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Cloning and Expression of ORF124 Koi Herpesvirus as a Vaccine

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Abstract

Koi herpesvirus (KHV) which also known as *Cyprinid herpesvirus* 3 (CyHV-3), Koi herpes-like virus, and carp interstitial nephritis gill necrosis virus (CNGV), caused significant morbidity and mortality in koi and common carp (*Cyprinus carpio*). The case fatality rate of this disease is 80–100%. Glycoprotein has been used for vaccine development as sub unit vaccine against viruses. The aim of this research was to clone and express membrane glycoprotein ORF124 KHV as a candidate of recombinant vaccine. ORF124 KHV gene was successfully cloned into pBSKS and sequenced. Result showed that ORF124 KHV (isolate from Indonesia) had 100 % similarity with *Cyprinid herpesvirus* 3 strain TUMST1 (from Japan), 99% similarity with *Koi herpesvirus* strain KHV-U (from USA) and *Koi herpesvirus* strain KHV-I (from Israel). Prediction analysis of T and B cell epitopes showed that ORF124 KHV protein had 14 and 11 T cell epitopes (IAd, Rothbard/Taylor pattern), and had 10 B cell epitopes, suggested that the protein can be used as a vaccine candidate. ORF124 gene has been expressed in *Escherichia coli* under pET32-a(+)vector.

Keywords : *Koi herpesvirus*, ORF124, vaccine, glycoprotein

Introduction

Common carp (*Cyprinus carpio carpio*) is the most widely cultivated fish for human consumption mainly in Asia, Europe, and the Middle East. The koi (*Cyprinus carpio koi*) subspecies is cultivated as an expensive, beautiful, and colorful pet fish for personal pleasure or competitive showing (Costes *et al*., 2009). One of problems on culturing those fishes is diseases. Koi herpesvirus (KHV), also known as Cyprinid herpesvirus 3 (CyHV-3), Koi herpes-like virus, and carp interstitial nephritis gill necrosis virus (CNGV) caused significant morbidity and mortality both in common carp and koi carp (Hedrick *et al*., 2000, Michel *et al*., 2010, Ilouze *et al*., 2011). The first outbreak of KHV was reported in 1998 and confirmed in 1999 in Israel. Since then, other cases have been confirmed in United States, Europe and Asia (Hedrick *et al*, 2000). KHV in Indonesia, having started in the area of Blitar, East Java in March 2002. Since then, it has spread rapidly through Java island, causing very high mortality (80-90%). From Java island the KHV was spread into Sumatra, Borneo and other islands (Sunarto *et al*, 2005).

A comparison of virion polypeptide and genomic restriction fragment of seven geographically diverse isolates of KHV indicated that with one exception they represented a homogeneous group (Gilad *et al*., 2003). Comparison of the full sequences of three isolates (from Japan, KHV-J; the United States, KHV-U and Israel, KHV-I)

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revealed more than 99% similarity. The genomes are highly similar to each other at the sequence level, with KHV-U and KHV-I more closely related to each other than either is to strain KHV-J (Aoki *et al*., 2007). Bigare *et al* (2009) studied using two molecular marker presented the genetic variation of KHV-U/KHV-I and KHV-J on 42 samples of infected carps from France, The Netherlands and Poland. They found that among those samples, both the U/I and J genotypes were identified, but also a third genotype representing a genetic intermediate between U/I and J for one of the two molecular markers was found from Netherlands and Poland.

Analysis on ORF2 membrane protein of KHV, showed that KHV Indonesian strain from Lake Toba was close related to KHV-J (Murwantoko, 2009). Sequence analysis of two variable regions between ORF29 and ORF31 (marker I) and near the start of ORF 133 (marker II) indicated that all Indonesian isolates displayed a marker I allele (I^{++}) previously identified only in isolates of the Asian lineage. However, in the marker II region, all Indonesian isolates displayed the II^{-} allele, which has been reported previously only amongst isolates of the European lineage, and nine of these displayed a mixed genotype ($II^{+}II^{-}$). The $I^{++}II^{-}$ genotype has not been reported previously and appears to represent a new intermediate lineage that may have emerged in Indonesia (Sunarto *et al*., 2011). Genotyping of KHV by Multiple locus VNTR analysis (MlVA) using eight loci had been applied on 38 samples collected in Indonesia, France and the Netherlands. The result indicated that globally, the isolates grouped in two main genetic clusters, each one divided in two subgroups including either KHV-U/I or KHV-J. Interestingly, Indonesian strains were rather distant from KHV-J isolate (Avarre *et al*., 2011).

