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Cloning and expression of gene encoding P23 protein from *Cryptosporidium parvum*

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ABSTRACT

We cloned the *cp23* gene coding P23 (glyco)protein from *Cryptosporidium parvum* isolated from Thua Thien Hue province, Vietnam. The coding region of *cp23* gene from *C. parvum* is 99% similar with *cp23* gene deposited in NCBI (accession number: U34390). SDS-PAGE and Western blot analysis showed that the *cp23* gene in *E. coli* BL21 Star™ (DE3) produced polypeptides with molecular weights of approximately 37, 40 and 49 kDa. These molecules may be non-glycosylated or glycosylated P23 fusion polypeptides. Recombinant P23 protein purified by GST (glutathione S-transferase) affinity chromatography can be used as an antigen for *C. parvum* antibody production as well as to develop diagnostic kit for *C. parvum*.

Key words: *cp23* gene, *Cryptosporidium parvum*, P23 protein

Introduction

Cryptosporidium parvum is a protozoan parasite that infects the intestinal epithelium of humans and animals causing the enteric disease. Cryptosporidiosis is a cause of life-threatening disease in immunodeficient people and it has been estimated that 10-15% of patients (or higher) died as a result of infection with *Cryptosporidium* in the world (Jenkins & Fayer, 1995; Wang et al., 2009).

To detect *C. parvum* infection, several methods were developed such as sedimentation-flotation technique (McNabb et al., 1985), oocysts staining (O'Donoghue, 1995), PCR amplification (Laxer et al., 1991). However, these approaches could not be applied widely because of its high cost (Wang et al., 2009). Rapid immuno-chromatographic assays for detecting infections with *C. parvum* in fecal samples were evaluated. The sensitivity (75%) and specificity (100%) of this assay were relatively high compare with modified Ziehl-Neelsen staining method (Klein et al., 2009; Luginbühl et al., 2005).

A 23 kDa (glyco)protein of *C. parvum* (P23 protein) is a sporozoite surface protein that is geographically conserved among isolated *C. parvum* strains. The P23 protein was identified as an antigen with neutralization-sensitive epitopes. Therefore, it is considered to be a potential candidate for the development of an effective vaccine against cryptosporidiosis, as well as for the diagnosis of cryptosporidiosis by ELISA since it is likely to detect *C. parvum* in various geographical regions (Perryman et al., 1999).

In this work, we report the cloning and expression of gene encoding P23 protein from *C. parvum* isolated in Thua Thien Hue province, Vietnam. Our recombinant P23 protein will be used as an antigen for production of anti-P23 antibody and to

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develop diagnostic kit for *C. parvum* based on immunoassays in the future.

Materials and Methods

Detection of *C. parvum* oocysts

Bovine fecal samples were collected from barns in Thua Thien Hue province, Vietnam. Sample preparation for detection of *C. parvum* oocysts was performed as described by Wang et al (2009).

The oocysts of *C. parvum* were detected by the modified Ziehl-Neelsen (MZN) method. The oocysts stained red on a pale green background and they appear as small discs with 4-6 µm in diameter, but the degree and proportion of staining varies with individual oocysts (World Organisation for Animal Health, 2008).

DNA isolation and amplification of cp23 gene

Genomic DNA of *C. parvum* was isolated by QIAamp DNA Stool Mini Kit (Qiagen, Max-Volmer-Straße, Hilden). Genomic DNA was then used as template in PCR amplification with the specific primers (Cp23F: 5'-ACGGATCCAAAAATGGGTTGTT-3' and Cp23R: 5'-ACGGATCCTAATTTAGGCATCA-3'), which designed base on the 23 kDa protein synthetic region of the *cp23* gene from NCBI (accession number: U34390). GGATCC sequence was designed for *Bam*HI cloning site in pGEX 4T-3 expression vector.

