CHAPTER III

RESULTS

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

3.1.1 Preparative production of recombinant proteins

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 (Anurakolan Thesis, 2001). Recombinant proteins have been produced in the baculovirus expression system at level ranging between 0.1 % and 30 % of total insect cell protein. Sf9 cells were infected with each recombinant baculovirus for 1 hour and incubated at 27 °C for 2-3 days. During incubation the Sf9 cells were checked for sign of infection by light microscope. The infected Sf9 cells were enlarged in size (about 2 fold) and a large nuclei were visible. The infected cells and the supernatant were harvested 60-72 hours post infection (hpi) and the sample was analyzed by SDS-PAGE. The infected cells were resuspended and subjected to 15 % SDS-PAGE. Analysis of protein from 6XHis Tag full-length anti-LPS factor (HFLALF) and 6XHis Tag NH2-terminal truncated anti-LPS factor (HΔNALF) infected cells on Coomassie blue stained SDS-PAGE gels revealed over expression of a protein band of approximately 15 kDa, which was not observed in uninfected cell lysates (mock infected cells) (Figure 3.1).

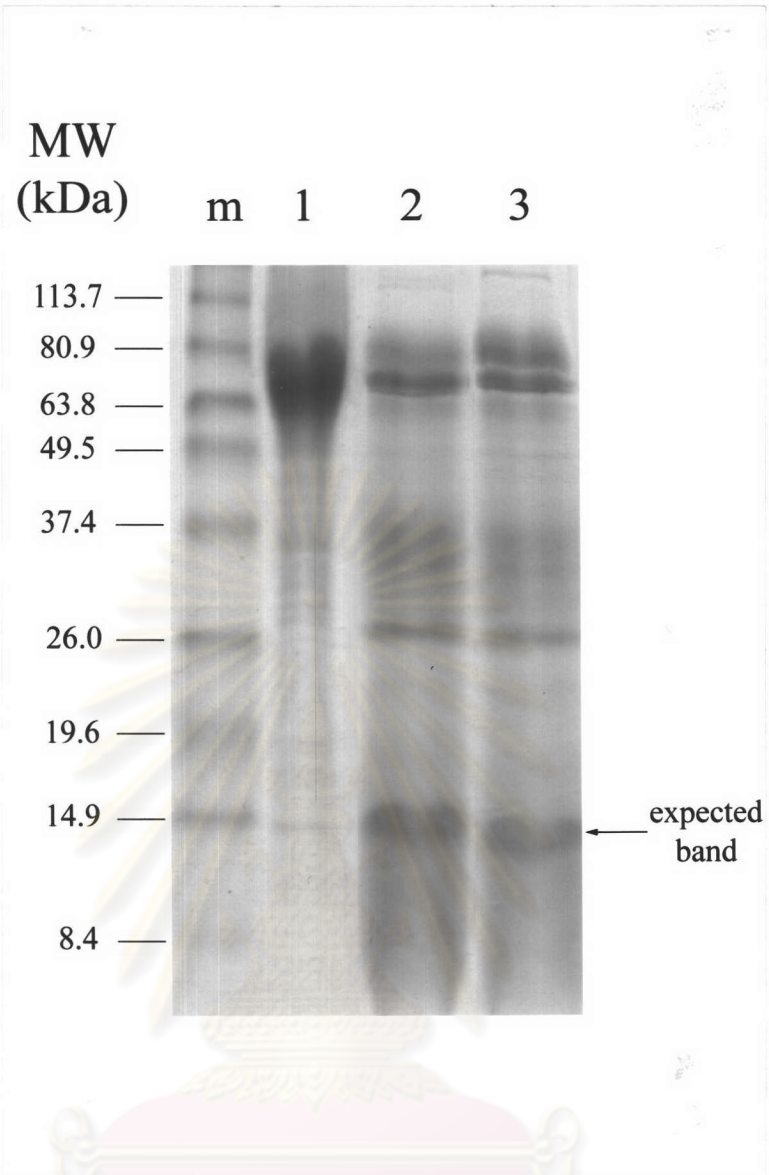

Figure 3.1 Expression of anti-LPS factor in Sf9 insect cells analyzed by SDS-PAGE. A 72 hours post infection (hpi) of infected and mock infected cell lysate of 5 µl was subjected to 15 % SDS-PAGE.

Lane m : Prestain standard molecular weight marker

Lane 1 : mock infected cells

Lane 2 : infected cells with virus harboring the full length anti-LPS gene

Lane 3 : infected cells with virus harboring the NH_2-terminal truncated anti-LPS factor

← ARROW indicates the over expressed proteins

3.1.2 Detection recombinant protein by western blot

To confirm of the result from SDS-PAGE, western blot analysis, using Anti-His antibody and second antibody linked with enzyme horse-radish peroxidase (HRP) was performed. The His-tag protein bands were detected with colorimetric method using diaminobenzidine (DAB) as substrate. There was no protein band observed from uninfected cells and cells infected with both virus strains, but there was protein band observed for positive control in lane 4 (Figure 3.2). This indicated that there was no expression or very low level of expression of anti-LPS protein in host cells, the expected band that seen in SDS-PAGE was not anti-LPS protein. Thus the 2 strains of virus, 6XHis Tag full length and the 6XHis Tag NH_2-terminal truncated anti-LPS factor, were examined for the existence of anti-LPS gene in viral genome.

3.1.3 Analysis of Anti-LPS factor gene by dot blot

The existence of anti-LPS gene in viral DNA was determined by southern blot analysis. The HΔNAL virus with various dilution, was blotted on nylon membrane. The particles were lysed to release viral DNA onto the membrane and [γ-^{32}P]dCTP-labeled anti-LPS factor gene fragment was used as probe. Anti-LPS probe hybridized to viral DNA at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and the maximum dilution was 10^{-5} to 10^{-6} fold dilutions from virus stock (Figure 3.3). The result indicated that ΔNAL anti-LPS gene existed in viral DNA but there was no expression of protein when insect cells were infected with this virus stock. The next step is to try to use a different eukaryotic expression system. The yeast *Pichia pastoris* expression system was chosen. The advantage of expression of protein in yeast was low cost and the expressed proteins are secreted into the culture medium.

