, Serdang, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia
Authors' full names: Lau Han Yih, Habibuddin Hashim and Tan Chon Seng
E-mail: hylau@mardi.gov.my

This organism is heat tolerant and can induce infected citrus plants grown in warm temperatures (27–32 °C) or in cool climate to produce symptoms. Trees infected with greening disease become stunted and bear multiple off-season flowers. Common symptoms on the leaves are yellowing of the veins and adjacent tissues, followed by yellowing or mottling of the entire leaf. Diseased leaves become hardened and curl outward, while young leaves developed after premature defoliation are relatively smaller and slender. Besides, the fruits of infected trees are small and irregularly shaped (Halbert and Manjunath 2004; Gottwald et al. 2007). Sometimes, symptoms of the disease can be confused with symptoms of zinc deficiency, which may affect the strategies taken to manage the disease (Sankaran et al. 2010).

The disease is transmitted by a psyllid vector, *Diaphorina citri* (Halbert and Manjunath 2004) and the spread of the disease could also result from the widespread use of infected planting materials. All of the commercial citrus cultivars grown in the country, including *Citrus suhuiensis* cv. Limau Madu are susceptible to the disease. Therefore, greening disease is a major causal factor for the declining of citrus cultivation in the country (Teo 1995).

Utilization of disease free planting materials is recommended for new planting areas and replacement of infected plants in the existing orchards (Ibrahim et al. 2003). This will delay the onset of greening disease in the field, provided a proper management of vector population in the field is practised to arrest the incoming of disease inoculums. Hence, efficient and cheap detection methods need to be developed to detect disease presence in the field and to index planting materials prior to their distribution.

The existing methods of *Ca*. L. asiaticus detection include the use of 16S rRNA gene-based Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis (Jagoueix et al. 1996), electron microscopy (Lafleche and Bove 1970), dot-blot hybridization (Garnier and Bove 1993), nested PCR and TaqMan® PCR (Lin et al. 2010). Currently, polymerase chain reaction (PCR) is used to detect infection and index planting materials in certain nurseries in Malaysia. Even though the technique is highly efficient, it is laborious and expensive, making large scale adoption of the technique impractical.

Enzyme-Linked Immuno-Sorbant Assay (ELISA) is relatively cheaper and can be used to index large volumes of test materials (Omrani et al. 2009). However, ELISA needs antibodies against the pathogen. The greening causing organism, *Ca*. L. asiaticus is restricted to the phloem of infected plants and cannot be cultured, making extraction of this polyclonal antigen from plant samples difficult. Hence, the recombinant protein of the pathogen should be explored to facilitate developing ELISA detection of the pathogen.

Several of the important attributes of the candidate molecules for antibody development in bacterial detection purposes are the presence of highly conserved regions in the species. These attributes are expressed on the surface of pathogens (antigen), so that the antibody can easily recognize them. Bacterial outer membrane proteins (Omps) possess these characteristics and therefore have potential for antibody production (Khushiramani et al. 2007). This study was conducted to produce a recombinant protein of *Ca*. L. asiaticus partial outer membrane (Omp) to be used as the candidate antigen for antibody production.

Materials and methods

Plant materials and DNA preparation

Citrus midribs from *Ca*. *L. asiaticus* infected and uninfected citrus plants (*Citrus suhuiensis* cv. Limau Madu) were collected from MARDI station, Jerangau, Terengganu, Malaysia. The samples were stored at −80 °C until further use. Genomic DNA was

extracted from the midribs using Murray and Thompson's method (1980).

Molecular cloning of the Omp gene

The primers used for cloning of the Omp gene were designed based on sequence of the outer membrane protein (Omp) of *Ca*. L. asiaticus (Accession No. AY842432) obtained from the National Center for Biotechnology Information (NCBI) Gene bank (www.ncbi.nlm.nih.gov). The sequence of the forward and reverse primers used were Omp F1 5´-TGA GGA TCC ATA TTT TTT AGG-3´ and Omp R1 5'- AGT GTC GAC CAT GCG ATT ACC TAT ACG-3' containing *BamH*I and *Sal*I restriction sites respectively.

PCR was performed in a 20 µl reaction volume containing 1.25U of *Taq* polymerase, 2 µl buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$), 100 ng template DNA, 200 µmol/litre of each dNTP, and 25nmol/litre of each primer. PCR was run in a programmable thermocycler (Biometra) with the following protocol (Sambrook et al. 2000): initial denaturation at 95 °C for 10 min, denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min followed by extension at 72 °C for 1 min for 30 cycles. The process was ended with a final extension at 72 °C for 10 min. The PCR amplified product was resolved in 1% agarose electrophoresis and analyzed using Gene Flash Syngene Bio Imaging.