Immunization is one way to prevent fish from KHV disease. Immunological studies by "naturally resistant" and immunization of attenuated virus have shown a significant elevation of the humoral response to vaccination. Furthermore, both viral inoculation and challenge resulted in very high anti-viral resistance and low mortality (Ronen *et al*., 2003). Adkison *et al*. (2005) also showed that survivor of KHV outbreak showed high titer antibody against KHV. The passive immunization could protect carp from KHV infection. Administration of live attenuated vaccine has a risk that could mutate into virulent virus (Yasumoto *et al*., 2006, Michel *et al*., 2010) . A formalin-inactivated KHV trapped within a liposomal compartment have been developed. This vaccine can be used for oral immunization in fish food and produced protection efficacy for carp is 70% (Yasumoto *et al*., 2006). Recombinant vaccine has several advantages such more secure because there is no risk of pathogen (Anderson, 2000).

Herpesviruses employ viral glycoproteins to enter cells, a process that involves fusion of the virion envelope with cellular membranes. Glycoprotein membrane has an important role in the fusion process (Farnsworth *et al*., 2007). ORF124 KHV gene encoded membrane glycoprotein (Aoki *et al*., 2007). In this study we explored the potency of ORF124 KHV as a candidate vaccine.

Materials and Methods

Genomic DNA extraction

The gill tissue of common carp (*Cyprinus carpio*) was originally from Yogyakarta, Indonesia. A ten to thirty miligram of gill were homogenized on 400 µl of TNE buffer (1 M Tris-HCl (pH 7.5), 5 M NaCl, 0.5 mM EDTA) using pestle on 1.5 ml. Proteinase K (10 mg/ml) as 3 µl and RNAse (10 mg/ml) as 3 µl were added into mixture followed by incubation 42^{o}C for 2-4 h. After incubation, the mixture was extracted using phenol-chloroform-isoamyl-alcohol, and the aqueous phase was collected. The DNA was precipitated with ethanol absolute and washed with 70% ethanol. DNA pellets was suspended with TE buffer (100 mM Tris-HCl, 10 mM EDTA, pH 7.6).

Primer design

Primers were designed based on Genbank data of *Cyprinid herpesvirus* 3, *complete genome* (NC_009127) to amplify ORF 124 of KHV. The forward primer, KHV-ORF124 F (5' GGA GAA TTC ATG GGA CCT TTG ACC ATC 3') from up stream part containing start codon with addition *Eco*R1 site. Reverse primer, KHV-ORF R (5' AGT AAG CTT TCA CTT GAG CTC GCC GTT G 3') from down stream part containing *Hind*III site.

DNA amplification

The amplification of ORF124 KHV was done using isolated DNA from gill as template with commercial PCR kit as reagent. The PCR reaction was conducted as follow one cycle of 95°C for 4 min, 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, followed one cycle with 72°C for 7 min. After amplification, the PCR products were electrophoreted on a 1.5% agarose-TAE (40 mM Tris-Acetate pH 8.3, 1 mM EDTA) gel and stained with ethidum bromide. The presence of amplicons were observed using UV transiluminator.

Cloning of ORF124 KHV in cloning vector

The PCR products were purified using phenol-chloroform and followed by digestion with *Eco*R1 and *Hind*III. After digestion, the PCR products were agarose electrophoreted and DNA bands were recovered from agarose. Purified PCR fragment were ligated into pBSKS (Stratagene) which digested by the same restriction enzyme using T4 DNA ligase (Toyobo) at 16°C for overnight. Ligation mixture were transformed into *Escherichia coli* DH5α using heat shock at 42°C for 90 sec followed incubation on ice. The bacteria were cultured on LB agar plate containing 50 µg/ml ampicillin for overnight. The colonies were cultured in LB broth containing ampicillin and cultured at 37°C for overnight. The plasmids were isolated from the bacteria using minipreparation of alkali lysis methods (Sambrook and Russel, 2011). The presence of recombinant plasmids were analyzed by restriction enzyme digestion.

Sequencing and DNA analysis

The recombinant plasmids were purified using PEG/NaCl method and sequenced using T3 and T7 primer with Big Dye terminator v3.1 (Applied Biosystem). The PCR products were applied into ABI310 sequencer. DNA sequences from those two primers were overlapped to determine complete sequences. DNA sequences were analyzed using BLAST to looking for the homology with the data on Genbank. The T cell epitopes were predicted by Genetyx, and the B cell epitopes were predicted by BcePred software (Saha and Raghava, 2004).

