

## ISOLATION OF LYSOZYME LIKE PROTEIN FROM CROCODILE LEUKOCYTE EXTRACT (*Crocodylus siamensis*)

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### ABSTRACT

In mammals, white blood cells (neutrophils, macrophages and natural killer cells) contain components of immune response such as antimicrobial proteins and antimicrobial peptides. A variety of proteins and peptides are effector molecules able to kill microbial pathogens. This study aims to screen and purify antimicrobial substances from crocodile leukocyte extract. The crude leukocyte extract showed antibacterial activity toward *Pseudomonas aeruginosa*, *Pseudomonas aeruginosa* ATCC 2785, *Salmonella typhi*, *Salmonella typhi* B, *Salmonella typhi* ATCC 5784, *Vibrio cholerae*, *Bacillus megaterium*, *Staphylococcus epidermidis* and *Candida albicans*. The crude extract was purified by anion exchange column chromatography (FPLC) and 4 protein peaks (P1-P4) were obtained. Protein fractions (P1-P3) showed antibacterial and antifungal activity like the crude leukocyte extract. Protein fraction (P4) displayed specific activity against to *Pseudomonas aeruginosa* ATCC 2785 and *Vibrio cholerae*. The leukocyte extract and protein fraction (P1) were characterized by zymogram refolding gel. Leukocyte extract demonstrated clear zones of inhibition at band molecular weight about 21 and 15 kDa. Antimicrobial activity and MW of these proteins are similar to that of lysozyme. When determined by western immunoblotting using anti-hen lysozyme as primary antibody, the protein of MW at 14-15 kDa could cross react with anti-hen lysozyme. In addition, the result from MALDI-TOF MS-MS confirmed that the 14-15 kDa protein was lysozyme. This result indicated that antimicrobial substance in crocodile leukocyte extract was lysozyme-like protein.

**KEYWORDS:** antimicrobial protein, white blood cell, *Crocodylus siamensis*

### 1. INTRODUCTION

Antimicrobial substances have been isolated from numerous types of organisms, ranging from bacteria to plants and animals [1]. Antimicrobial substances show their efficacy in killing or inhibiting microorganisms such as bacteria, fungal cells or enveloped viruses [2]. Mammalian leukocytes (neutrophils, macrophages, eosinophils, basophils and natural killer cells) are one source of antimicrobial substances [1].

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They express a variety of proteins and peptides as effector molecules that are able to kill or to inactivate microbial pathogens. Mammalian leukocytes contribute many different species of antimicrobial substances such as  $\alpha$ -defensin,  $\beta$ -defensin, even minor variations in peptide structure can influence activity. A systematic understanding of the relationship between peptide structure and lactoferrin, lysozyme, cathelicidins, and chemokines [3-9]. Many antimicrobial peptides display activity against gram-positive and gram-negative bacteria, yeasts and fungi, and even certain enveloped viruses and protozoa. Other peptides are more restricted in their spectrum of activity; lysozyme) or is dependent on specific recognition of bacterial macromolecules (*e.g.*, the bactericidal permeability-inducing protein, BPI) [10]. Synergistic interactions between two antimicrobial peptides in the frog skin, magainin 2 and PGLa (antibiotic peptide in magainin family), have also been reported [11]. In addition to their action on microbes, some antimicrobial peptides can function as regulatory molecules in the host. For example, *in vitro* studies suggest that defensins attract phagocytes and lymphocytes to sites of infection, inhibit the release of cortisol from adrenal cells, induce the proliferation of fibroblasts and modify ionic fluxes in epithelial cells [12]. However, most research to date has focused on searching for peptides, which possess antimicrobial activity without causing resistance in targeted microorganisms. Crocodilians are known to live with opportunistic bacteria without overt physiological effects. While crocodilians are not completely immune to microbial infection, these species do exhibit remarkable resistance. The immune system of crocodilians has not been well characterized, but several reports [13-26] have described the efficacy of alligator serum in fighting bacteria, viruses and amoeba. Recently, Merchant *et al.* [13] proposed that the complement systems of alligator are effective in killing bacteria. In addition, leukocyte extract from the American alligator has been shown to have a broad spectrum of antibiotic effects on bacteria, fungi and viruses. However, no research has previously been reported on antimicrobial substance from leukocyte extracts of *Crocodylus siamensis*. In this study, we have purified and characterized one antimicrobial substance from crocodile leukocyte.

## 2. MATERIALS AND METHODS

### 2.1 Collection of Blood from *Crocodylus siamensis* Shiracha Modar Farm

Adult crocodiles were cables snare and unconscious with electric spark. Blood samples drawn from the supravertebral branch of the internal jugular vein [17-18] using 3.8 cm 18 gauge needles and 10 mL syringes, were transferred immediately to 15 mL sterile centrifuge tubes containing 1 mL of 0.5 M EDTA [19]. Centrifuge tubes were kept in ice box. Whole blood from 8 adult *Crocodylus siamensis* (>1.5 m) was pooled. The volume of blood collected from each crocodile depended on the size of the animal.