The PCR product was first cloned into pJET1.2 PCR cloning vector (Fermentas) according to the manufacturer's instructions. Ten microliters of the ligation reaction was then transformed into XL1-Blue competent cells. The colonies containing the recombinant plasmid were identified and confirmed by PCR. Purified plasmids from the selected clones were then outsourced to 1st BASE Laboratories Sdn. Bhd. for sequencing using T7 promoter and reverse priming site primers. The obtained sequences were then analyzed using NCBI BLAST (Basic Local Alignment Search Tool) program (http://blast.ncbi.nlm.nih.gov).

Construction of the expression vector pRSETB-Omp

Four positive clones were picked and cultured in 2 ml of Luria-Bertani (LB) broth medium with 100 µg/ml ampicillin for propagation of plasmids. The plasmids were isolated using alkaline lysis protocol (Birnboim and Doly 1979). The *Omp* gene from one of the positive clones was released by restriction enzyme digestion with *Bam*HI and *Sal*I and further sub-cloned into an expression vector pRSET B (Invitrogen). One microliter of the purified recombinant plasmid (pRSET/Omp) was then transformed into chemically competent expression host cells, *E. coli* JM109 (DE3) using heat shock. The recombinant transformants were selected using ampicillin (100 µg/ml) on LB agar plates.

Expression of the recombinant Omp gene in E. coli

The transformed *E. coli* JM109 (DE3) with pRSET/Omp was cultured in 5 ml 2x Yeast Extract and Tryptone (YT) broth medium containing 100 µg/ml ampicilin at 37 °C for 16 h. One milliliter of this culture was inoculated into 100 ml of the 2xYT broth medium containing 100 µg/ml ampicillin in a shake flask and continually grown at 37 °C. Once the optical density (OD) of the cultures had reached 0.6–0.7 at 600 nm (OD_{600}), cells were induced with 0.4 mM isopropyl thiogalactoside (IPTG). Whole cell lysates of the bacteria were prepared after 1h, 3 h, 5 h and overnight of the IPTG induction. *E. coli* JM109 (DE3) host cells with only pRSET B plasmid were used as control.

Purification of the Omp recombinant protein

The bacterial culture was harvested by centrifugation at 6,000xg for 10 min at 4 °C and the cell pellet was resuspended in 15 ml of guanidinium lysis buffer, pH7.8 (6 M guanidine hydrochloride, 20 mM sodium phosphate pH 7.8, 500 mM NaCl) and incubated at 4 °C overnight. The lysate was

centrifuged at 6,000xg for 10 min to pellet the cellular debris and the supernatant was transferred to a fresh tube. Ten mililitres/ml of lysate solution was mixed well with 4 ml nickel-chelating resin (ProBond^{TM} purification system, Invitrogen) by gentle agitation.

The resin was settled by gravity in a column and the supernatant was aspirated. The column was washed twice with 4 ml denaturing binding buffer, pH 7.8 (8 M urea, 20 mM sodium phosphate pH 7.8, 500 mM NaCl) followed by 4 ml denaturing wash buffer, pH 6.5 (8 M urea, 20 mM sodium phosphate pH6.0, 500 mM NaCl). The protein was eluted by adding 8 ml denaturing elution buffer, pH4.0 (8 M urea, 20 mM sodium phosphate pH 4.0, 500 mM NaCl).

SDS PAGE and Western blot analysis

The purified recombinant Omp protein was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Using prepared anti-cMyc antibody against the recombinant Omp protein tagged with cMyc, the expression of the recombinant protein was analysed by Western blot according to standard protocol (Sambrook et al. 2000). A volume of 40 µl of protein sample was mixed well with an equal amount of 2x SDS loading buffer, and boiled for 10 min. To analyze the recombinant protein, 20% SDS polyacrylamide separating gel with 4% stacking gel was used. The cell lysates of uninduced *E. coli* BL21 containing expression vector was also analysed as a negative control. After electrophoresis, the gel was stained with Coomassie brilliant blue R250 to visualize the protein bands.

After SDS-PAGE, protein bands were transferred onto a polymer of vinylidene fluoride (PVDF) membrane which was treated with blocking solution (4% skim milk) for 15 min. The membrane was incubated with the anti-cMyc antibody with gentle agitation for 2 h and further incubated for 2 h with anti-mouse IgG alkaline phosphatase conjugate. After washing 3x, the membrane was incubated in the substrate solution (5-bromo-4-chloro-3-indolyl phosphate) until the bands were visualized.

Results and discussion

Cloning and sequencing of the Omp gene

Genomic DNA of *C. suhuiensis* and *Ca.* L. asiaticus were isolated together from the citrus midrib due to the non-culturable characteristic of the bacteria. To study Omp from *Ca.* L. asiaticus, the gene was PCR amplified using genome DNA as template. The PCR primers amplified a 877 bp fragment of Omp gene of *Ca.* L. asiaticus (*Figure 1*) which is the expected size of the partial Omp DNA sequence from the gene bank.