Cloning of ORF124 KHV gene in expression vector

ORF124 KHV was excised from pBSKS by digestion with *Eco*R1 and *Hind*III and then ligated into pET-32(a), which digested by the same restriction enzymes. *E. coli* DH5α was transformed with ligation mixture in same method above. The colonies were screened to obtain pET-32(a)-ORF124 KHV by plasmid digestion. The selected pET plasmid was transformed into *E. coli* BL21cd to express protein.

Expression and purification of recombinant ORF124 KHV

A colony E. *coli* BL21cd containing pET-32(a)-ORF124 KHV was cultured in 5 ml LB broth contain ampicillin (50 µg/ml) and shaking in 37°C incubator for overnight.

The 5 ml of overnight cultured was inoculated into 500 ml of LB broth and incubated on 37°C. After density of bacteria reached OD600 at 1.0, IPTG was added to final concentration 1 mM and continued for incubation. The bacteria were harvested at 0 h, 3 h, 4.5 h and 6 h after IPTG induction. The bacteria were sonicated and the proteins from bacteria were analyzed using 10% SDS-PAGE (Sambrook and Russel, 2001). Purification of recombinant protein was

done using Ni-NTA agarose (Qiagen). The bacteria were homogenized in PBS, than applied into Ni NTA column. Column was washed with PBS, then eluted with increasing concentration of imidazole in PBS. The eluents from the steps were collected and analyzed by SDS-PAGE (Sambrook and Russel, 2001).

Results and Discussion

Koi herpes virus (KHV) caused significant morbidity and mortality both in common carp and koi carp (Hedrick *et al*., 2000, Michel *et al*., 2010, Ilouze *et al*., 2011). KHV infection may produce severe gill lesions which exhibit as gill mottling with red and white patches due to necrosis (death) of the gill tissue (Hartman *et al*., 2008). In this experiment, the carp from Yogyakarta showing white patches was used. DNA was extracted from those gills and used as template for the amplification experiment. The amplification by PCR using KHV-ORF124 F/KHV-ORF124 R primer was successfully produced single band at around 800 bp in size (Figure 1A). This result showed that the primers are working well to amplify ORF124 KHV, as the amplicon size was in range of the prediction, the distance between two primers in DNA sequence from Genbank (NC_009127). This result also confirmed that carp sample was positively infected by KHV, supporting the clinical signs showing white patches in gills.

The PCR products were digested with *Eco*RI and *Hind*III and purified. This purified DNA fragments were ligated to pBSKS vector. Transformation of ligation mixture into *E. coli* DH5α produced some colonies in LB agar containing ampicilin. The plasmids were isolated from the bacteria. Evaluation the recombinant plasmids were done by digestion with *Eco*R1 and *Hind*III. The result showed that two bands were appeared. The lower band around 800 bp in size was appear from colony 1, 2, 3 and 5 (Figure 1B). This band was in same position with the PCR product. Whereas the upper bands were came from the pBSKS with 2961 bp in size. This result showed that KHV ORF124 was successfully cloned.

The recombinant plasmids were purified and sequenced using T3, a reverse primer and T7, a forward primer. The sequencing results contained a part of plasmid and inserted DNA. Those sequences also contained overlapped part of inserted DNA and used to construct whole inserted DNA sequences as shown in Figure 2. The figure showed that DNA was inserted on multiple cloning sites (MCS) of pBSKS between *Eco*RI and *Hind*III