The PCR component consisted of 2 µl DNA template, 12.5 µl 2× GoTag PCR master mix (Promega, Madison, WI), and 10 pmol each primer. Distilled water was added to a final volume of 25 µl. After 3 min of genomic DNA denaturation at 94°C, 40 cycles of 1 min for denaturation at 94°C, 45 seconds for annealing at 45°C and 1 min for polymerization at 72°C were carried out in the iCycler thermocycler (Bio-Rad, Hercules, CA). In the final cycle, the temperature of 72°C was held for an additional 7 min. The PCR products were purified by Quantum Prep™ Freeze N Squeeze DNA Gel Extraction Spin Columns Kit (Bio-Rad) after electrophoresis on 2% agarose gel.

Cloning cp23 gene

PCR products (*cp23* gene) were ligated into pGEM®-T Easy vector (Promega), the ligation component consists of 50 ng vector, 5 µl buffer, 3 unit T4 DNA ligase, and 25 ng PCR product. Distilled water was added to a final volume of 10 µl. The ligation was incubated at 25°C for 1 h and at 4°C overnight. Ligation products were transformed into chemically competent *E. coli* DH5α cells by heat-shock method. The presence of the insert was determined by colony direct PCR followed by 2% agarose gel electrophoresis. Positive colonies were cultured on 5 ml of LB medium supplemented with 50 µg/ml ampicillin for biomass production. Recombinant vector (pGEM®-T Easy/*cp23*) was then isolated by PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, CA). The PCR product was sequenced by the dideoxyterminator method on the 3130 Genetic Analyzer (Applied Biosystems®).

Expression of P23 protein in E. coli

The *cp23* gene was cut from pGEM-T Easy/*cp23* vector by *Bam*HI, and purified by Wizard® SV Gel and PCR Clean-Up System Kit (Promega), they were then ligated into *Bam*HI site of pGEX 4T-3 expression vector harboring glutathione S-transferase (GST) gene (GE Healthcare, Björkgatan, Uppsala). The ligation component consists of 0.5 µg vector, 5 µl 2× buffer, 4 µg DNA, 3 unit T4 DNA ligase, and distilled water was added to a final volume of 10 µl. The ligation was incubated at 4°C for overnight.

E. coli BL21(DE3) cells containing recombinant pGEX 4T-3/*cp23* vector was cultured in 400 ml LB medium supplemented with 50 µg/ml ampicillin on a rotation shaker with speed of 200 rpm at 37°C to an OD_{600} value (cell density) of 0.6. Isopropylthio-β-galactoside (IPTG) was added to a final concentration of 1 mM for induction and the culture was then incubated at 25°C for 4 h. The cell biomass was harvested by centrifugation at 10 000 rpm/4°C for 10 min, resuspended in 8 ml TNE buffer (50 mM Tris.HCl pH 7.5, 100 mM NaCl, 2 mM EDTA) and kept on ice bath for 1 hour. Total soluble protein was obtained after sonication for 5 min and centrifugation at 10 000 rpm/4°C for 5 min. Expression levels of P23 protein were determined by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE, 12% w/v). The gel slice was then stained with Coomassie Blue R-250 and the image was analyzed by Quality One software (ver 4.1, Bio-Rad).

Western blot

Total soluble protein was separated on a SDS-12% PAGE and then electroblotted to a nitrocellulose membrane. Western blot was performed using WesternDotTM 625 Western Blot Kit (Invitrogen) according to the manufacturer's instructions. Anti-P23 antibody was produced in mice by our laboratory (Immunology Laboratory, Institute of Biotechnology, Hue University). The image was collected under UV Transilluminator system (DyNA Light, Labnet,

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Edison, NJ) and analyzed by Quality One software (ver. 4.1, Bio-Rad).

Protein purification

The P23 protein was purified by Glutathione Sepharose™ 4B Purification System (GE Healthcare) according to the manufacturer's instructions. Eight milliliters of cell lysate was added to 2 ml prepared glutathione sepharose 4B and incubated for 30 min at room temperature, the mixture was then transferred to a 15-ml Falcon tube. The glutathione sepharose 4B resin was collected by centrifugation at 500× g for 5 min, washed by adding 5 ml 1× PBS and centrifuged at 500× g for 5 min again, repeat washing step 3 times. P23 protein was eluted by adding 1 ml elution buffer, incubated at room temperature for 30 min and centrifuged at 500× g for 5 min, repeat eluting step 2 times. Eluted protein from each step was mixtured to run SDS-PAGE.