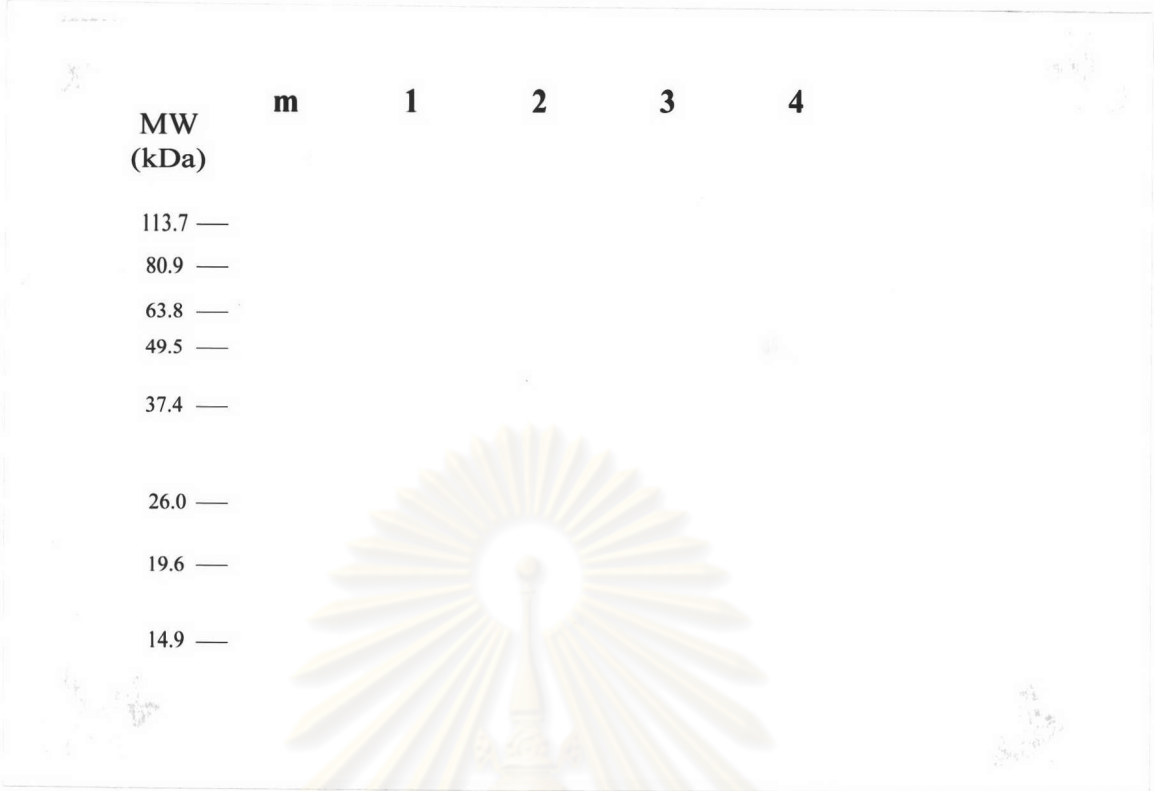

Figure 3.2 Western blot analysis of anti-LPS factor expressed in Sf9 insect cells. A 72 hours post infection (hpi) of infected cell lysate was subjected to 15 % SDS-PAGE, blotted to nitrocellulose membrane and incubated with anti-6xhis antibody and then goat-anti-mouse antibody linked with horse-radish peroxidase(HRP). Detection was achieved by colorimetric method using diaminobenzidine (DAB) as substrate.

Lane m : Prestain standard molecular weight marker

Lane 1 : mock infected cells

Lane 2 : infected cells with virus harboring the 6XHis Tag full length anti-LPS gene

Lane 3 : infected cells with virus harboring the 6XHis Tag NH_2-terminal truncated anti-LPS factor

Lane 4 : positive control [6XHis-tag major royal jelly protein (Chanprapa Injongjirak)]

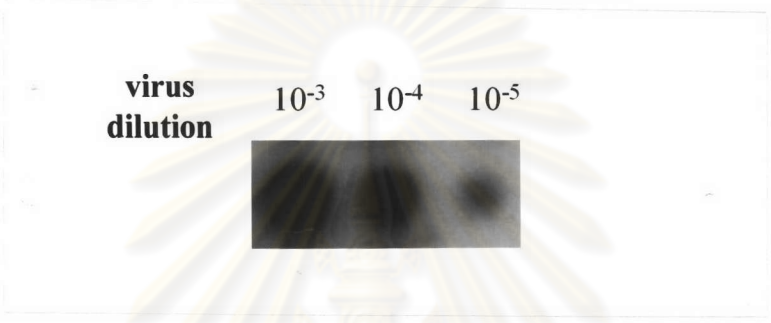

Figure 3.3 Dot blot analysis of anti-LPS factor gene in the HΔNAL virus genome. HΔNAL virus was blotted onto nylon membrane and hybridized with anti-LPS factor gene fragment labeled by random-primed [γ-^{32}P]dCTP-labeled

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3.2 Expression of 6X His Tag NH_2-terminal truncated anti-LPS factor in the yeast (*Pichia*) expression system

3.2.1 Construct of transfer vector

3.2.1.1 Preparation of 6X His-Tag NH_2-terminal truncated anti-LPS factor gene

6X His-Tag NH_2-terminal truncated anti-LPS factor (HΔNAL) gene was prepared by polymerase chain reaction (PCR) using p6XHAL in previous study as a template, which had 6 histidine residue at NH_2 terminal. Oligonucleotide primers incorporating 5' *Eco* RI (5'GCGAATTCATGTCCCTATAGATC-3') and 3' *Xba* I (5'CGTCTAGAAAAAGGCCTATGAGC -3') cleavage sites was used for the PCR. The PCR product was separated on 1.5% agarose gel to determine the size at a specific 441 bp fragment (Figure 3.4).The HΔNAL fragment contains an ORF of 399 bp encoding 133 amino acids (Figure 3.5) The predicted molecular weight of the protein is 15 kDa. The 442 bp HΔNAL fragment gene was used for the construction of the transfer vector.

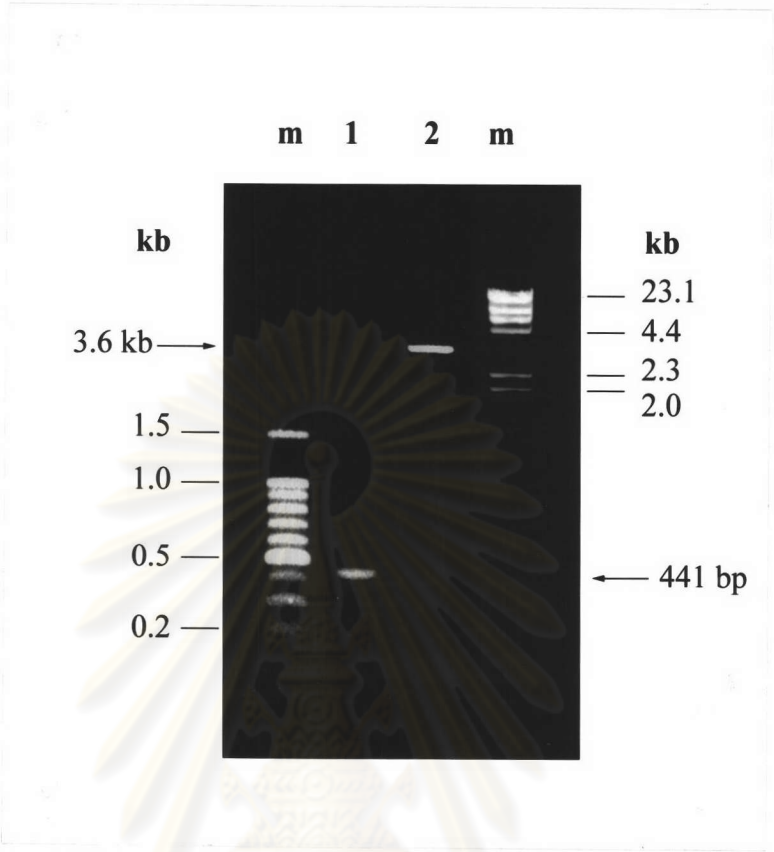

Figure 3.4 Agarose gel electrophoresis of 6X His-Tag NH2-tenninal truncated anti-LPS factor (HΔNAL) gene amplified by PCR, using pΔNAL7 as a template and oligonucleotide primers incorporating 5' *Eco* RI (5' GCGAATTCATGTCCCCTATAGATC -3') and 3' *Xba* I (5'- CGTCTAGAA AAAGGCCTATGAGC -3') cleavage sites. The PCR product and plasmid pPICZαA was run on a 1.5 % agarose gel at 100 volts for 1 hour.