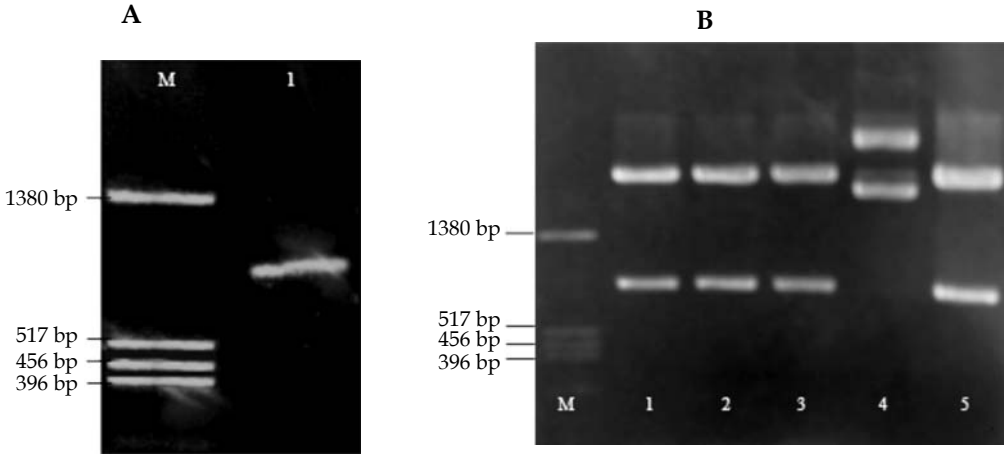

Figure 1. Agarose electrophoresis. a. PCR product of ORF124 KHV (lane 1) showed one band around 800 bp in size. B. Evaluation of pBSKS recombinant. Plasmids were digested with *Eco*RI and *Hind*III, upper band from pBSKS, lower band from inserted DNA. M: DNA size marker BSM13/*Hinf*1 with indicated size.

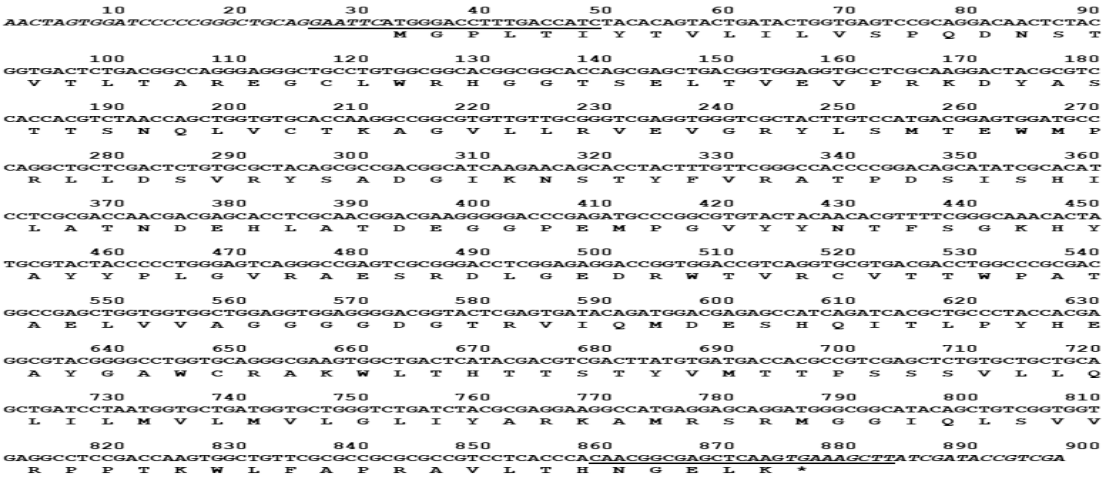

Figure 2. Sequences of inserted DNA in pBSKS. Sequences in italic indicate part of MCS of pBSKS. Sequences with underlines indicate sequences of primers containing of *Eco*R1 and *Hind*III sites. The predicted amino acids were indicated bellow second nucleotide of codon.

restriction sites. DNA insert itself composed 846 bp and predicted to encode 281 amino acid.

The inserted DNA sequences were analyzed using BLAST. BLAST analysis showed only 3 entries have homology with the sequences. The sample showed 100% homology with DNA genomic Cyprinid herpesvirus 3 strain TUMST1 (AP008984.1) from Japan and 99% homology with Koi herpesvirus strain KHV-U (DQ657948.1) from USA and KHV-I (DQ177346.1) from Israel. The number of entries, by inputting koi herpesvirus or cyprinid herpesvirus 3 in NCBI, was more than 250 entries. This result indicated that, this analyzed gene of KHV was less analyzed. The most analyzed gene in KHV is thymidine kinase, with more than 65 entries in Genbank.

The result showed that this sequenced have 100% homology with ORF124 of KHV TUMST1 (KHV-J) from Japan and 99% homology with KHV-U from USA and KHV-I from Israel. This result was matched to previous study on KHV in Indonesia by Murwantoko (2009), Sunarto *et al*. (2011) that KHV in Indonesia was closer to Asia or Japan strain than to other strains. For further discussion, Indonesian KHV ORF124 was used to describe this sequence.