### 2.2 Isolation of Leukocytes

Whole blood in centrifuge tubes were put on ice overnight during which the erythrocytes settled to the bottom of tubes, the interphase layer containing the leukocytes was removed using sterile pipettes. The leukocytes were collected by centrifugation at 800x g (25 °C) for 20 mins. The contaminating red blood cells were lysed by addition of 0.83% ammonium chloride solution to the leukocyte suspension at a ratio of 3:1 [1]. The white blood cells were collected by centrifugation at 800xg for 15 mins (4 °C). The cell pellet was gently re-suspended in normal saline and again centrifuged at 800 x g (25 °C) for 20 mins. Pellets of white blood cells were then kept in centrifuge tubes at -70 °C prior to use.

### 2.3 Leukocyte Extract

A crude extract from the white blood cells of crocodile was obtained using a method previously described [1]. White blood cells were frozen at  $-70^{\circ}\text{C}$  overnight for breaking cell after that re-suspended in 0.01 M acetic acid and sonicated to release the antimicrobial substances. The solution containing the antimicrobial substances were collected ( $12,000\times g$ , 20 mins,  $4^{\circ}\text{C}$ ). The supernatant was collected and centrifuged again at  $12,000\times g$  for 20 mins,  $4^{\circ}\text{C}$ . These supernatants were kept in micro centrifuge tubes.

### 2.4 Determination of Protein Concentration

Protein concentration was determined by the Bradford method [20] using bovine serum albumin as standard.

### 2.5 Purification of Antimicrobial Substances

Anion-exchange chromatography was used to separate the components present in the crude extract, based on the reversible adsorption of the anionic substance to the immobilized ion-exchange groups of opposite charge. Crude protein  $50\text{ }\mu\text{L}$  ( $46.25\text{ }\mu\text{g}$ ) was put on Pharmacia ( $1\times 20\text{ cm}$ ) column and ran by biologic duo flow program of FPLC. The anionic compounds bound to the cationic resin and the non-anionic molecules were washed through with 25 mM Tris – HCl pH 8.1 (Tris (Hydroxymethyl) methylamine, AR (BDH)) then the anionic molecules were eluted with 0.5 M NaCl (AR, AnalaR<sup>R</sup>) in 25 mM Tris – HCl pH 8.1. The solutions were pumped through the column at a rate of 60 mL/h and the absorbance at a wavelength of 280 nm was monitored using FPLC. The fractions (3 mL/fraction) corresponding to the peaks were collected, concentrated by freeze dry and assayed for antimicrobial activity.

### 2.6 Tris- tricine SDS-PAGE

Leukocyte extract and protein fractions from Anion-exchange column chromatography using Fast performance liquid chromatography system (FPLC) were separated by Tris tricine SDS-PAGE as described by Schagger and von Jagai [21] using 16.5% separating gel, 10% spacer gel and 4% stacking gel in an AE-6440 gel electrophoresis instrument (Atto, Japan). The gel was stained with Coomassie blue G-250.

### 2.7 Zymogram Refolding Gel

*Micrococcus lysodeikticus* (SIGMA) whole cells were labeled with the vinyl-sulfone reactive dye, Remazol Brilliant Blue (RBB) as for the synthesis of RBB-labeled starch and the refolding gel was performed according to the procedure of Hardt *et al.* [22]. Polyacrylamide gel electrophoresis (PAGE) was performed using a 4% (w/v) stacking gel and a 15.0% (w/v) resolving gel containing 0.1% (w/v) blue *M. lysodeikticus* cells. Polyacrylamide gels and buffers contained 0.1% (w/v) SDS. Samples  $10\text{ }\mu\text{L}$  ( $9.25\mu\text{g}$ ) were mixed in 2x sample buffer without reducing agent (62.5 mM Tris-HCl buffer, pH 6.8, 0.006% (w/v) bromophenol blue as tracking dye, 20% (v/v) glycerol, 2% (w/v) SDS). Electrophoresis was performed using Atto electrophoresis cell (Atto, Japan) at a constant 120 V for 1.46 hrs. The gel was washed twice with 0.1 % triton x 100 in 50 mM PB buffer pH 7.0 for 3-5 hours to remove SDS and gently shaken at  $37^{\circ}\text{C}$ . After SDS wash out, proteins refolded and re-function of antimicrobial activity. Proteins could digest cell wall of *Micrococcus lysodeikticus*. The activities of antimicrobial proteins were seen as the clear zone in an otherwise opaque bluish gel.

## 2.8 Molecular Weight Determination by SDS-PAGE

To examine the molecular mass of leukocyte extract protein preparations, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed as described by Laemmli [23] using 15% acrylamide gel. The protein bands were stained with Coomassie brilliant blue R-250 (CBB). Phosphorylase B (97 kDa), bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and  $\alpha$ -lactalbumin (14.4 kDa) were used as standards.