Lane m (right) : DNA marker *(λDNA/Hind* III)

Lane m (left) : Standard DNA ladder (100 bp marker)

Lane 1 : amplified HΔNAL gene products

Lane 2 : plasmid pPICZαa

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1 GCGAATTCATGTCCCCTATAGATC ATG GGA CAT CAT CAT CAT CAT CAC GGA AGG AGA 57
                           M   G   H   H   H   H   H   H   G   R   R

58 AGG GCC AGT GTT GCG GCG GGA ATT TTG GTC CCT CGT GGA AGC CCA GGA CTC 108
   R   A   S   V   A   A   G   I   L   V   P   R   G   S   P   G   L

109 GAT GGC ATA TGC TCG ATC GAG CGG ATG TGG GAG GCT GTG GCA GCG GCC GTC 159
    D   G   I   C   S   I   E   R   M   W   E   A   V   A   A   A   V

160 GCC AGC AAG ATC GTA GGG TTG TGG AGG AAC GAG AAA ACT GAA CTT CTC GGC 210
    A   S   K   I   V   G   L   W   R   N   E   K   T   E   L   L   G

211 CAC GAG TGC AAG TTC ACC GTC AAG CCT TAT TTG AAG AGA TTC CAG GTG TAC 261
    H   E   C   K   F   T   V   K   P   Y   L   K   R   F   Q   V   Y

262 TAC AAG GGG AGG ATG TGG TGC CCA GGC TGG ACG GCC ATC AGA GGA GAA GCC 312
    Y   K   G   R   M   W   C   P   G   W   T   A   I   R   G   E   A

313 AGC ACA CGC AGT CAG TCC GGG GTA GCT GGA AAG ACA GCC AAA GAC TTC GTT 363
    S   T   R   S   Q   S   G   V   A   G   K   T   A   K   D   F   V

364 CGG AAA GCT TTC CAG AAA GGT CTC ATC TCT CAA CAG GAG GCC AAC CAG TGG 414
    R   K   A   F   Q   K   G   L   I   S   Q   Q   E   A   N   Q   W

415 CTC AGC TCA TAG GCCTTTTTCTAGACG 441
    L   S   S   *
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Figure 3.5 Nucleotide sequence and deduced amino acid sequence of 6X His-Tag NH_2-terminal truncated anti-LPS factor (HΔNAL) gene amplified by PCR, using pΔNAL7 as a template and oligonucleotide primers incorporating 5' *Eco* RI (5'GCGAATTCATGTCCCTATAGATC -3') and 3' *Xba* I (5'CGTCTAGAA AAAGGCCTA TGAGC -3') cleavage sites. The specific 441 bp PCR product contains an ORF of 399 bp encoding 133 amino acids. Nucleotide (upper) and deduced amino acid sequence (lower) are shown. The double underlined corresponds to 5' *Eco* RI site and the single underlined nucleotides correspond to 3' *Xba* I site.

3.2.1.2 Preparation of 6X His-Tag NH_2-terminal truncated anti-LPS factor (ΔNAL) transfer vector

The amplified 6X His-Tag NH_2-terminal truncated anti-LPS factor (HΔNAL) fragment was digested with restriction enzymes *Eco* RI and *Xba* I then purified with QIAquick gel extraction kit (QIAGEN) and further ligated into pPICZαA/*Eco* RI /*Xba* I vector. The ligation mixture was transformed into *E.* coli JM 109 cells by electroporation. The recombinant clones were first selected with low salt-LB agar plates containing 50 mg/ml zeocin and further detected by extracting plasmid from selected clones and digested with restriction enzymes *Eco* RI and *Xba* I. The digested reaction mixture was run on 1.5% agarose gel to determine the size at about 441 bp (Figure 3.6). The result in Figure 3.6 indicated that these clones contain HΔNAL gene.

3.2.1.3 Transformation of recombinant plasmid with 6X His-Tag anti-LPS factor gene into yeast cells

The recombinant transfer vector of HΔNAL gene (pHΔNAL) was extracted by using QIAprep plasmid purification kit (QIAGEN). Plasmid pHΔNAL was digested with restriction enzyme *Pme* I to linearlize and then transformed into *Pichia pastoris* KM71 cells by electroporation. The electroporated cells were spread on YPD agar plate and single colonies were picked to detect whether HΔNAL gene has integrated into the yeast chromosome by colonies PCR using yeast chromosome as template and oligonucleotide primers 5' *AOX1* (5'-GACTGGTTCAATTGACAAGC-3') and 3' *AOX1* (5'-GCAAATG GCATTCTGACATCC-3'). The resulting products PCR from clone 1 to 28 were analysed by 1.2% agarose gel electrophoresis and the specific DNA fragment of 1069 bp was obtained (Figure3.7). This indicated that the clones capable for amplified specific DNA fragment of 1069 bp contain HΔNAL gene, which were found clone 2-5, 7-20 and 28.

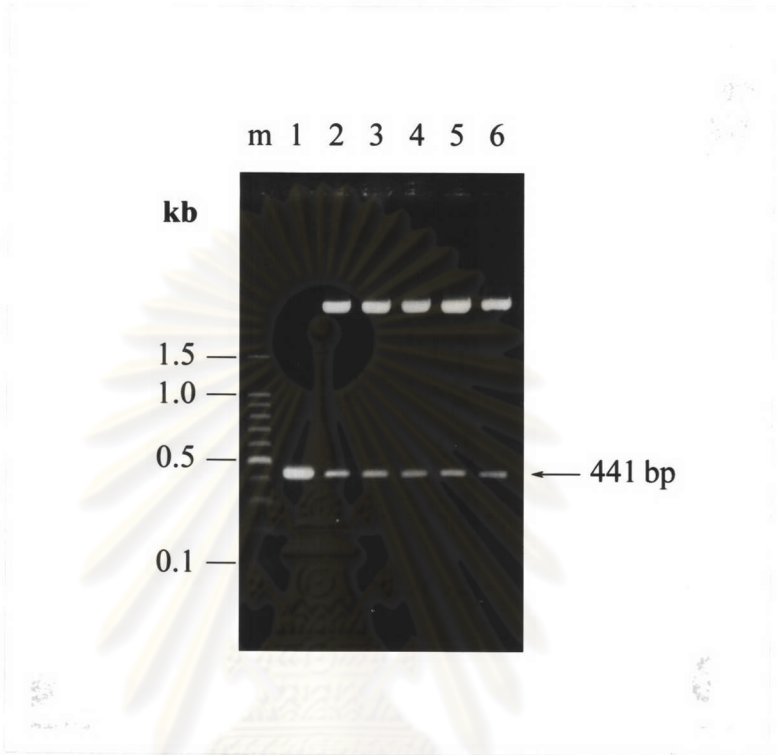

Figure 3.6 Agarose gel electrophoresis of recombinant HΔNAL transfer vector digested with restriction enzyme *Eco* RI and *Xba* I. The DNA was run on 1.5 % agarose gel at 100 volts for 1 hour.

Lane m : Standard DNA ladder (100 bp marker)

Lane 1 : Amplified HΔNAL gene products

Lane 2-6 : Plasmid from selected clone digested with *Eco* RI and *Xba* I

Figure 3.7 Screening of yeast clones for insertion of gene by colonies PCR, using genomic DNA from yeast clones as a template and oligonucleotide primers 5' AOX1 and 3' AOX1. The PCR product was run on a 1.2 % agarose gel at 100 volts for 1 hour.