Using whole virion, KHV showed strong antigenic indicated by high titer of antibodies found in immunized fish and high protection against KHV infection (Ronen *et al*., 2003; Adkison *et al.,* 2005; Yasumoto *et al.*, 2006). An immune response was directed by antigen with multiple epitopes (Baratawidjaya, 2002). So the 281 amino acid as result of Indonesia KHV ORF124 was analyzed on T cell epitope prediction performed using program based on IAd and Rothbard/Taylor pattern with Genetyx software and analysis of B cell epitope with BcePred (Saha and Raghava, 2004). IAd pattern based on the amino acid sequences that are generally identified IAd molecules of Major Histocompatibility Complex (MHC). Rothbard/Taylor pattern based on amino acid sequences generally recognized by T cell.

The result of T and B cell prediction of the Indonesian KHV ORF124 was presented in Table 1. Indonesian KHV ORF124 was predicted had 14 epitopes (IAd) and 11 epitopes (Rothbard/Taylor) of T cell; and 10 epitopes of B cell. Those result implied that the protein could be recognized by T and B cell lymphocytes and produced immune respons, so that it may be used as a vaccine candidate.

Vaccination into fishes can be done by protein or DNA vaccination. Protein

Table 1. Prediction of T cell and B cell epitopes of Indonesian KHV ORF124

T cell epitope prediction				B cell epitope prediction	
IAd pattern		Rothbard/Taylor pattern			
Amino acid position	Sequence	Amino acid position	Sequence	Amino acid position	Sequence
4-9	LTIYTV	62 - 66	GVLLR	13-18	VSPQDN
9-14	VLILVS	77 - 81	EWMPR	29-35	CLWRHGG
19-24	STVTLT	81 - 84	RLLD	32-38	RHGGTSE
107-112	ISHILA	109 - 113	HILAT	46-53	KDYASTTS
108-113	SHILAT	117 - 120	HLAT	118-124	LATDEGG
161-166	VRCVTT	184 - 187	RVIQ	145-151	GVRAES
171-176	AELVVA	203 - 206	GAWC	152-158	RDLGEDR
211-216	LTHTTS	209 - 212	KWLT	176-182	AGGGGDG
212-217	THTTST	240 - 243	GLIY	186-192	QMDESHQ
220-225	MTTPSS	246 - 249	KAMR	246-252	KAMRSRM
239-244	LGLIYA	271 - 275	RAVLT		
242-247	IYARKA				
257-262	LSVVRP				
270-275	PRAVLT				

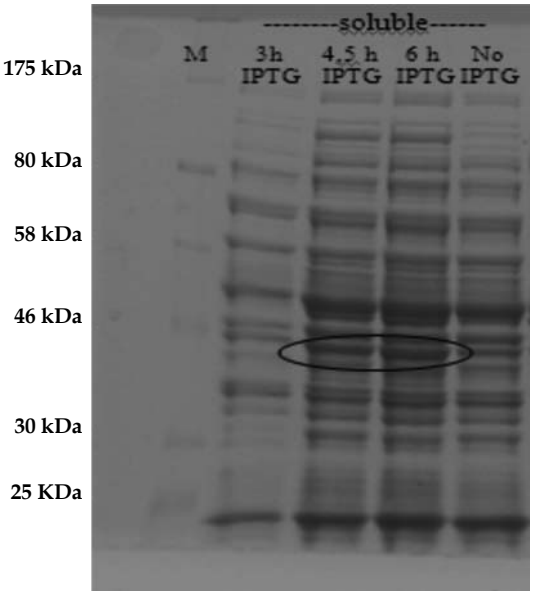

Figure 3. Expression of pET-32(a)-ORF124 KHV in *E.coli* BL21cd (DE3). The bacteria were cultured and induced for protein expression by adding IPTG. The bacteria were harvested before induction, 3 h, 4.5 h and 6 h after induction. The soluble fractions of those bacteria were analyzed on SDS-PAGE. The specific induced protein bands are indicated.