After the bacteria transformation, four colonies were picked from the transformed XL1-blue, plasmids were isolated and the DNA encoding the Omp gene was sequenced (*Figure 2a*). The nucleotide sequence of the Omp gene was found to be 100% identical with the *Ca.* L. asiaticus species from Thailand (Accession

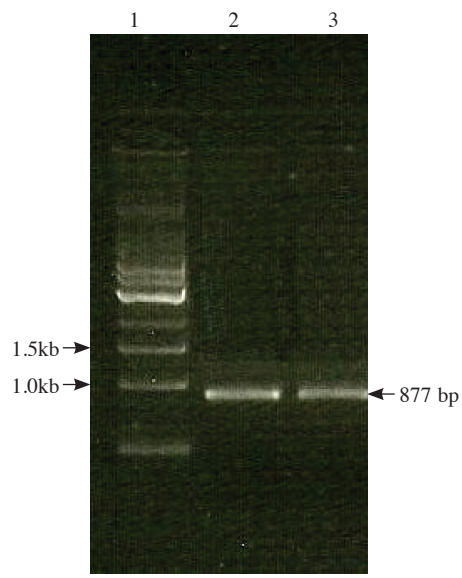

Figure 1. PCR amplification of partial Omp gene. Lane 1, molecular wt marker (1kb ladder); Lanes 2–3, PCR product from ***Ca.*** *L. asiaticus*

```
tgaggatccatatttttagggagtcctatatccgcgggttttgatctccaaaaaacc
 E  D  P  Y  F  L  G  S  P  I  S  A  G  F  D  L  Q  K  T
catcttgaagatggctctcttgacataaatgatgaatctgctgctgtacgtatgata
H  L  E  D  G  S  L  D  I  N  D  E  S  A  A  V  R  M  I
gttcctattactgaaagcatatcgacaagttttaagtatgatcttaggtttttacaa
V  P  I  T  E  S  I  S  T  S  F  K  Y  D  L  R  F  L  Q
tatggcgctatatcagaaaaagaaaagatcccttcgatatatacaacgttaatagaa
Y  G  A  I  S  E  K  E  K  I  P  S  I  Y  T  T  L  I  E
catggaaaattcagcagccattctatttcccaaagtatcatctataatacactagat
H  G  K  F  S  S  H  S  I  S  Q  S  I  I  Y  N  T  L  D
aacccaattgtgccacgtaaaggcatgttgatatcatcttcttatgattatgcaggt
N  P  I  V  P  R  K  G  M  L  I  S  S  S  Y  D  Y  A  G
tttggaggagattctcaatatcatcggattggatctcgagcatcgtatttttatctt
F  G  G  D  S  Q  Y  H  R  I  G  S  R  A  S  Y  F  Y  L
ctatcagatgattctgatattgtcggttctttacgatttggatatggatgtgtcatt
L  S  D  D  S  D  I  V  G  S  L  R  F  G  Y  G  C  V  I
cctagcaataaaaatttgcaattgtttgatcagttctcagtgagttcgaattattat
P  S  N  K  N  L  Q  L  F  D  Q  F  S  V  S  S  N  Y  Y
ctgaggggatttgcatataagggtataggtccgcgtgtggataagaaatatgcgatt
L  R  G  F  A  Y  K  G  I  G  P  R  V  D  K  K  Y  A  I
ggaggtaagatttattcgtctgcaagtgcagcagtgagttttcccatgcctcttgtt
G  G  K  I  Y  S  S  A  S  A  A  V  S  F  P  M  P  L  V
cctgaaagggctggtttgcgtggtgctttttttgttgattctgcgactctttatgca
P  E  R  A  G  L  R  G  A  F  F  V  D  S  A  T  L  Y  A
aatcatgttgctctcggtgccgataagctggaagggaatgattctttctggcgtgtt
N  H  V  A  L  G  A  D  K  L  E  G  N  D  S  F  W  R  V
tctactggagtagaaataatgtggaattctccactcgggatgatgggtgtctattat
S  T  G  V  E  I  M  W  N  S  P  L  G  M  M  G  V  Y  Y
ggtataccattgcgtcaccgagagggtgataaaattcagcagtttggttttcgtata
G  I  P  L  R  H  R  E  G  D  K  I  Q  Q  F  G  F  R  I
ggtaatcgcatggtcgacact

G  N  R  M
```

*Figure 2a. Nucleotide and amino acid sequence of the **Ca.** L. asiaticus Omp gene (AY842432). The sites used to design the primers are underlined and restriction enzyme sites are shown in bold*

no. AY842432) in the NCBI database (*Figure 2b*).