Lane m : Standard DNA ladder (100 bp marker)

Lane 1-17 : Amplified HΔNAL gene with α-factor signal sequence products from yeast clones

3.2.2 Expression of recombinant HΔNAL yeasts

Recombinant proteins have been produced in the *Pichia* expression system. *Pichia pastoris* is a methylotrophic yeast, which metabolized methanol as its sole carbon source. The first step in metabolizing methanol is oxidation of methanol by enzyme alcohol oxidase. The promoter regulating the production of alcohol oxidase is the one used to drive recombinant protein expression in *Pichia*, so methanol was used to induce the expression of recombinant protein. Recombinant yeasts were inoculated in BMGY media then cells were harvested and concentrated to induce expression in BMMY media. Every 24 hours, added 100 % methanol to a final concentration of 0.5% to maintain induction and transfer 1 ml of expression culture to analyze expression levels in supernatant. The supernatant of 6 days were subjected to 12% tricine SDS-PAGE and gel stained by silver staining method (Appendix C). The gel revealed an obvious protein band of approximately 15 kDa but it was also found in the clone with vector only (control) (Figure 3.8). The observed band of approximately 18 kDa in clone 12 at 5 day and 6 day, which was not observed in the control. From the result we can not identify the expressed recombinant HΔNAL. All the clones were tested for antibacterial activity to confirm the expression of recombinant protein.

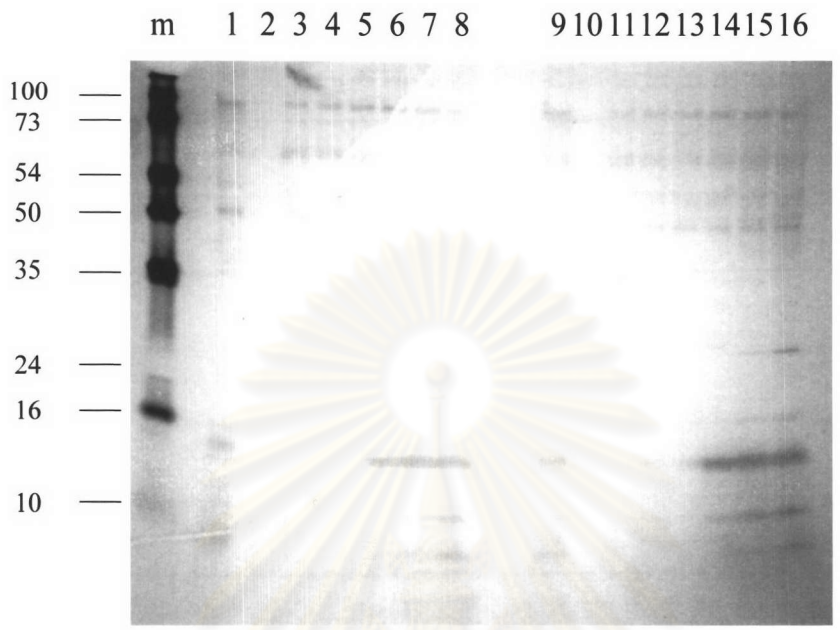

Figure 3.8 Expression of recombinant HΔNAL in *Pichia* supernatant analyzed by Tricine-SDS-PAGE. A collected of *Pichia* supernatant every 24 hours was subjected to 12 % Tricine-SDS-PAGE and stained by silver staining method.

Lane m : Prestain molecular weight standard marker

Lane 1, 9 : supernatant of clone with vector only at day 6 (control)

Lane 2-8 : A collected of clone 11 supernatant from day 0 to 6

Lane 10-16 : A collected of clone 12 supernatant from day 0 to 6

3.2.3 Analysis of recombinant HΔNAL by antibacterial activity

The crude proteins from expression culture supernatant at day 6 of clones 2-5, 7-20, 27 and 28 were tested for antibacterial activity at various concentration against *Escherichia coli* strain XL-I blue by measuring bacterial growth at OD 600. The bacterial growth was compared with supernatant of clone with vector only at day 6 as control and lysozyme as positive control. The bacterial cells were diluted in poor broth medium to OD 600 of 0.00. The crude protein samples were added 2, 4, 6, 8 and 10 µl to 100 µl of initial cells and make final volume to 110 µl each well with 20 mM sodium phosphate buffer pH 7.4. The reactions were incubated at 30 °C with shaking for 18 hours then measured optical density at 600 nm. The results show 4 clones including clone number 11, 12, 14 and 18 were likely to inhibit growth of *E. coli* strain XL-I blue and these clones had percent inhibition more than 30% (Table 3.1). Lysozyme can completely inhibit at 4 µM. The selected 4 clones were prepared for further purification by affinity column chromatography.

Table 3.1 Percent inhibition of expression culture supernatant at day 6 clone 2-5, 7-20 and 28 against *Escherichia coli* strain XL-I blue

Clone number	Volume(µl)					
	0	2	4	6	8	10
2	-	-	8.0	12.2	15.6	11.6
3	-	-	2.7	9.4	19.1	15.4
4	-	-	15.0	16.9	11.3	15.8
5	-	2.2	12.8	27.7	19.1	10.8
7	-	-	11.8	14.6	12.1	6.6
8	-	-	18.2	16.4	8.7	4.6
9	-	-	7.0	9.9	9.1	15.4
10	-	-	20.9	4.7	1.3	-
11	-	-	14.4	40.4	40.3	36.1
12	-	-	12.8	43.2	53.3	52.7
13	-	13.4	17.1	5.2	0.9	5.4
14	-	16.4	36.9	28.6	39.8	32.0
15	-	5.2	26.2	4.2	8.7	6.6
16	-	-	42.3	0.5	6.9	3.3
17	-	18.7	25.1	-	4.3	7.1
18	-	-	39.0	44.1	41.6	30.7
19	-	-	35.8	18.3	15.6	11.2
20	-	-	32.1	35.7	30.7	17.4
28	-	27.6	17.1	2.8	5.6	2.5

	Concentration (µM)					
	0	1	2	4	8	16
lysozyme	-	-	33.7	100.0	100.0	100.0

$$\text{Percent inhibition} = \frac{(OD_{600}\ \text{control} - OD_{600}\ \text{sample})}{OD_{600}\ \text{control}} \times 100$$

- indicated the value was less than zero

3.3 Purification of anti-LPS factor from recombinant yeasts

3.3.1 Purification of anti-LPS factor from recombinant yeasts by affinity column chromatography

The supernatant of expression culture at day 6 of clone 11, 12, 14 and 18 was dialyzed against working buffer and filtrated through 0.45 μm column filter. The samples were applied to Hitrap chelating column as described in section 2.7.3. The chromatogram of clone 11, 12, 14 and 18 are shown in Figure 3.9, 3.10 3.11 and 3.12, respectively. The unbound proteins were eluted from Hitrap chelating column with working buffer. The bound proteins were then eluted with 0.5 M imidazole. The unbound and bound fractions of every clone were dialyzed against with 20 mM sodium phosphate buffer and then lyophilized (clone 11 was lost during dialyzing step, therefore only 3 clones were used for further steps). The lyophilized samples were dissolved with deionized water and then subjected to 12% tricine SDS-PAGE and gel stained by silver staining method (Figure 3.13) and bound proteins were tested for antibacterial activity against *E. coli* strain XL-I blue (Table 3.2). The result from tricine SDS-PAGE gel shows that HΔNAL size was about 24 kDa.