## 2.9 Western Immuno - blotting

Purified peptides were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% polyacrylamide gel. For transfer, the proteins from SDS-PAGE were electrophoresed onto nitrocellulose membrane by established techniques [24]. Electrophoresis was conducted in transfer buffer for 1 h at 120 mA and 25°C, the resulting transfer marker membrane was stained with amido black, and the sample membrane was soaked in blocking solution (5% skimmed milk in TBST buffer) at room temperature for 1 hr. Next, the membrane containing proteins was incubated with the first antibody (anti hen egg white lysozyme antibody from mice) diluted using blocking solution (antibody: blocking solution, 1:50) at room temperature for 1 h. After rinsing the membrane three times for 5 mins each with TBST (10 mM Tris-HCl, 150 mM NaCl containing 0.05% tween 20), the membrane was incubated in alkaline phosphatase-goat anti-mouse IgG (1:500 in TBST) for 1 hr at room temperature. The membrane was again rinsed three times for 5 mins each with TBST, followed by TBS pH 8.0 (10 mM Tris-HCl containing 150 mM NaCl) three times, and then substrate buffer, pH 9.5 (100 mM Tris-base, 100 mM NaCl containing 50 mM  $MgCl_2 \cdot 6H_2O$ ) two times for 30 secs each at room temperature. Visualization of any immunoreaction was carried out by incubating the membrane in a substrate solution containing 30  $\mu$ l p-Nitroblue tetrazolium chlorides (NBT, Bio-Rad) and 30  $\mu$ l 5-Bromo-4-chloro-3-indolyl phosphates (BCIP, Bio-Rad) in 5 ml substrate buffer, pH 9.5 for at least 5 mins. The color reaction was stopped by transferring the membrane into water.

## 2.10 Microorganisms

Gram positive bacteria 11 stains such as *Staphylococcus aureus*, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* TISTR 5049, *Staphylococcus epidermidis*, *Bacillus subtilis* TISTR 008, *Bacillus subtilis*, *Bacillus megaterium*, *Xanthomonas* sp., *Micrococcus luteus*, *Streptococcus pneumoniae* DMS 5851, *Bacillus cereus*, *Aeromonas hydrophilla*, Gram negative bacteria 12 stains *Escherichia coli*, *Escherichia coli* O157:H7, *Pseudomonas aeruginosa*, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 27736, *Salmonella typhi*, *Salmonella typhi* B, *Vibrio cholera*, *Salmonella typhi* ATCC 5784, *Salmonella paratyphi* B., *Salmonella paratyphi* A and fungi, *Candida albicans* were used for testing antimicrobial activity.

## 2.11 Antimicrobial Assays

Antibacterial activity was assessed using the disc diffusion assays. The disc diffusion assay was performed using nutrient agar, as described previously [25]. Antibacterial activity was tested with both Gram-negative and Gram-positive bacteria, including *C. albican*. Bacteria at mid-log ( $OD_{600}$  0.5-0.6) phase 100  $\mu$ l was pipette and swabbed to 20 mL nutrient agar. Sterile paper disc (3mm) were placed on the gels and test sample (30  $\mu$ l, 28 $\mu$ g) was added diffusion into each paper disc. Dishes were incubated for 12–24 hours at 37 °C. Antimicrobial activity was assessed by the clearing zone around the paper disc. The broad-spectrum antibiotic streptomycin (10 mg/mL) and 0.01 M acetic acid sterile were used as positive and negative controls, respectively.

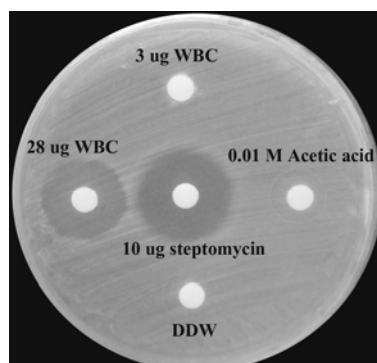
### 2.12 Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF-MS-MS)

Peptide masses were determined by MALDI-TOF mass spectrometry. Coomassie-stained peptides were excised, washed three times in 50% acetonitrile/50%  $\text{NH}_4\text{HCO}_3$ , and lyophilized. Peptides were subjected to in-gel reduction with 10 mM dithiothreitol at 56°C for 1 hrs and alkylation with 100 mM iodoacetic acid for 30 mins at room temperature, followed by digestion with 0.5 g trypsin (Roche Diagnostics). Isolated peptides were washed and concentrated in C18 Zip Tips (Millipore, Bedford, MA) according to the manufacturer's protocol, mixed with 4-hydroxy cinnamic acid matrix, and spotted onto a platform. MALDI-TOF was performed on an Applied Biosystems DE STR mass spectrometer (Applied Biosystems, Foster City, CA). Each sample was spiked with an internal control for angiotension and renin tetradecapeptide. Data were analyzed using the Paws program (<http://prowl.rockefeller.edu>).