Expasy Protscale online software (http://www.expasy.ch/tools/protscale.html) was used to analyse the cloned Omp amino acid sequence. The Omp partial sequence consisted of 289 amino acids with an estimated molecular weight of 32 kDa. The isoelectric point (pI) of the protein was estimated to be 6.68. The hydrophobicity test results from Kyte and Doolittle (1982) scale (*Figure 3*) showed that the protein was more hydrophilic where it was bound to the water molecules with the hydrophilic heads on the surface of the membranes. The hydrophilic Omp expressed on the surface of pathogens have high potential to produce specific antibodies (Khushiramani et al. 2007).

One positive clone was selected for recombination into pRSET B expression vector (Invitrogen). Four positive clones were picked and screened for the insert using the same primers and PCR conditions. Positive colonies showed the 877 bp amplicon. The clone was named pRSETB/Omp. The purified pRSETB/Omp plasmid was digested with two internal restriction enzyme cutting sites, *Xho*I and *Hind*III. Two DNA fragments, 500 bp and 3.2 kb were obtained after the digestion (*Figure 4*) which indicated the right cloning orientation of the partial Omp gene.

```
                1396                                                    1447
Thai(AY842432): --TTTAGTGTTGAGGATCCATATTTTTTAGGGAGTCCTATATCCGCGGGTT
Malaysia      : ----------TGAGGATCCATATTTTTTAGGGAGTCCTATATCCGCGGGTT
                1448                                                    1499
Thai(AY842432): TTGATCTCCAAAAAACCCATCTTGAAGATGGCTCTCTTGACATAAATGATG
Malaysia      : TTGATCTCCAAAAAACCCATCTTGAAGATGGCTCTCTTGACATAAATGATG
                1500                                                    1551
Thai(AY842432): AATCTGCTGCTGTACGTATGATAGTTCCTATTACTGAAAGCATATCGACAA
Malaysia      : AATCTGCTGCTGTACGTATGATAGTTCCTATTACTGAAAGCATATCGACAA
                1552                                                    1603
Thai(AY842432): GTTTTAAGTATGATCTTAGGTTTTTACAATATGGCGCTATATCAGAAAAAG
Malaysia      : GTTTTAAGTATGATCTTAGGTTTTTACAATATGGCGCTATATCAGAAAAAG
                1604                                                    1655
Thai(AY842432): AAAAGATCCCTTCGATATATACAACGTTAATAGAACATGGAAAATTCAGCA
Malaysia      : AAAAGATCCCTTCGATATATACAACGTTAATAGAACATGGAAAATTCAGCA
                1656                                                    1707
Thai(AY842432): GCCATTCTATTTCCCAAAGTATCATCTATAATACACTAGATAACCCAATTG
Malaysia      : GCCATTCTATTTCCCAAAGTATCATCTATAATACACTAGATAACCCAATTG
                1708                                                    1759
Thai(AY842432): TGCCACGTAAAGGCATGTTGATATCATCTTCTTATGATTATGCAGGTTTTG
Malaysia      : TGCCACGTAAAGGCATGTTGATATCATCTTCTTATGATTATGCAGGTTTTG
                1760                                                    1811
Thai(AY842432): GAGGAGATTCTCAATATCATCGGATTGGATCTCGAGCATCGTATTTTTATC
Malaysia      : GAGGAGATTCTCAATATCATCGGATTGGATCTCGAGCATCGTATTTTTATC
                1812                                                    1863
Thai(AY842432): TTCTATCAGATGATTCTGATATTGTCGGTTCTTTACGATTTGGATATGGAT
Malaysia      : TTCTATCAGATGATTCTGATATTGTCGGTTCTTTACGATTTGGATATGGAT
                1864                                                    1915
Thai(AY842432): GTGTCATTCCTAGCAATAAAAATTTGCAATTGTTTGATCAGTTCTCAGTGA
Malaysia      : GTGTCATTCCTAGCAATAAAAATTTGCAATTGTTTGATCAGTTCTCAGTGA
                1916                                                    1967
Thai(AY842432): GTTCGAATTATTATCTGAGGGGATTTGCATATAAGGGTATAGGTCCGCGTG
Malaysia      : GTTCGAATTATTATCTGAGGGGATTTGCATATAAGGGTATAGGTCCGCGTG
                1968                                                    2019
Thai(AY842432): TGGATAAGAAATATGCGATTGGAGGTAAGATTTATTCGTCTGCAAGTGCAG
Malaysia      : TGGATAAGAAATATGCGATTGGAGGTAAGATTTATTCGTCTGCAAGTGCAG
                2020                                                    2071
Thai(AY842432): CAGTGAGTTTTCCCATGCCTCTTGTTCCTGAAAGGGCTGGTTTGCGTGGTG
Malaysia      : CAGTGAGTTTTCCCATGCCTCTTGTTCCTGAAAGGGCTGGTTTGCGTGGTG
                2072                                                    2123
Thai(AY842432): CTTTTTTTGTTGATTCTGCGACTCTTTATGCAAATCATGTTGCTCTCGGTG
Malaysia      : CTTTTTTTGTTGATTCTGCGACTCTTTATGCAAATCATGTTGCTCTCGGTG
                2124                                                    2175
Thai(AY842432): CCGATAAGCTGGAAGGGAATGATTCTTTCTGGCGTGTTTCTACTGGAGTAG
Malaysia      : CCGATAAGCTGGAAGGGAATGATTCTTTCTGGCGTGTTTCTACTGGAGTAG
                2176                                                    2227
Thai(AY842432): AAATAATGTGGAATTCTCCACTCGGGATGATGGGTGTCTATTATGGTATAC
Malaysia      : AAATAATGTGGAATTCTCCACTCGGGATGATGGGTGTCTATTATGGTATAC
                2228                                                    2279
Thai(AY842432): CATTGCGTCACCGAGAGGGTGATAAAATTCAGCAGTTTGGTTTTCGTATAG
Malaysia      : CATTGCGTCACCGAGAGGGTGATAAAATTCAGCAGTTTGGTTTTCGTATAG
                2280                                                    2294
Thai(AY842432): GTAATCGCATGTAG
Malaysia      : GTAATCGCATG---
```