Results

Cloning cp23 gene

According to Perryman et al (1996), the cDNA of *cp23* gene has a full-length of 602 bp with a coding region of 336 bp (from nucleotide 111 to 446). The coding frame of *cp23* gene has 345 bp in length including two *Bam*HI ends was amplified by specific primers.

Genomics DNAs from 10 oocysts of *C. parvum* were used as DNA templates for *cp23* gene isolation by PCR amplification. Figure 1 showed bands were of expected size of approximately 350 bp, this size is similar with the coding frame of *cp23* gene.

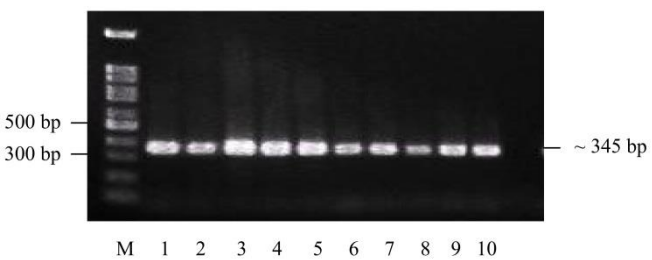

Figure 1. *PCR products with specific primers of cp23 gene. M: DNA size marker (GeneRuler 100 bp DNA Ladder). 1-10: C. parvum strains isolated from Thua Thien Hue province.*

PCR products were cloned into pGEM-T Easy vector, and one of them was sequenced. The results showed PCR product has 345 bp in length and high similarity with *cp23* gene from NCBI (99%) (Figure 2). The difference of nucleotide 327 between our *cp23* gene and *cp23* gene from NCBI did not change the deduced amino acid sequence (Figure 3).

```
Query   1    ATGGGTTGTTCATCATCAAAGCCAGAAACTAAAGTTGCTGAAAATAAATCTGCAGCAGAT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U34390  1    ATGGGTTGTTCATCATCAAAGCCAGAAACTAAAGTTGCTGAAAATAAATCTGCAGCAGAT  60

Query   61   GCTAACAAACAAAGAGAATTAGCTGAAAAGAAGGCTCAATTAGCCAAGGCTGTAAAGAAT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U34390  61   GCTAACAAACAAAGAGAATTAGCTGAAAAGAAGGCTCAATTAGCCAAGGCTGTAAAGAAT  120

Query   121  CCAGCTCCAATCAGCAACCAAGCTCAACAAAAGCCAGAAGAACCAAAGAAGTCCGAGCCT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U34390  121  CCAGCTCCAATCAGCAACCAAGCTCAACAAAAGCCAGAAGAACCAAAGAAGTCCGAGCCT  180

Query   181  GCTCCCAATAATCCTCCAGCTGCTGATGCACCAGCAGCCCAAGCTCCTGCTGCCCCTGCT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U34390  181  GCTCCCAATAATCCTCCAGCTGCTGATGCACCAGCAGCCCAAGCTCCTGCTGCCCCTGCT  240

Query   241  GAACCTGCTCCACAGGATAAGCCAGCTGATGCCCCAGCTGCTGAAGCTCCAGCTGCTGAA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U34390  241  GAACCTGCTCCACAGGATAAGCCAGCTGATGCCCCAGCTGCTGAAGCTCCAGCTGCTGAA  300

Query   301  CCTGCTGCTCAACAAGACAAGCCAGCCGATGCCTAA  336
             |||||||||||||||||||||||||| |||||||||
U34390  301  CCTGCTGCTCAACAAGACAAGCCAGCTGATGCCTAA  336
```

