CHAPTER IV

DISCUSSION

Anti-LPS factor was initially characterized in horseshoe crabs *Limulus polyphemus* and *Tachypleus tridentatus* (Aketagawa et al., 1986 and Muta et al., 1987). It is a small basic protein found in hemolymph, which binds and neutralizes bacterial endotoxin (LPS) and has a strong antibacterial effect especially on the growth of Gram-negative R-type bacteria (Morita et al., 1985). From Expressed Sequence Tags (ESTs) analysis of *P. monodon* hemocytes, several cDNA clones which showed homology to anti-LPS factor from horseshoe crab were isolated. These clones were redundant indicating the abundance of mRNA of anti-LPS factor in *P. monodon* hemocytes. The entire amino acid sequence of the protein shows 57 % homology to anti-LPS factor from the Atlantic horseshoe crab, *Limulus polyphemus.* The amino acid sequences alignment of anti-LPS factor purified from the horseshoe crabs, *L. polyphemus* and *T. tridentatus,* and the black tiger shrimp *P. monodon* deduced amino acid sequence using the clustalX program showed the two conserved cysteine residues and conserved clusters of positively charged residues between the 2 cysteine residues. The cysteine residues are necessary for disulfide bridge formation in the three dimensional structure and the positively charge residues are involved in the binding with phosphate groups in lipid A portion of LPS, respectively (Hoess et al., 1993). The NH_2-terminal region of the putative anti-LPS factor contains an extra 26 amino acid residues which was proposed to be a signal peptide for protein transport. This region is also highly hydrophobic, which is similar to those found in the horseshoe crab proteins. These results suggested that the structure, function and mechanism of action of anti-LPS factor protein from *P. monodon* should be similar to that of the homologous found in horseshoe crabs.

4.1 Expression of 6XHis Tag anti-LPS factor in the baculovirus expression system

The baculovirus expression system was chosen because the antibacterial activity of the expressed protein, anti-LPS factor, maybe toxic to host cells of bacterial expression system. The host of baculovirus was insect, which was an arthropod as same as shrimp, consequently the expression and modification of gene products are probably more similar to *P. monodon* than other eukaryotic expression system. This system offers a number of advantages, including high expression level, limitless size of the expressed protein, efficient cleavage of signal peptides , post-translational modifications and corrected protein folding.

Two versions of the gene expressing 6XHis Tag full-length and 6XHis Tag NH_2-terminal truncated derivative of the protein were constructed in the baculovirus expression system previously. Histidine tag was added to proteins for mainly to ease the purification step and tagging can be helpful for stabilizing proteins or enhancing their solubility. Poly-histidine tags has been carried out successfully using a number of expression systems including bacteria (Chen and Hai, 1994; Rank et al., 2001), yeast (Borsing et al. 1997; Kaslow and Shiloach, 1994), mammalian cells (Janknecht et al., 1991; Janknecht and Nordheim, 1992), and baculovirus-infected insect cells (Kuusinen et al., 1995; Schmidt et al., 1998).

In this study, expression of anti-LPS factor both full-length and NH_2-terminal truncated were observed as an over express protein band at 15 kDa (Figure 3.1), while there was no protein band observed in uninfected cells. Protein extracted from cells infected with both virus strains did not show any antibacterial activity, no inhibition was observed (data not shown). To investigate whether the absence of the recombine protein was due to the lost of the recombinant gene, the existence of anti-LPS gene in viral DNA was determined by dot blot analysis. The result showed that the anti-LPS gene was still present in baculovirus genome. These results indicate that there might be

very low level of expression or no express at all despite the presence of anti-LPS gene in the recombinant baculovirus genome.

There are many factors affecting protein expression level in baculovirus such as, virus passage. Virus stocks are prepared by infecting cells at low MOI (<1) [multiplicity of infection,(plaque-forming unit/cell number)] and harvest supernatant 4-5 days post infection (1 virus passage). It is critical to use a low MOI because passaging the virus at high MOI increase the number of virus with extensive mutations in their genome (O'Reilly et al., 1992). The number of mutant virus is increased by serial passage, so virus stock is maintained in a low passage of seed stock and produce large scale of working stocks. In this study, we used recombinant virus passage 2 as seed stock virus and working stocks virus were passage 3 and 4. Multiple passages of virus stock may cause mutant virus and lost of inserted gene, thus dot blot hybridization was used to detect anti-LPS gene in virus genome. From the result there were signal at 10^{-4}-10^{-6} dilution which is a small group of virus but they can give a signal, this indicates that most of virus had anti-LPS gene in genome but they got no expression.

The causes of low level or no expression of recombinant anti-LPS maybe because of, first sf9 cells kept for too long or continuous culture for more than a year may lost the ability to express foreign proteins efficiently (O'Reilly et al., 1992). Second, expressed protein is driven by polyhedrin promoter, if there is a point mutation in the polyhedrin promoter, this may reduce or lost mRNA of expressed protein. Third, anti-LPS is likely to be a membrane bound or membrane insertion protein from ability to lyse red blood once they have been impregnated with LPS. This type of proteins and secreted proteins may produced at lower levels than proteins that remain in the cytoplasm or targeted to the nucleus (Kidd and Emery, 1993). The forth, anti-LPS may be mildly toxic to the insect cell. The expression level of any protein is due to the nature of the gene and the encoded protein.