Figure 2b. Comparative DNA sequence analysis of Omp for ***Ca.*** *L. asiaticus from Malaysia and Thailand (AY842432) using CLUSTALW*

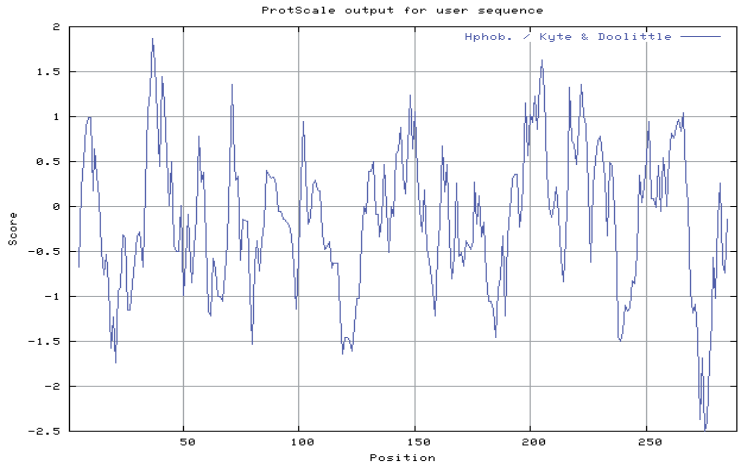

Figure 3. Hydropathicity plot of Omp amino acid sequence

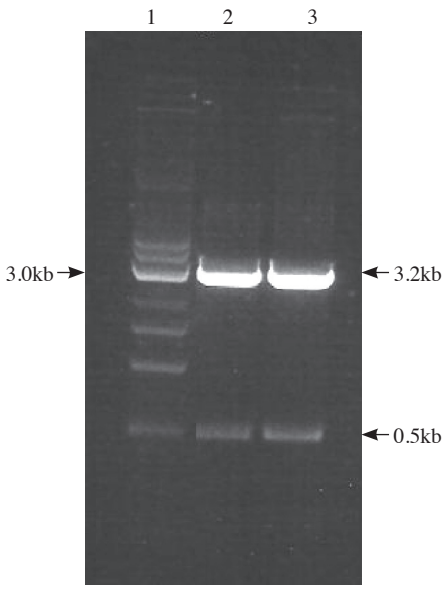

Figure 4. pRSETB/Omp plasmid digestion with two internal restriction enzymes (XhoI and HindIII) to check cloning orientation. Lane 1, molecular wt marker (1kb ladder); Lanes 2 and 3, pRSETB/Omp plasmid digestion

Construction of the expression vector

One of the positive pRSETB/Omp clones with *Bam*HI and *Sal*I flanking the cloning sites was used for the construction of the expression vector. All the elements necessary for the expression were included in the 6xHis upstream with the start codon and cMyc region downstream (*Figure 5*).

His-tagged Omp was purified by using a nickel chelating column and cMyc-tagged Omp to serve the purpose of Omp expression confirmation by anti-cMyc detection.

Expression of the Omp

The molecular mass of the recombinant protein obtained was approximately 30kDa using 12% SDS-PAGE which is the expected size of the protein based on the DNA sequence. Based on the western blot analysis, the amount of protein expressed was dramatically increased after induction with IPTG. The overnight protein expression gave the highest concentration of the partial Omp protein and the protein stability was maintained after overnight incubation (*Figure 6*).