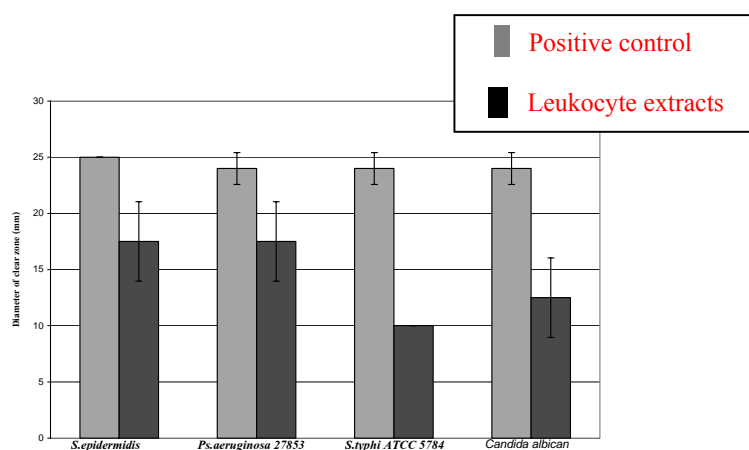
## 3. RESULTS AND DISCUSSION

### 3.1 Screening Crocodile Leukocyte Extract for Antimicrobial Activity

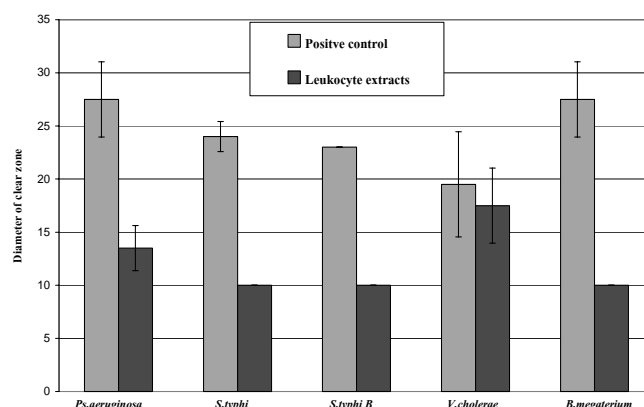
Antimicrobial activity of crocodile leukocyte extract was determined using 23 bacteria and 1 yeast as test organisms. Using a sensitive disc diffusion assay method, leukocyte extract was tested at a concentration of 28  $\mu\text{g}/\mu\text{l}$ . The data presented in Figures 1-3 demonstrate the dose-dependent activity of antimicrobial substance as measured by the size of the clear zone surrounding the sample disc. Crocodile leukocyte extract showed antimicrobial activity against 10 microorganism strains such as *Staphylococcus epidermidis*, *Bacillus megaterium*, *Micrococcus luteus* (gram positive), *Pseudomonas aeruginosa*, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi*, *Salmonella typhi* B, *Vibrio cholerae* and *Salmonella typhi* ATCC 5784 (gram negative bacteria), including *Candida albican* (fungus) only concentration was tested. An appreciable zone of clearing was apparent at 28  $\mu\text{g}$ . At concentrations of leukocyte extract less than 28  $\mu\text{g}$  weakly inhibited growth of microorganism. The diameters of clearing were dose-dependent, demonstrating log-linear relationships in the range tested typical of antimicrobial peptides and proteins. From report of Merchant *et al.* [16], alligator leukocyte extract was effective as an antibacterial agent against seven of seven Gram negative and two of four Gram-positive species tested. Among the Gram-negative bacterial species, the extract exhibited strong activities against *S. flexneri* and *C. freundii*, and moderate activity against *E. coli*, *Ps. aeruginosa*, and *S. choleraesuis*. The extract exhibited high antibacterial activity against the Gram-positive *S. faecalis* and *S. pyogenes*, but no antibacterial activities were observed for *S. aureus* and *S. epidermidis*. Leukocyte extract from the alligator displayed moderate antiviral activity against HIV-1IIB and HSV-1HF. However in this experiment does not assay antimicrobial activity for virus. This result indicated that leukocyte extract of *Crocodylus siamensis* contained antimicrobial substances. These antimicrobial substances were purified further with anion exchange column chromatography.



**Figure 1** Antimicrobial activity of *Crocodylus siamensis* leukocyte extract against *Vibrio cholerae*. Positive control is streptomycin; Negative control is 0.01 M acetic acid and sterile double distilled water (DDW). Concentration of crocodile leukocyte (WBC) extract used for assay activity as 28 µg/µl.