Figure 2. *Multiple alignment of the mature peptide region of cp23 gene from NCBI (accession number: U34390) and that of our cp23 gene.*

```
Query     1  MGCSSSKPETKVAENKSAADANKQRELAEKKAQLAKAVKNPAPISNQAQQKPEEPKKSEP
U34390    1  MGCSSSKPETKVAENKSAADANKQRELAEKKAQLAKAVKNPAPISNQAQQKPEEPKKSEP

Query   601  APNNPPAADAPAAQAPAAPAEPAPQDKPADAPAAEAPAAEPAAQQDKPADA
U34390   60  APNNPPAADAPAAQAPAAPAEPAPQDKPADAPAAEAPAAEPAAQQDKPADA
```

Figure 3. *Amino acid sequence of mature peptide region of cp23 gene from NCBI (accession number: U34390) and that of our cp23 gene.*

Expression of cp23 gene

The *cp23* gene was cut from recombinant pGEM T-Easy/*cp3* vector by *Bam*HI and inserted into pGEX 4T-3 expression vector. The pGEX 4T-3/*cp23* vector was transformed into *E. coli* BL21(DE3) cells and positive colonies from selection culture were checked by PCR. Our result showed that a DNA band occurred with the expected size of 345 bp (Figure 4), this suggested that the pGEX 4T-3/*cp23* vector successfully transformed into *E. coli* cells.

Induction was then performed at low temperature of 25°C to avoid formation of inclusion bodies (Makrides, 1996). Expression of the *cp23* gene was expected to produce protein of approximately 49 kDa (23 kDa of P23 protein and 26 kDa of glutathione S-transferase (GST) tag fussion fragment). The results of SDS-PAGE and Western blot showed that there are three protein bands with molecular weights approximate of 49, 40 and 37 kDa (Figure 5).

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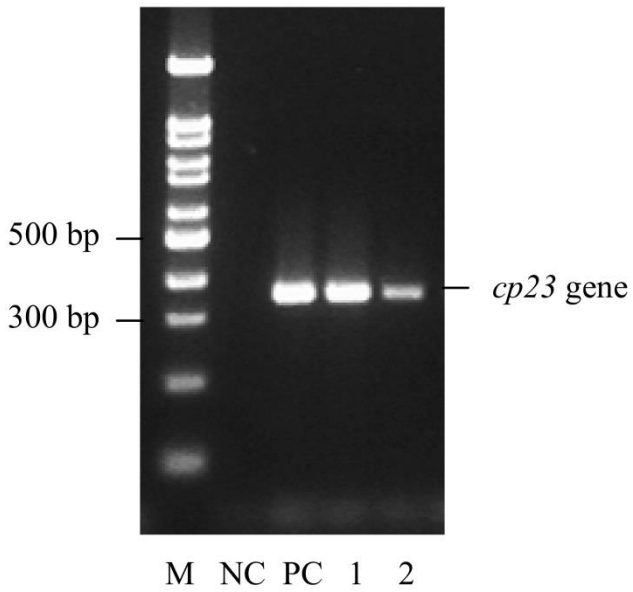

Figure 4. *Determination of cp23 gene in transformed E. coli BL21 (DE3) cells. M: DNA size marker, NC: negative control (non-transformed E. coli BL21(DE3) cells), PC: positive control (PCR product from pGEM T-Easy/cp23 vector), 1-2: PCR products from transformed E. coli BL21(DE3) cells containing pGEX 4T-3/cp23 vector.*

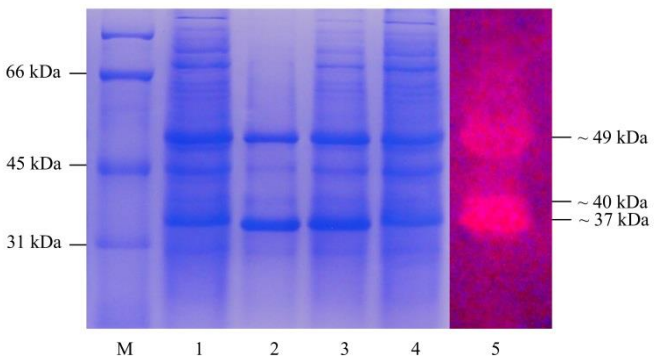

Figure 5. *Expression of P23 polypeptides in E. coli BL21(DE3) cells. M: protein weight standard, 1-4: SDS-PAGE of P23 fusion polypeptides after IPTG induction from 2-8 h for transformed E. coli BL21(DE3) cells, 5: Western blot analysis after 6 h of IPTG induction.*