Purification of the Omp recombinant protein

Candidatus L. asiaticus Omp is an insoluble protein where the recombinant protein remains in the pellet after the cell lysis process (*Figure 7*). ProBond purification system (Invitrogen) was used to purify the insoluble recombinant *Ca*. L. asiaticus Omp protein under denaturing conditions. The high affinity of nickel-chelating resin in the purification system is effective towards recombinant fusion proteins

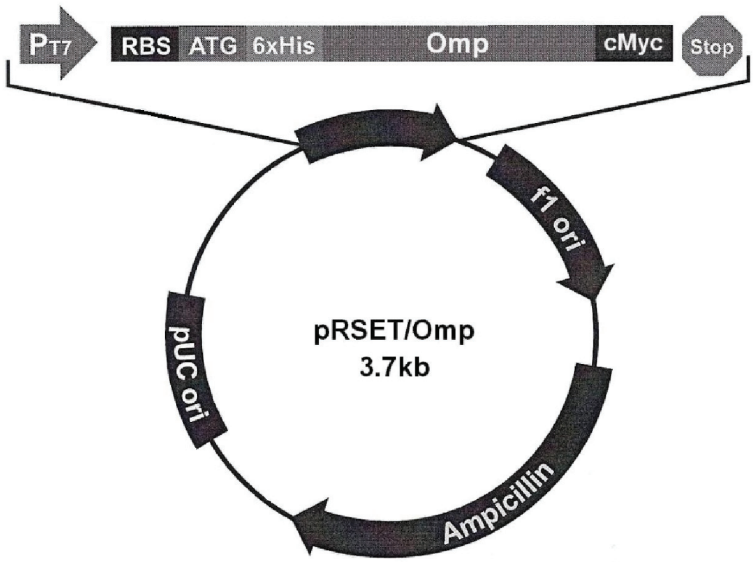

Figure 5. Construction of the recombinant vector containing the cassette for Omp expression

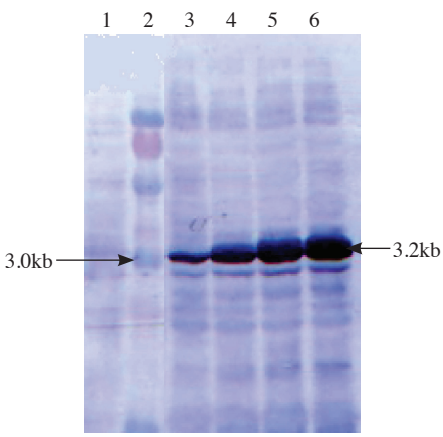

Figure 6. Western blot analysis after expression of recombinant OmpCL protein
Lane 1 = non-recombinant JM109 with IPTG induction
Lane 2 = molecular wt marker (PageRuler^{TM} Prestained Protein Ladder Plus)
Lanes 3–6 = recombinant JM109 after 1h, 3h, 5h and overnight of IPTG induction

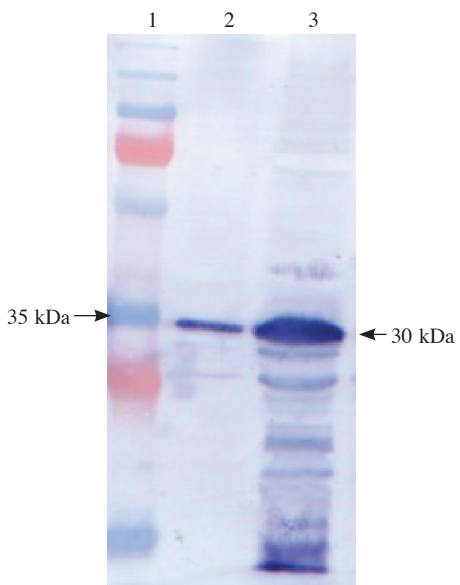

Figure 7. Western blot analysis for recombinant Omp solubility test
Lane 1 = molecular wt marker (PageRulerTM Prestained Protein Ladder Plus);
Lane 2 = soluble protein;
Lane 3 = insoluble protein

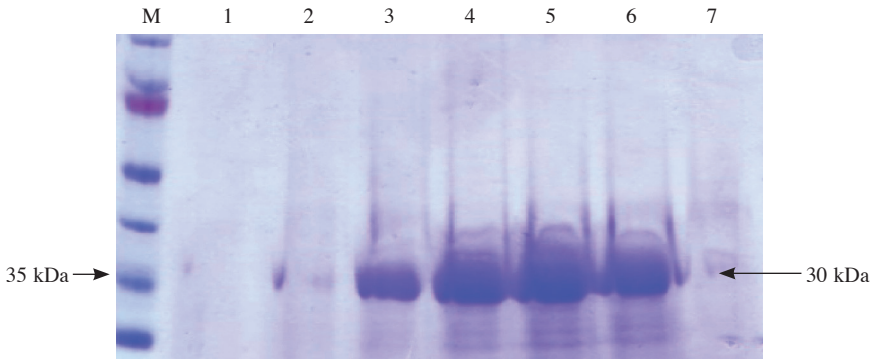

Figure 8. SDS-PAGE analysis of purified His-tagged OmpCL protein fractions (F1-F7) under denaturing conditions.
Lane M, protein marker;
Lane 1 = F1 of OmpCL protein
Lane 2 = F2 of OmpCL protein
Lane 3 = F3 of OmpCL protein
Lane 4 = F4 of OmpCL protein
Lane 5 = F5 of OmpCL protein
Lane 6 = F6 of OmpCL protein
Lane 7 = F7 of OmpCL protein