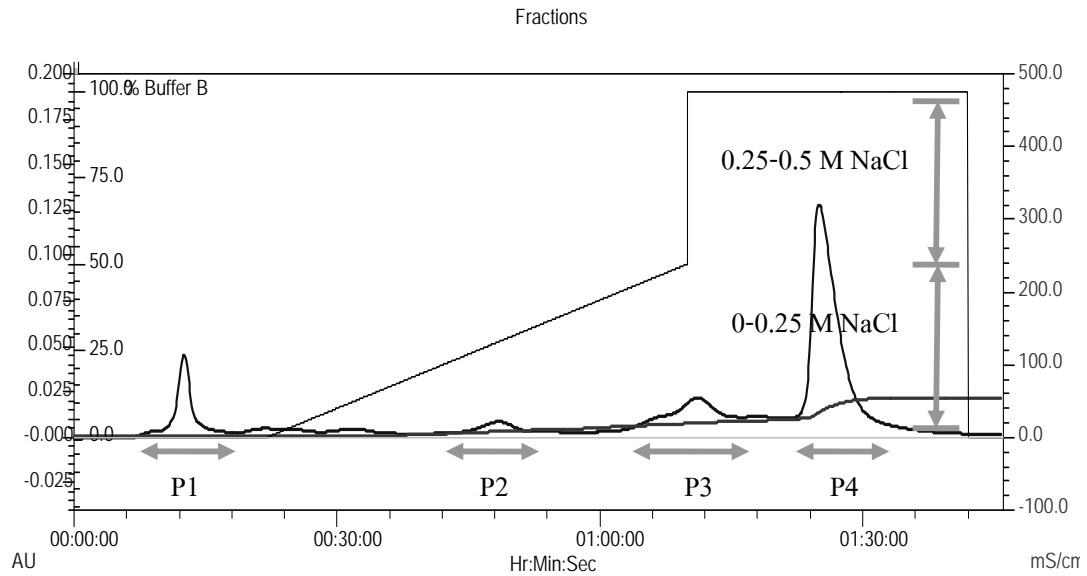
**Figure 2** Antibacterial activity of crocodile leukocyte extract by disc diffusion assay. The dispersion represents the mean  $\pm$  SD of triplicate samples. Light bar is a positive control (10 µg Streptomycin). Dark bar is a leukocyte extract (28 µg/µl). *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi* ATCC 5784, *Candida albicans* were used as test organisms.



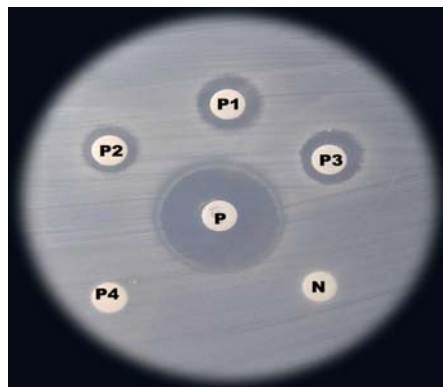
**Figure 3** Antibacterial activity of crocodile leukocyte extract by disc diffusion assay. The dispersion represents the mean  $\pm$  SD of triplicate samples. Light bar is a positive control (10  $\mu$ g Streptomycin). Dark bar is a leukocyte extract (28  $\mu$ g/ $\mu$ l). *Pseudomonas aeruginosa*, *Salmonella typhi*, *Salmonella typhi B*, *Vibrio cholerae*, *Bacillus megaterium* were used as test organism.

### 3.2 Purification of Antimicrobial Substances from Crocodile Leukocyte Extract by Anion Exchange Column Chromatography

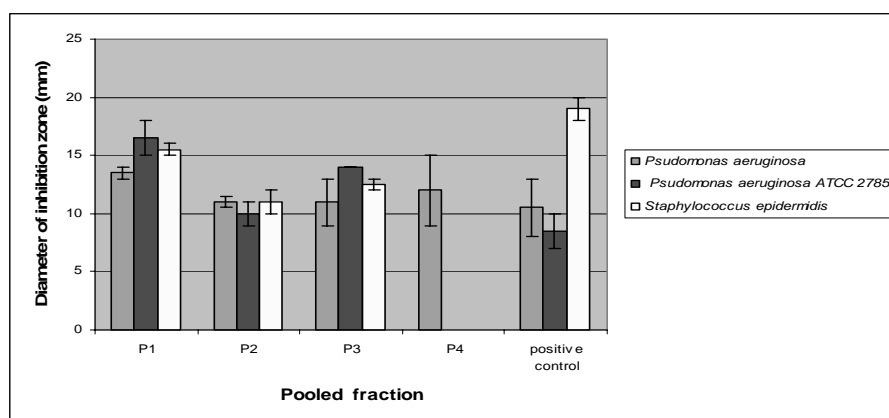
Anion exchange chromatography was used to separate proteins present on the leukocyte extract. One volume of 25 mM Tris-HCl buffer, pH 8.1, was added to the extract to equilibrate the protein sample before separation. After removal of some precipitated proteins, the leukocyte proteins (0.925  $\mu$ g/ $\mu$ l, inject 50  $\mu$ l/time) were first separated in the FPLC system using a Q Sepharose Fast Flow column. In the first step, anion exchange column (20 x 1 cm) was equilibrated with 25 mM Tris-HCl (pH 8.1). The column was washed with the 0.5 M NaCl in same buffer and eluted according to the time program (data not show). The elution profile of the leukocyte proteins is shown in Figure 4. Four chromatographic protein peaks were obtained from the leukocyte extract with high yield shown in peaks 1 and 4. Antimicrobial activity of each protein peak was determined by disc diffusion assay as shown in Figure 5. Proteins P1-P4 showed antimicrobial activity in Figures 6-7. P1, P2 and P3 could act against a broad spectrum of organisms [*Staphylococcus epidermidis*, *Bacillus megaterium*, *Micrococcus luteus* (gram positive), *Pseudomonas aeruginosa*, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi*, *Salmonella typhi B*, *Vibrio cholerae* and *Salmonella typhi* ATCC 5784 (gram negative bacteria), including *Candida albican* (fungus)]. In contrast, protein P4 acted specifically against to *Pseudomonas aeruginosa* and *Vibrio cholerae*. P1 fraction consists of cationic molecule because they could not bind the resin in column; they were eluted from column first. From diameter clearing zone, P1 has strong antimicrobial activity than other peak. Recent studies described, cationic antimicrobial peptide shown high antibacterial activity against board spectrum of microorganism [27]. However P4 was last eluted from column. So P4 contain strong anionic antimicrobial substance which found a little in nature. For the next experiments P1-P4 were separated by tricine -SDS PAGE.