From the results, recombinant yeast clone 12 produced the most recombinant HΔNAL proteins, thus this clone was selected to produce the recombinant protein in large scale for further purification. The proteins concentration of expression culture in 6^{th} day of clone 12 was determined by Gene Quant Pro (Amersham) and 50 ml of expression culture contained 1035 mg proteins with 635 units [1 unit = 1 ΔOD 600 (ΔOD 600 calculate from OD 600 of control – OD 600 of sample)] thus, the specific activity of the recombinant HΔNAL was 0.61 unit/mg protein. The sample was dialyzed and applied to Hitrap chelating column as above. The bounded fraction were pooled, dialyzed, lyophilized to reduce volume and dialyzed against working buffer. The protein remained from this step was 6.5 mg with 259.5 units and the specific activity of the recombinant HΔNAL was 39.9 unit/mg protein. The recombinant HΔNAL was purified about 65.5 folds and recovery was about 40.9% compare with crude expression culture.

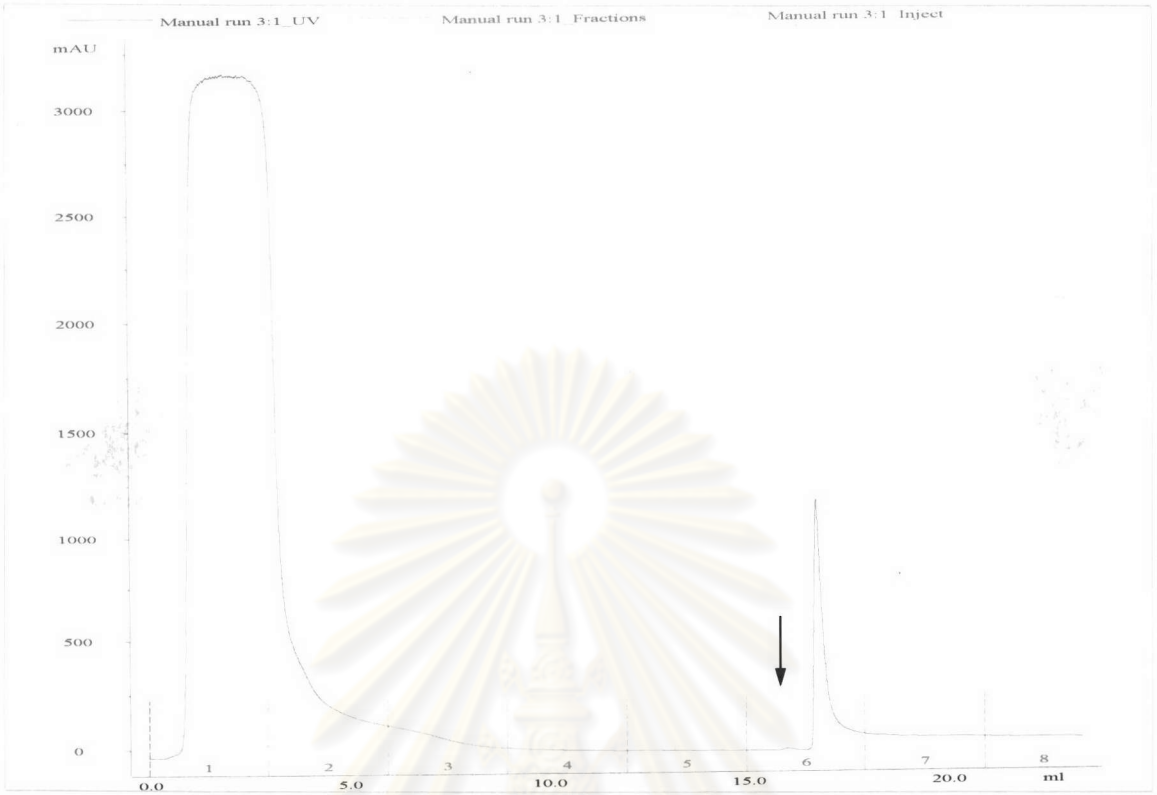

Figure 3.9 Purification of recombinant HΔNAL from clone 11 by Hitrap chelating column. The supernatant of expression culture at day 6 of clone 11 was applied to Hitrap chelating column (1 ml) and washed with 20 mM sodium phosphate buffer with 0.5 M NaCl, pH 7.4 until A_{280} decreased to base line. Elution of bound proteins was made by 0.5 M imidazole in the same buffer at the flow rate 1 ml/min. 4 ml fraction was collected. The blue line was A_{280} and red dash line and number indicate each fraction. Arrow indicates where elution started

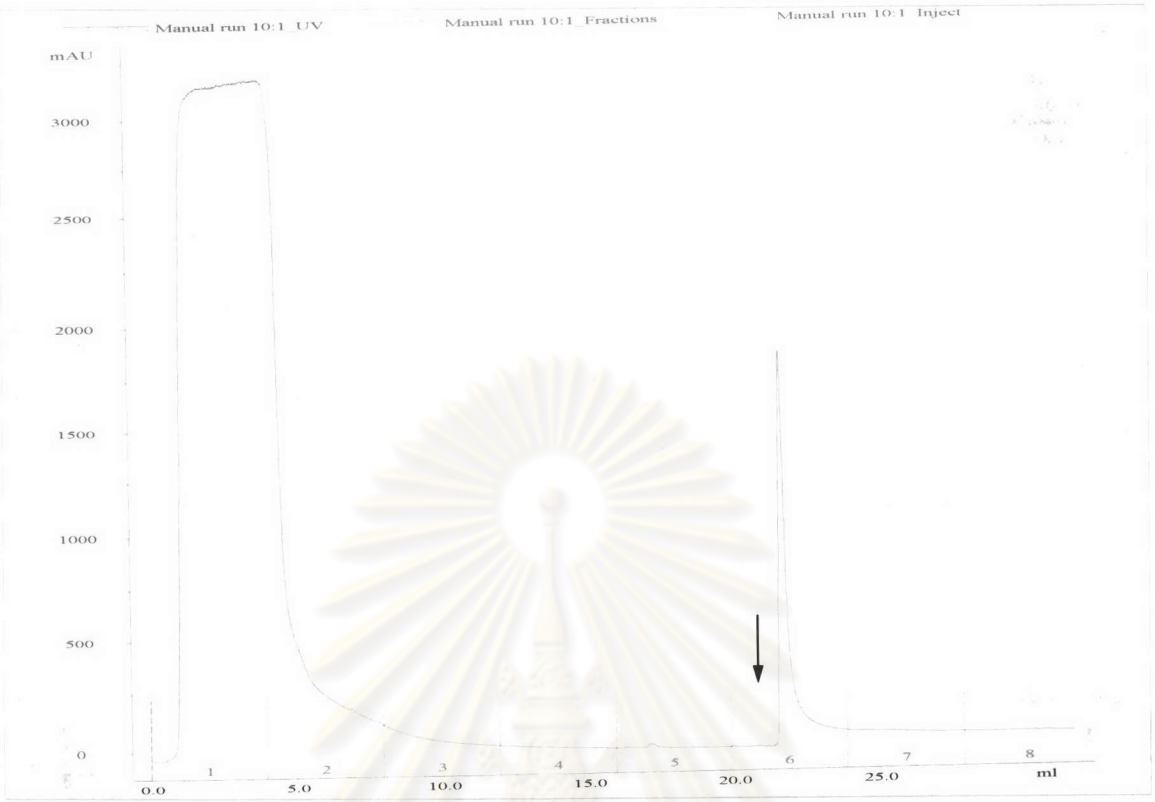

Figure 3.10 Purification of recombinant HΔNAL from clone 12 by Hitrap chelating column. The supernatant of expression culture at day 6 of clone 12 was applied to Hitrap chelating column (1 ml) and washed with 20 mM sodium phosphate buffer with 0.5 M NaCl, pH 7.4 until A_{280} decreased to base line. Elution of bound proteins was made by 0.5 M imidazole in the same buffer at the flow rate 1 ml/min. 4 ml fraction was collected. The blue line was A_{280} and red dash line and number indicate each fraction. Arrow indicates where elution started