In *C. parvum*, the *cp23* gene encodes a polypeptide chain with 111 amino acids (molecular weight approximate of 11.3 kDa). After P23 protein was synthesized, a *N*-glycosylation was performed to produce a glycoprotein with molecular weight approximate of 23 kDa (or 27 kDa in some cases based on electrophoresis condition) (Priest et al., 1999; Wang et al., 2009). In this study, the P23 polypeptide chain may be glycosylated or not. If it was glycosylated, molecular weight (fusion protein) will be approximate of 49 kDa or 40 kDa (glycosylated in a diffirent form); if not, molecular weight (fusion polypeptide) will be approximate of 37 kDa (11.3 kDa of P23 polypeptide and 26 kDa of GST tag).

In some previous reports, P23 glycoprotein from *C. parvum* were expressed successfully in *E. coli* based on different expression systems such as pET 30a(+) (Liu et al., 2010), pGEX 4T (Bonafonte et al., 2000), pGEX 4T-2 (Borad et al., 2012; Priest et al., 1999), or pGEX-5X-2 vector (Shahbazi et al., 2009; Shayan et al., 2008). Another host also was used for expression of P23 protein to be *Toxoplasma gondii* (Shirafuji et al., 2005). All of them are glycosylated forms with different molecular weight, i.e. 43 kDa (Shahbazi et al., 2009) or 46 kDa (Bonafonte et al., 2000; Wang et al., 2009) (fusion protein), 23 kDa (Inpankaew et al., 2009; Shahbazi et al., 2009; Shirafuji et al., 2005) or 27 kDa (Liu et al., 2010; Priest et al., 1999) (non-fusion protein). The difference in non- or fusion proteins indicated that P23 protein may be glycosylated in different forms. Other antigenic *C. parvum* protein also expressed in *E. coli* to be CP15 (Jenkins & Fayer, 1995).

The lysate of induced *E. coli* BL21(DE3) cells containing pGEX-4T-3/*cp23* vector were purified by GST chromatography using glutathione sepharose 4B resin. The results of SDS-PAGE showed two strong protein bands have molecular weights of approximately 37 kDa and 49 kDa, another one is very weak (appx. 40 kDa) (Figure 6).

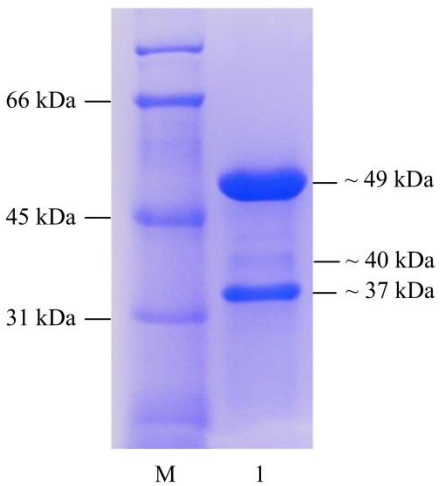

Figure 6. *Protein purification by GST chromatography. M: protein weight standard, 1: P23 polypeptides.*

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Conclusion

A *cp23* gene coding P23 protein from *C. parvum* was cloned and its sequence has 99% similarity with the *cp23* gene from NCBI. The *cp23* gene in *E. coli* BL21(DE3) produced polypeptides with molecular weights of approximately 37, 40 and 49 kDa; they may be non-glycosylated and glycosylated fusion polypeptides. Western blot analysis showed that 3 fusion polypeptide chains have a GST tag and an epitope for immunological response. Protein purification using GST affinity chromatography was harvested two strong bands (37 and 49 kDa).

Acknowledgement

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