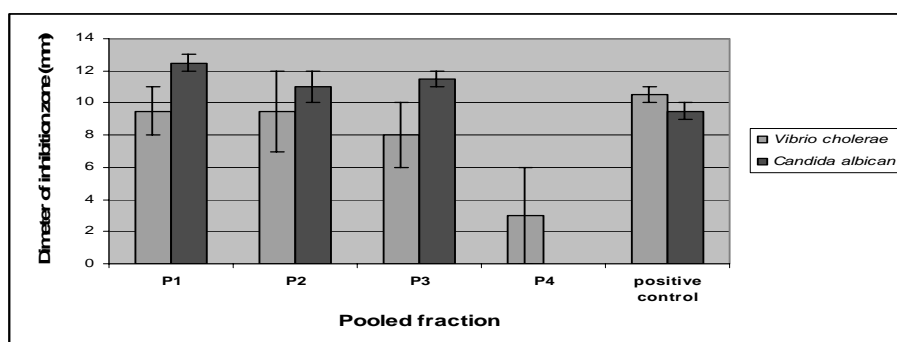
**Figure 4** Anion exchange chromatography on a Q sepharose Fast Flow column of crocodile leukocyte extract preparation containing 46.25  $\mu$ g of proteins was applied to the column Q sepharose Fast Flow column (20 x 1 cm i.d.)



**Figure 5** Antimicrobial activity of protein fractions (P1, P2, P3, P4) from anion exchange column chromatography. P: Positive control: Streptomycin, N: Negative control: Tris-HCl pH 8.1+ 0.5 M NaCl. *Candida albicans* was used as test organisms



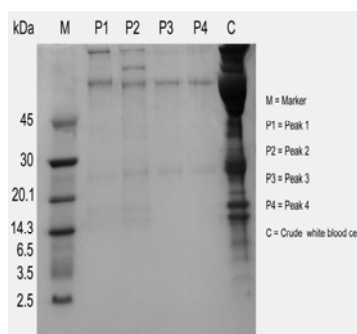
**Figure 6** Antibacterial activity of the partially purified proteins from anion exchange column by disc diffusion assay. The dispersion represents the mean  $\pm$  SD of triplicate samples. *Pseudomonas aeruginosa*, *Pseudomonas aeruginosa* ATCC2785 and *Staphylococcus epidermidis* were used as test organisms.



**Figure 7** Antibacterial activity of the partially purified proteins from anion exchange column by disc diffusion assay. The dispersion represents the mean  $\pm$  SD of triplicate samples. *Vibrio cholerae* and *Candida albicans* were used as test organisms.

### 3.3 Tricine SDS-PAGE Analysis of Protein Fractions P1-P4

The protein components of the fractions were examined using Tris-tricine SDS-PAGE data shown in Figure 8. The P1-P4 fractions contained the proteins with the molecular weight ranging from 14 kDa to over 97 kDa. However, the peptides or proteins with the molecular weight less than 14 kDa were not found due to low sensitivity of detection method. Nevertheless P1 fraction consists of strong antimicrobial activity, P1 was chose for studying in next step. An antimicrobial activity of P1 was determined using zymogram refolding gel. This experiment was used assay antimicrobial activity on gel which contain *M. lysodeikticus* cell. After finish separation, SDS was washed out from gel using 1% Triton X 100 in 50 mM phosphate buffer pH 7.0. Proteins refold and re-function again. The clearing zone of digestion *M. lysodeikticus* cell wall was appeared on local of antimicrobial protein.



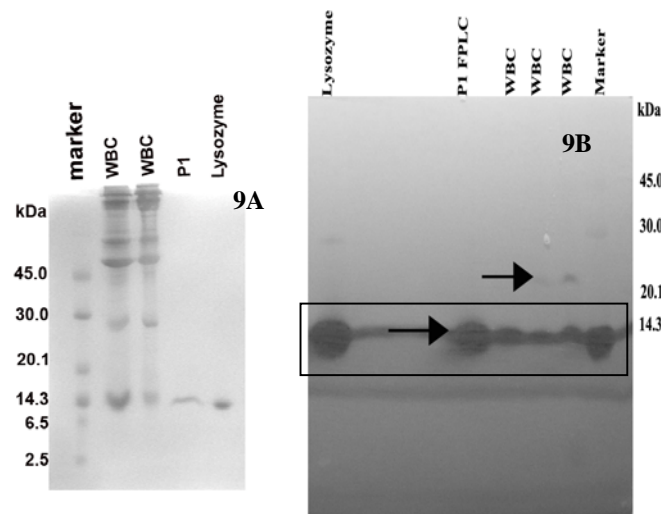
**Figure 8** Tris–Tricine SDS–PAGE (16.5% T, 3% C with a 5-mm spacer gel 10% T, 6% C) analysis of partially purified fraction (P1-P4). Staining with Coomassie G-250.