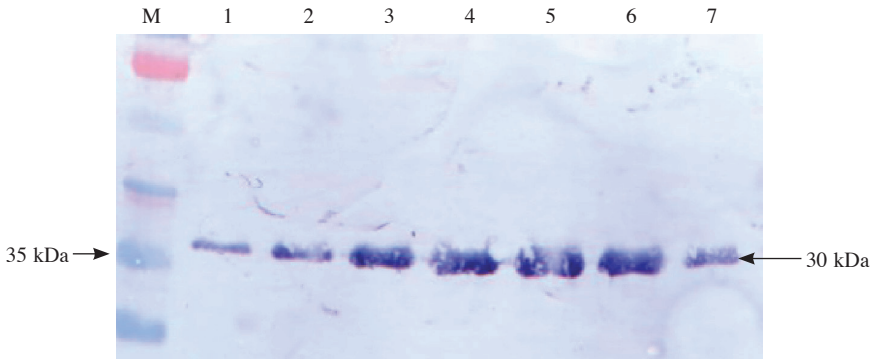

Figure 9. Western blot analysis of purified OmpCL protein fractions (F1-F7) under denaturing conditions with anti-cMyc antibody
Lane M, protein marker;
Lane 1 = F1 of OmpCL protein
Lane 2 = F2 of OmpCL protein
Lane 3 = F3 of OmpCL protein
Lane 4 = F4 of OmpCL protein
Lane 5 = F5 of OmpCL protein
Lane 6 = F6 of OmpCL protein
Lane 7 = F7 of OmpCL protein

containing 6xHis residues at N-terminal. Polyhistidine tagging is the option for purifying recombinant proteins in denaturing conditions because its mode of action is dependent only on the primary structure of proteins and the histidine binding is based on the pH instead of imidazole binding.

The cell lysate was loaded into the nickel-chelating resin column and washed with denaturing wash buffer (pH 6.5)

3x before eluting the purified protein with elution buffer (pH 4.0). Affinity chromatography removed all the major contaminating bands and impurities after non-specific elution with higher pH of washing buffer. Seven fractions (F1-F7) of the protein elution were collected and run on the SDS-PAGE gel. The purified protein was successfully eluted from the column at fractions 3 to 6. The size of the purified protein on SDS-PAGE gels was estimated to be approximately 30kDa (*Figure 8*), which is similar to the theoretical size deduced from the amino acid sequence.

Approximately 3 mg of purified Omp per liter of medium was obtained. In order to prove that the purified protein was the recombinant Omp, western blot was carried out using the anti-cMyc antibody. From the western blot analysis, the size of the recombinant protein was approximately 30 kDa which was similar to the estimated size (*Figure 9*).

Conclusion

Partial Omp gene of *Ca*. L. asiaticus of 877 bp length was successfully cloned into pRSET B expression vector and the clone was named pRSET/Omp clone. The pRSET/Omp plasmid was transformed into the expression host *E. coli* JM109 (DE3) followed by the expression of recombinant Omp protein with 0.4 mM IPTG induction at 37 °C. This study showed that the overnight protein expression after IPTG induction gave the highest concentration of the 30 kDa recombinant protein. The insoluble recombinant Omp protein was purified under denaturing conditions and approximately 3 mg of Omp was obtained from 1 liter of medium culture. This 30 kDa recombinant protein will be used in future studies to obtain the antibodies against *Ca*. L. asiaticus.

Acknowledgement

This study was supported by the Science Fund Grant (02-03-08-SF0021) from the Ministry of Science, Technology & Innovation, Malaysia (MOSTI) and Wang Rizab MARDI (JP-RB-0064). The authors would like to thank Ms Kamariah Mohamed from Horticulture Research Centre, MARDI Jerangau for supplying the citrus leave samples.

References

Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res*. 7(6): 1513–1523

Bové, J.M. (2006). Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. *J. Plant Pathol*. 88: 7–37

Garnier, M. and Bové, J.M. (1993). Citrus greening disease. *Proc. 12th Conference of the International Organization of Citrus Virologists*. University of Califonia, Riverside, p. 212–219

Gottwald, T.R., Da Graca, J.V. and Bassanezi, R.B. (2007). Citrus huanglongbing the pathogen and its epidemiology, and impact. *Plant Healthy Progress*. doi:10.1094/PHP-2007-0906-01-RS

Hajivand, S., Thohirah, L.A., Kamaruzaman, S. and Siti, N.A.A. (2011). Potential use of selected citrus rootstocks and interstocks against HLB disease in Malaysia. *Crop Protection* 30: 521–525