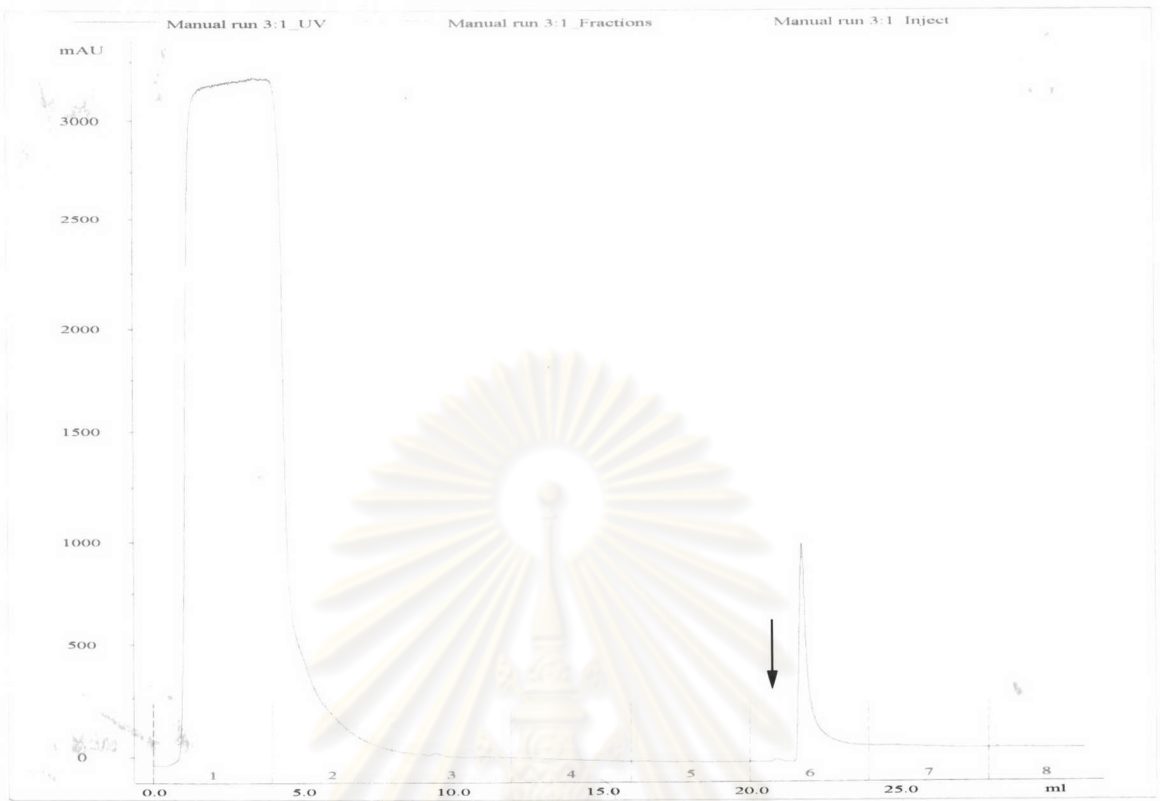

Figure 3.11 Purification of recombinant HΔNAL from clone 14 by Hitrap chelating column. The supernatant of expression culture at day 6 of clone 14 was applied to Hitrap chelating column (1 ml) and washed with 20 mM sodium phosphate buffer with 0.5 M NaCl, pH 7.4 until A_{280} decreased to base line. Elution of bound proteins was made by 0.5 M imidazole in the same buffer at the flow rate 1 ml/min. 4 ml fraction was collected. The blue line was A_{280} and red dash line and number indicate each fraction. Arrow indicates where elution started

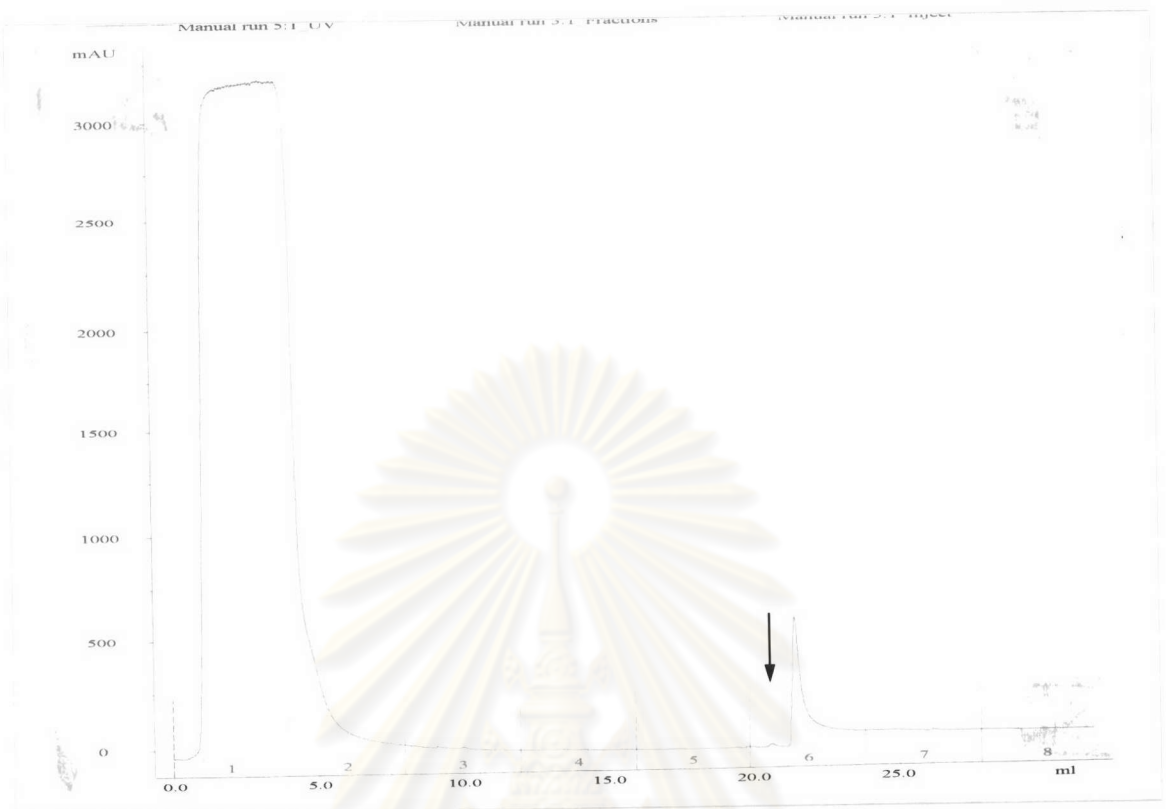

Figure 3.12 Purification of recombinant HΔNAL from clone 18 by Hitrap chelating column. The supernatant of expression culture at day 6 of clone 18 was applied to Hitrap chelating column (1 ml) and washed with 20 mM sodium phosphate buffer with 0.5 M NaCl, pH 7.4 until A_{280} decreased to base line. Elution of bound proteins was made by 0.5 M imidazole in the same buffer at the flow rate 1 ml/min. 4 ml fraction was collected. The blue line was A_{280} and red dash line and number indicate each fraction. Arrow indicates where elution started