### 3.4 Identification Antimicrobial Substance from P1 by Zymogram Refolding Gel

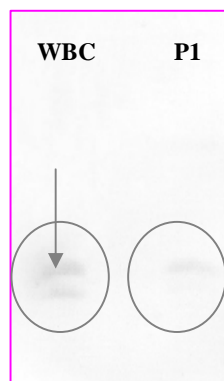
The ability of P1 protein to kill microorganism's cell was determined using zymogram refolding gel. Significant zones of lyses were observed with 10 µg/µl. After gel was washed using 1% triton X -100 in 50 mM phosphate buffer pH 7.0 which cause SDS out of proteins. Proteins on gel refolded and acted antimicrobial activity again. The result shown proteins of P1 fraction molecular weight around 15 kDa, (Figure 9A) could digest cell wall of *Micrococcus lysodeikticus* data shown in Figure 9B. Antimicrobial activity of protein in white blood cell extract was found two locals that were protein MW 23 and 15 kDa. However P1 fraction only found antimicrobial activity of protein MW 15 kDa which possibility protein MW 21 kDa contained in other peaks (P2, P3 or P4). This result demonstrates that antimicrobial activity of P1 fraction got from proteins MW 15 kDa. Activity of these proteins (MW 15 and 21 kDa) is similar to enzyme lysozyme [28] because lysozyme could digest β-1-4-glycosidic linkage on bacteria cell wall, especially cell wall of *M. luteus* [29] and molecular weight of proteins in crude (MW 21) and P1 fraction (15 kDa) similarity lysozyme type G [30] and lysozyme type C [29], respectively. From result, protein in P1 fraction was analyzed by western immuno –blotting compared with hen egg white lysozyme that is a type C lysozyme [29].

### 3.5 Western Immune Blotting Assay

Proteins from P1 fraction were identified by Western immuno blotting in comparison with leukocyte extract. Anti-hen lysozyme was used as primary antibody and anti mouse Ig G linked with alkaline phosphatase was used as secondary antibody. The result showed that polyclonal anti-hen lysozyme antisera showed cross-reactivity to crocodile leukocyte extract and P1 fraction, the result shown in Figure 10. They have strong signal. This protein of molecular weight around 14-15 kDa was lysozyme like protein. However protein molecular weight about 21-23 kDa was not interacted with anti hen egg white lysozyme (lysozyme type C). Recent report of Thammasirirak *et al.* [28] showed structure and amino acid composition of Goose type lysozyme differenced from C type lysozyme which thus anti lysozyme type C could not bound. The possibility protein 21 kDa from white blood cell may be goose type lysozyme which will be studied in further experiment.



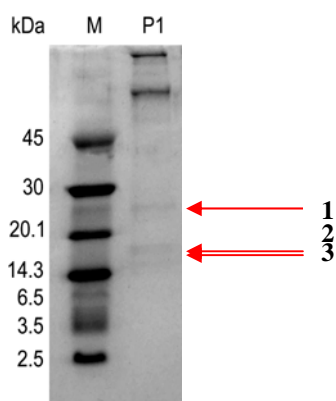
**Figure 9** (A) 15 % SDS-PAGE using separated proteins and peptides from P1 and crocodile leukocyte extract, 120 volt, 106 mins. (B) Identification of antimicrobial protein from fraction P1 and compared with leukocyte extract using zymogram refolding gel.



**Figure 10** Western immuno-blotting using anti hen lysozyme antibody was first antibody (1<sup>st</sup> antibody)

### 3.6 Matrix-assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF-MS-MS)

The protein fractions were separated by Tris-tricine SDS-PAGE. The band proteins were sending to analyze primary structure by MALDI TOF MS - MS. The result of MALDI-TOF-MS-MS from protein band 1, 2 and 3 (Figure 11) were searched on data base of Mascot search, the identity of proteins as showed in Table 1. MALDI TOF mass spectrum of protein band 1 (MW 21 kDa), protein band 2 (MW 15 kDa) and protein band 3 (MW 14 kDa) were not shown. Protein band 1 (MW 21 kDa) was digested and separated by high performance liquid column chromatography (HPLC). This protein composed 7 fragments (data not show). After that these fragment were analyzed using mascot program. Proteins molecular weight around 21-25 kDa were choose from data base, result shown in Table 1. Protein band 1 was similar to Casein alphaS1, PSB3 BOVIN Proteasome subunit beta type 3, glutathione peroxidase (EC 1.11.1.9). Protein band 2 and 3 determined as same as protein band 1. Protein band 2 was similar to hemoglobin beta subunit (Hemoglobin beta chain, Beta-globins) of alligator mississippiensis, lysozyme protein of *Gallus gallus* and mannose-binding lectin precursor (*Dendrobium officinale*). From the report of Craig *et al.* [31] showed antimicrobial activity of human hemoglobin which inhibited growth of microorganism. The result of western immuno-blotting, we used polyclonal antibody which has specificity to many epitope. Structure of hemoglobin may be consisting of epitope which could interact with anti hen egg white lysozyme. Protein band 3 similar to lysozyme type C (E.C.3.2.1.17). However sequence of lysozyme protein in crocodile leukocyte extracts probably differed from lysozyme of *Gallus gallus*. This study is the first report that leukocyte extracts of *crocodylus siamensis* contained antimicrobial proteins, such as lysozyme like protein. More over, crocodile leukocyte extracts may be having other component which could be as antimicrobial substances but could not identify in this experiment.