Halbert, S.E. and Manjunath, K.L. (2004). Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: A literature review and assessment of risk in Florida. *Florida Entomologist* 87(3): 330–352

Ibrahim, O., Habibuddin, H., Shamsuddin, M.O., Tan, H.H., Nousa, N. and. Jatil Aliah, T. (2003). Development of disease free planting materials of citrus varieties for commercial planting. *Proc. national horticultural conference*, Bangi, Malaysia. 20–23 Oct. 2003, p.165–168. Serdang: MARDI

Jagoueix, S., Bové, J.M. and Garnier, M. (1996). PCR detection of the two 'Candidatus' liberobacter species associated with greening disease of citrus. *Molecular and Cellular Probes* 10: 43–50

Khushiramani, R., Girisha, S.K. and Karunasagar, I. (2007). Protective efficacy of recombinant OmpTS protein of *Aeromonas hydrophila* in Indian major carp. *Vaccine* 25: 1157–1158

Kyte, J. and Doolittle, R. (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol*. 157: 105–132

Lafleche, D. and Bové, J.M. (1970). Mycoplasmes dans les argumes attentis de "greening", de stubborn, ou des maladies similaries. *Fruits* 25: 455–465

Lin, H., Chen, C., Doddapaneni, H., Duan, Y., Civerolo, E.L., Bai, X. and Zhao, X. (2010). A new diagnostic system for ultra-sensitive and specific detection and quantification of *Candidatus Liberibacter asiaticus*, the bacterium associated with citrus Huanglongbin. *Journal of Microbiology Methods* 81: 17–25

Murray, H. and Thompson, W. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res*. 8: 4321–4326

Omrani, M., Ansari, M.H.K. and Agaverdizadae, D. (2009). PCR and ELISA methods (IgG and IgM): Their comparison with conventional techniques for diagnosis of *Mycobacterium tuberculosis*. *Journal of Biological Sciences* 12: 373–377

Ohtsu, Y. (1998). Recent progress on citrus greening research in Asia including a serological. *Proceeding of a regional workshop on disease management of banana and citrus through the use of disease-free planting materials*. Davao City, Philippines, p. 57–61

Sambrook, J., Maniatis, T. and Fritsch, E.F. (2000). *Molecular cloning: A Laboratory Manual*. 3rd ed. Cold Spring Harbor, New York, USA: Cold Spring Harbor Laboratory

Sankaran, S., Ehsani, R. and Etxeberria, E. (2010). Mid-infrared spectroscopy for detection of Huanglongbing (greening) in citrus leaves. *Talanta* 83: 574–581

Teo, C.H. (1995). Citrus greening and tristeza virus diseases in Sarawak, East Malaysia. Paper presented in International symposium on integrated management of insect-borne viral diseases of tropical fruits, Pingtung, Taiwan, ROC

Abstrak

Penyakit *greening* ialah penyakit utama yang menyerang tanaman limau di kebanyakan negara termasuk Malaysia. Penyakit ini disebabkan oleh sejenis bakteria, *Candidatus* Liberibacter asiaticus, yang tidak dapat dikultur dan kehadirannya hanya terhad kepada saluran floem tisu perumah sahaja. Kepekatannya yang amat rendah di dalam pohon yang dijangkiti menyukarkan usaha memperoleh kuantiti patogen yang mencukupi untuk dijadikan antigen bagi penghasilan antibodi terhadapnya. Dalam kajian ini, protein rekombinan membran luaran (Omp) separa telah dihasilkan untuk dijadikan sebagai calon antigen semasa pengeluaran antibodi poliklon. Gen pengekodan 'Omp' tersebut diperbesar dengan menggunakan DNA yang diekstrak daripada urat tengah daun pokok limau yang dijangkiti penyakit. Serpihan PCR daripada gen tersebut sepanjang 877 bp ditulenkan, dicerna dengan enzim penyekatan dan seterusnya diklon ke dalam plasmid vektor penglahiran bakteria, pRSET B. Selepas konstruk disahkan melalui penggunaan enzim penyekatan dan penjujukan DNA, penglahiran bakteria dijalankan dengan menggunakan *E. coli* JM109 (DE3) dan Omp rekombinan dirangsang dengan isopropil thiogalaktosida (IPTG). Saiz protein yang dihasilkan ialah 30 kDa seperti yang ditunjukkan melalui migrasi dalam gel elektroforesis menggunakan 10% sodium dodesil sulfat poliakrilamid (SDS-PAGE). Omp rekombinan ditulenkan dengan resin pengkelatan nikel dalam keadaan penyahaslian. Protein rekombinan yang diperoleh akan digunakan untuk menghasilkan antibodi terhadap patogen penyebab penyakit greening limau tersebut dalam kajian akan datang.

Accepted for publication on 15 July 2011