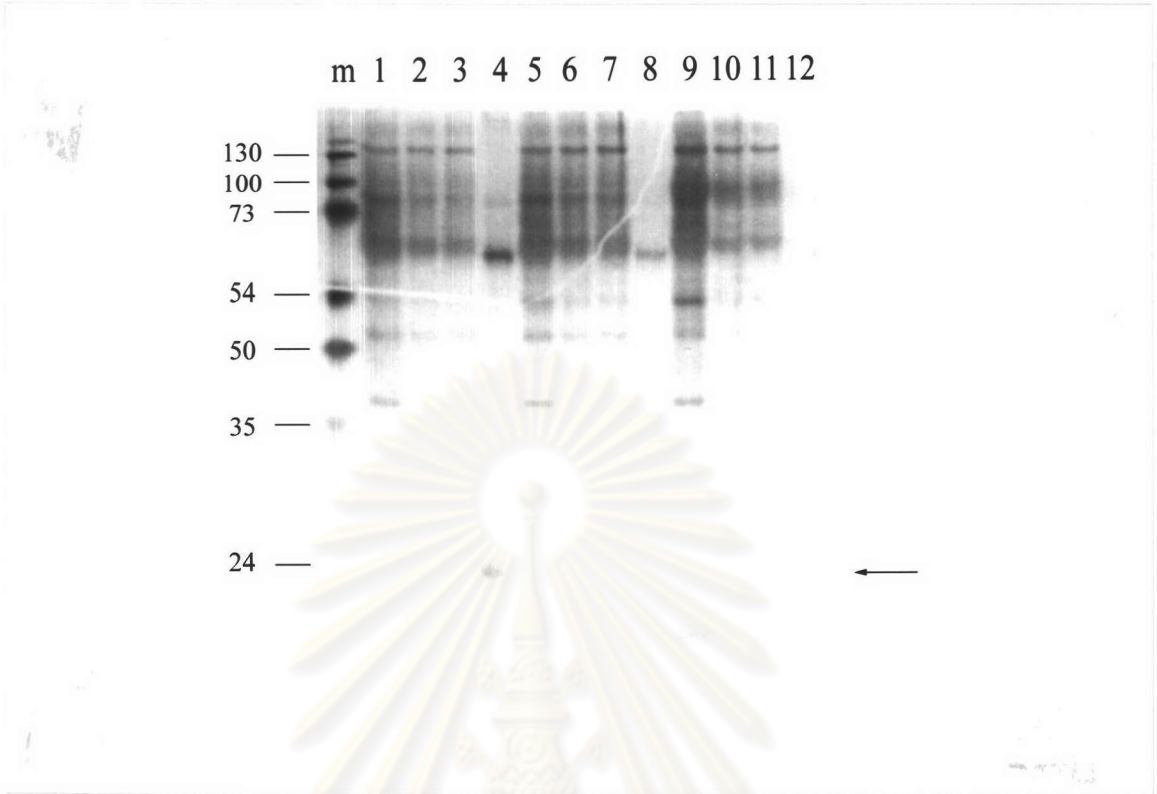

Figure 3.13 Purification of recombinant HΔNAL from clone 12, 14 and 18 supernatant analyzed by Tricine-SDS-PAGE. The supernatant from each step of purification from Hitrap chelating column was subjected to 12 % Tricine-SDS-PAGE and stained by silver staining method.

Lane m : Prestain molecular weight standard marker

Lanes 1, 5, 9 : supernatant of clone 12, 14 and 18 at day 6 respectively

Lanes 2, 6, 10 : dialysed supernatant of clone 12, 14 and 18 at day 6 respectively

Lanes 3, 7, 11 : unbound proteins of clone 12, 14 and 18 respectively

Lanes 4, 8, 12 : bound proteins of clone 12, 14 and 18 respectively

← ARROW indicates the recombinant HΔNAL

Table 3.2 Percent inhibition of bound proteins from Hitrap chelating column of clone 12, 14 and 18 against *Escherichia coli* strain XL-I blue

Clone number	Volume(µl)					
	0	2	4	6	8	10
12	-	26.87	54.01	63.85	67.53	71.78
14	-	21.64	45.99	52.58	50.65	47.30
18	-	11.94	39.04	49.30	46.75	44.40

	Concentration (µM)					
	0	1	2	4	8	16
lysozyme	-	7.46	44.39	100.00	100.00	100.00

$$\text{Percent inhibition} = \frac{(OD_{600}\ \text{control} - OD_{600}\ \text{sample})}{OD_{600}\ \text{control}} \times 100$$

- indicated the value was less than zero

3.3.2 Purification of anti-LPS factor from recombinant yeasts by gel filtration column chromatography

The pooled active fraction from Hitrap chelating column was further purified by Sephacryl S-100 HR column as described in section 2.7.4. The chromatogram profile is shown in Figure 3.14. There were 3 peaks eluted from column. They were pooled, dialyzed and lyophilized to reduce volume and then dissolved in 400 µl deionized water. The supernatant from each step of purification from Hitrap chelating column and Sephacryl S-100 HR column was subjected to 12 % Tricine-SDS-PAGE and stained by silver staining method (Figure 3.15). The result from gel is shown that Sephacryl S-100 HR column did not gave much purity for recombinant HΔNAL. The recombinant HΔNAL was found the most in peak 2. This step yielded 1.036 mg with 7.88 units and the specific activity of the recombinant HΔNAL was 7.61 unit/mg protein. The recombinant HΔNAL was purified about 12.5 folds and recovery was about 2.0 % compared with crude expression culture (Table 3.3).

The proteins from peak 2 was tested for antibacterial activity at various concentrations against 4 types of bacteria by measuring bacterial growth at OD 600. The bacteria used were Gram-negative bacteria *V. harveyi* 1526 and *E. coli* strainXL-I blue and the Gram-positive *S. aureus* and *M. luteus*. The bacterial growth was compared with supernatant of clone with vector only in 6^{th} days as control and lysozyme as positive control. The bacterial cells were diluted in poor broth medium to OD 600 of 0.001 except *V. harveyi* 1526 in poor broth with 2% NaCl. The crude protein samples were added 2, 4, 6, 8 and 10 µg to 100 µl of initial cells and made final volume to 110 µl each well with 20 mM sodium phosphate buffer pH 7.4. The reactions were incubated at 30 °C with shaking for 18 hours then measured optical density at 600 nm. The results showed there were strong antibacterial activity with Gram-nagative bacteria and weak activity with Gram-positive bacteria (Table 3.4). Lysozyme inhibited growth completely at 4 µM except *S. aureus* was at 8 µM.