**Figure 11** P1 fraction was separate by 16.5% tricine SDS PAGE. Protein band 1 MW 21 kDa (Narrow1), protein band 2 MW 15 kDa (Narrow 2), protein band 3 MW 14 kDa (Narrow 3) were identified peptide mass figure print by MALDI TOF MS MS.

**Table 1** Similarity of protein from the data base searching

Protein Band No.	Matched protein	Species	Theoretical MW (kDa)	Score	Accession number
1	1308122A casein alphaS1 R.YLGYLEQLLR.L R.YLGYLEQLLR.L R.FFVAPFPEVFGK.E	<i>Bos taurus</i> (cattle)	24.4356	20.18	225632
	PSB3_BOVIN Proteasome subunit beta type 3 R.LYIGLAGLATDVQTVQR.L	<i>Bos taurus</i> (cattle)	21.5044	10.26	730379
	glutathione peroxidase (EC 1.11.1.9), plasma,precursor K.FLVGPDGVPVMR.W K.FLVGPDGVPVMR.W	Mouse <i>Gallus-gallus</i>	25.4024	10.16	1589389
2	Hemoglobin beta subunit (Hemoglobin beta chain) (Beta- globin) R.QVAAALAAEYH	<i>Alligator mississippiensis</i>	16.6100	20.16	122556
	Lysozyme (E.C.3.2.1.17) Mutant With Ile 55 Replaced By Leu, Ser 91 Replaced By Thr, And Asp 101 Replaced By Ser (I55I,S91t,D101s) R.NTDGSTDYGLLQINSR.W	<i>Gallus gallus</i> (chicken)	14.2999	10.26	809243
	mannose-binding lectin precursor [Dendrobium officinale]. K.DGNLVLYSKPIFATGT NR.F K.DGNLVLYSKPIFATGTNR.F K.DGNLVLYSKPIFATGTNR.F	<i>Dendrobium officinale</i>	18.5379	30.30	55824596
3	Lysozyme (E.C.3.2.1.17) Mutant With Ser 91 Replaced By Thr (S91t) R.HGLDNYR.G K.FESNFNTQATNR.N	<i>Gallus-gallus</i>	14.3271	20.21	442980
	Lysozyme (E.C.3.2.1.17) Mutant With Ile 55 Replaced By Leu, Ser 91 Replaced By Thr, And Asp 101 Replaced By Ser (I55I,S91t,D101s) R.NTDGSTDYGLLQINSR.W	<i>Pelodiscus sinensis</i> (Chinese softshell turtle)	14.2991	10.25	809243
	LYSC_TRISI Lysozyme C (1,4-beta-N-acetylmuramidase C) R.GDQSTDYGILQINSR.W	<i>Pelodiscus sinensis</i> (Chinese softshell turtle)	14.7316	10.24	47117005

#### 4. CONCLUSIONS

Study antimicrobial activity from crocodile leukocyte extract (*Crocodylus siamensis*), Leukocyte extract of crocodiles able to inhibit growth of 10 strain microorganisms. Many strain were pathogen such as *Salmonella typhi*, *Salmonella typhi B*, *Vibrio cholerae* and *Candida albicans*. *Pseudomonas aeruginosa* and *Vibrio cholerae* were sensitive with antimicrobial substance in leukocyte extract. Next antimicrobial substance was purified by anion exchange column chromatography. The elution profile showed 4 protein peaks such as P1, P2, P3, P4. Molecular weight of proteins in every peaks was from 14 kDa to 97 kDa after separated by 15 % tricine-SDS PAGE. P1-P3 able to kill microorganism similarity with leukocyte extract, except P4 contains specific antimicrobial activity to *Vibrio cholerae* and *Pseudomonas aeruginosa*. The strong antimicrobial activity was found in P1. The result of refolding gel method demonstrated that crude and P1 compose proteins which kill microorganism molecular weight about 21 and 14 kDa. Molecular weight of these protein similar to lysozyme type G and C respectively, so western immuno- blotting was used for confirm proteins molecular weight 15 kDa of P1. The result showed that antimicrobial protein at MW 15 kDa was lysozyme liked protein. However antimicrobial protein MW 21 kDa can not detect because limited about antibody. After protein band MW 15 kDa was identified by MALDI TOF MS MS. The protein band MW 15 kDa similarity with lysozyme type C and hemoglobin. These results indicated that crocodile leukocyte extract consist of antimicrobial protein such as lysozyme liked protein. However, other antimicrobial protein and peptide will be identified in further experiment.

#### 5. ACKNOWLEDGMENTS

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