4.2 Expression of 6X His Tag anti-LPS factor in the yeast (*Pichia*) expression system

Yeast (*Pichia*) expression system is selected because, *Pichia pastoris* has many of the advantages of higher eukaryotic expression systems such as protein processing, protein folding, and posttranslational modification, while being as easy to manipulate as *E. coli* or *Saccharomyces cerevisiae*. It is faster, easier, and less expensive to use than other eukaryotic expression systems and generally gives higher expression levels. As a yeast, it shares the advantages of molecular and genetic manipulations with *Saccharomyces*, while *Pichia pastoris* has a strong inducible promoter. This inducible promoter is related to the fact that *Pichia pastoris* is a methyltropic yeast. The first step in the utilization of methanol is the oxidation of methanol to formaldehyde and hydrogen peroxide (Ledeboer et al., 1985). This step is catalyzed by the enzyme alcohol oxidase. Alcohol oxidase has a poor affinity for O_2, and *Pichia pastoris* compensates by generating large amounts of the enzyme. The promoter regulating the production of alcohol oxidase is the one used to drive heterologous protein expression in *Pichia* (Cregg et al., 1985). *Pichia pastoris* grows on a simple mineral media and does not secrete high amounts of endogenous protein. Therefore the heterologous protein secreted into the culture is relatively pure and purification is easier to accomplish (Faber et al., 1995)

In this study, 6XHis Tag NH_2-terminal truncated derivative of anti-LPS factor was constructed using a vector with signal sequence (α factor) and histidine tag to help during the purification step. From the expression result, the bands which increase in the intensity after induction as the time increase was found at the size about 14 and 17 kDa. However a band at 14 kDa in size was also present in control lane. We analysed the recombinant protein band using western blot analysis, there was no positive band observed from culture medium (data not shown). The final concentration of methanol from 0.5 to 1, 2 and 3 % was further used for induction in attempt to increase protein expression. There was no significant change in pattern and intensity of protein band (data

not shown). Therefore antibacterial activity was used to screen for positive clones to find a clone, which express the recombinant protein at high levels.

The low expression level observed maybe due to biological factors such as, mRNA stability, translational capacity, protein folding, protein secretion and protein degradation. The nature of anti-LPS factor is likely to be a membrane bound or membrane insertion protein, this may cause a low expression level as observed in other membrane proteins (Fryxell et al., 1995; Weiss et al. 1995).

4.3 Purification of anti-LPS factor from recombinant yeasts

The development of techniques and methods for the separation and purification of proteins has been an essential pre-requisite for many of the recent advancements in bioscience and biotechnology research. The global aim of a protein purification process is not only the removal of unwanted contaminants, but also the concentration of the desired protein and its transfer to an environment where it is stable and in a form ready for the intended application (Queiroz et al., 2001). The principal properties of enzymes that can be exploited in separation methods are size, charge, solubility and the possession of specific binding sites. Most purification protocols require more than one step to achieve the desired level of product purity. This includes any conditioning steps necessary to transfer the product from one technique into conditions suitable to perform the next technique. Each step in the process will cause some loss of product. Consequently, the key to successful and efficient protein purification is to select the most appropriate techniques, optimize their performance to suit the requirements and combine them in a logical way to maximize yield and minimize the number of steps required.

The expressed anti-LPS factor was purified by Ni-NTA column. It was purified 65.5 folds with 40.9 % recovery. In this step, 99.4 % of other proteins were removed. The 2 major proteins retained in the column was about 60 kDa and 24 kDa as seen in Figure 3.13. The gradient of imidazole from 0-500 mM was used to elute bound proteins. We lowered the slope gradient in the attempt to separate the 2 major proteins from each other. Although the slope of

imidazole gradient was reduced, the 2 proteins still copurified. The result from tricine SDS-PAGE gel suggested that 24 kDa is most likely to be anti-LPS but the predict size from amino acid sequence was about 15 kDa. The increase in size may due to anti-LPS factor's high positive charge and the added histidine tag. This positive charge may retard protein mobility and cause a shift in the protein band. It has also been reported that proteins of molecular weight below 15 kDa begin to behave unusually due to mass ratio that is different from larger proteins (Bollag et al., 1996).

Because of the different in size of 2 proteins, gel filtration chromatography was used for further purification. Gel filtration separates proteins with differences in molecular size. The technique is ideal for the final polishing steps in a purification when sample volumes have been reduced (sample volume significantly influences speed and resolution in gel filtration). Samples are eluted isocratically (single buffer, no gradient). Buffer conditions are varied to suit the sample type or the requirements for further purification, analysis or storage step, since buffer composition does not directly affect resolution. Proteins are collected in purified form in the chosen buffer. Sephacryl S-100 HR was selected which have resolution range from 1 000 to 100000 Da. The result from chromatogram (Figure 3.14) show there are 3 overlapping peaks and the peak with the 24 kDa protein, presumably anti-LPS was peak 2. This column purified the protein further12.5 folds and recovery was about 2.0 %.

Antibactrial activity in crude expression culture (Table 3.1) was observed that at 4, 6, 8 and 10 µl of crude expression culture add, the reduction in inhibitory effect, when high amount of crude protein was added, was due to the increasing of rich media in the test reaction.

The partial purified anti-LPS was tested for antibacterial activity with Gram negative and Gram positive bacteria. From results anti-LPS can inhibit both Gram negative and Gram positive bacteria, while the activity on Gram negative bacteria was better. Anti-LPS binds specifically with free LPS and

intact membrane that contain LPS thus most of Gram negative bacteria should be inhibited. However not all strains are inhibited because the different in cell wall composition of each strain of bacteria. Gram positive bacteria were inhibited because they may have some component on cell wall, which can bind with anti-LPS factor.

The identity of the partially purified protein needs to be further confirm by NH_2-terminal sequence or mass spectroscopy methods.