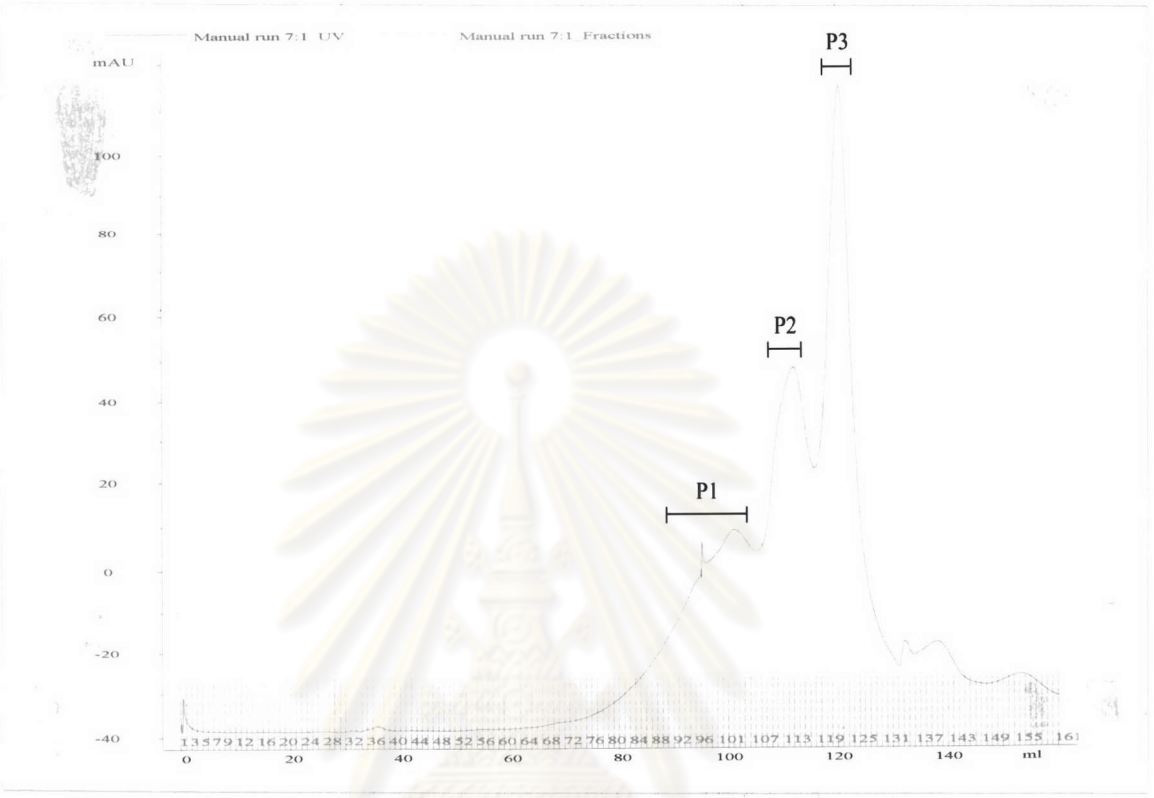

Figure 3.14 Purification of recombinant HΔNAL by Sephacryl S-100 HR column. The pooled active fraction from Hitrap chelating column was applied to Sephacryl S-100 HR column and washed with 20 mM sodium phosphate buffer with 0.5 M NaCl at the flow rate 0.5 ml/min. The fraction of 1 ml was collected. The blue line was A_{280} and red dash line and number indicate each fraction.

P1 the pool fraction of peak 1 (89-104)

P2 the pool fraction of peak 2 (108-114)

P3 the pool fraction of peak 3 (117-124)

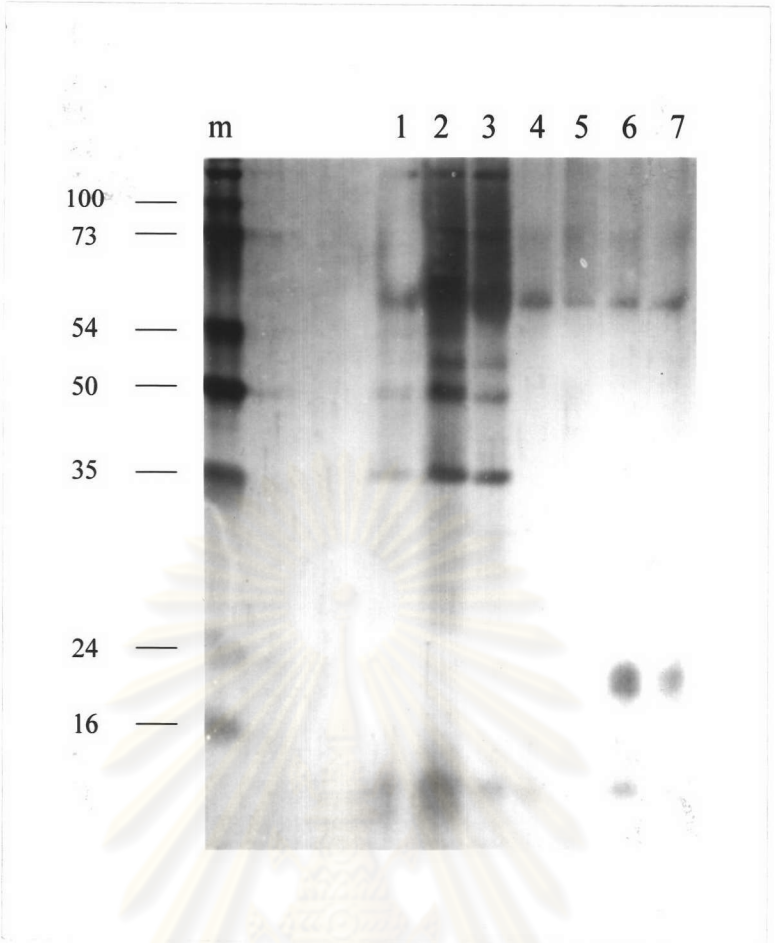

Figure 3.15 Purification of recombinant HΔNAL from each step of purification analyzed by Tricine-SDS-PAGE. The supernatant from each step of purification from Hitrap chelating column and Sephacryl S-100 HR column was subjected to 12 % Tricine-SDS-PAGE and stained by silver staining method.

Lane m : Prestain molecular weight standard marker

Lane 1 : supernatant of clone 12 at day 6

Lane 2 : loaded clone 12 at day 6

Lane 3 : unbound proteins from Hitrap chelating column

Lane 4 : bound proteins from Hitrap chelating column

Lane 5 : peak 1 from Sephacryl S-100 HR column

Lane 6 : peak 2 from Sephacryl S-100 HR column

Lane 7 : peak 3 from Sephacryl S-100 HR column

Table 3.3 Purification of 6XHis Tag NH_2-terminal truncated anti-LPS factor from *Pichia* expression system

Purificartion step	Total activity * (unit)	Total protein (mg)	Specific activity ** (unit/ mg protein)	% recovery	Purification fold
Crude culture medium	635.0	1035.0	0.6	100.0	1.0
Hitrap HP chelating column	259.5	6.5	40.0	40.9	65.5
S-100 HR column	7.9	1.0	7.6	2.0	12.5

* Total activity = (OD 600 of control – OD 600 of sample) x total volume

** Specific activity = $\frac{\text{Total activity}}{\text{Total protein}}$

Table 3.4 Percent inhibition of peak 2 proteins from Sephacryl S-100 HR column against 4 types of bacteria.

Bacterial strains	Protein concentration (μg)					
	0	2	4	6	8	10
V. harveyi	25.0	58.5	71.9	78.7	83.8	87.7
E. coli	-	25.4	53.5	69.5	78.4	81.7
S. aureus	-	1.1	7.3	15.0	10.5	14.3
M. luteus	-	-	24.4	30.9	36.4	39.8

Bacterial strains	Lysozyme concentration (μM)					
	0	1	2	4	8	16
V. harveyi	-	63.1	92.1	100.0	100.0	100.0
E. coli	-	55.2	78.6	100.0	100.0	100.0
S. aureus	-	16.8	32.9	57.7	100.0	100.0
M. luteus	-	65.9	80.4	100.0	100.0	100.0

$$\text{Percent inhibition} = \frac{(OD_{600}\ \text{control} - OD_{600}\ \text{sample})}{OD_{600}\ \text{control}} \times 100$$

- indicated the value was less than zero