vaccination is more applicable than DNA vaccination. Most of protein recombinants are produced under bacterial system. To produce KHV ORF124 protein, the ORF was sub cloned into pET32a(+). The pET system has been recognized as one of the most powerful methods for producing recombinant proteins in *E. coli*. The plasmid contain T7 lac promoter which can be induced by IPTG (Sambrook and Russel, 2001). Figure 3 showed protein profile of bacteria at 0 h, 3 h, 4.5 h and 6 h after induction with IPTG. A clear band was expressed and increase the intensity after 4.5 h and 6 h induction. Those results indicated that Indonesian KHV ORF124 protein can be expressed on bacterial system under pET32 expression vector. Indonesian KHV ORF124 protein obtained in this experiment was approximately 45 kDa in size. The pET-32(a) fusion protein contain 109 amino acids Trx-tag (approximately 12 kDa) and His-tag, S-tag for detection and purification. The total weight of those proteins was around 12-18 kDa. Sample ORF124 KHV has 281 amino acids and approximately 31 kDa in size. Total protein fusion produced in pET-32(a)-ORF124 KHV was approximately 43-49 kDa in size.

Ni-NTA agarose purification system was designed for purification of 6xHis-tagged recombinant protein expressed in the cells of bacteria, insects, and mammals. The pET-32(a)

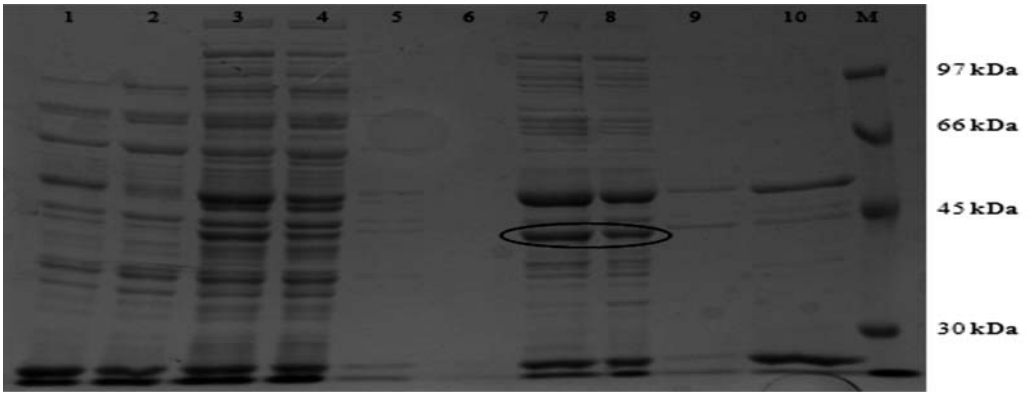

Figure 4. Purification of ORF124 protein with Ni-NTA agarose. The soluble fraction of *E. coli* BL21cd without pET (lane 1,2, 10) and containg pET-32(a)-ORF124 KHV (lane 3-10) were analyzed by SDS-PAGE. The protein of bacteria containing ORF124 before apply into Ni-NTA (lane 3, 4); flowed through column of PBS during early (lane 5) and last (lane 6) washing step: elution 1 with imidazol 25 mM (lane 7), elution 2 with imidazol 50 mM, (lane 8), elution 3 imidazol 200mM (lane 9). The protein of bacteria without pET also was applied into Ni-NTA, and finally eluted 200mM (lane 10). The specific protein bands are indicated.

contains His-Tag sequences, so that Ni-NTA can be used to purify ORF124 protein. The soluble fraction of the bacteria was flowed through Ni-NTA column, extensively washed with PBS and eluted with PBS containing imidazole. The fractions from those steps were analyzed with SDS-PAGE (Figure 4). The specific band, as a ORF124 protein was bound in Ni-NTA and be eluted at 25 and 50 mM of imidazol (Figure 4 lane 7 and 8). The specific band was not appeared in *E. coli* without the plasmid (Figure 4, lane 1, 2 and 10). Those results showed that KHV ORF124 protein can be purified using Ni-NTA system and gave opportunity to used it as a vaccine candidate.

In this experiment ORF124 KHV protein could be expressed in *E.coli,* but the expression was not as high as in other study, such as Murwantoko (2004). This low expression may be caused by the presence of hydrophobic protein in ORF24 KHV. Non polar amino acids cluster in the ORF124 KHV was the transmembrane region N terminal 1 to C terminal 15 (MGPLTIYTVLILVSP). Another cluster was found in the transmembrane from 233 to 244 amino acid residue (PSSSVLLQLILMVMVLGLIYA). One way to increase the level of expression by eliminating the hydrophobic region (Sambrook and Russel, 2001). So, to increase the level of expression of ORF124 KHV, it was necessary to remove those non polar amino acids clusters. Removing those parts may not affect to immunogenicity properties because only few amino acid from those clusters showing have epitopes prediction.

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