ฤทธิ์ต้านแบคทีเรียก่อโรคของพิษงูจงอางที่แยกส่วน

นางสาว อุษา ฤกษ์สิริกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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# ANTI-PATHOGENIC BACTERIAL ACTIVITIES OF FRACTIONATED VENOM OF KING COBRA (*OPHIOPHAGUS HANNAH*)

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A Thesis Submitted in Partial Fulfillment of the Requirements

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อุษา ฤกษ์สิริกุล : ฤทธิ์ต้านแบคทีเรียก่อโรคของพิษงูจงอางที่แยกส่วน. (ANTI-PATHOGENIC BACTERIAL ACTIVITIES OF FRACTIONATED VENOM OF KING COBRA (*OPHIOPHAGUS HANNAH*)) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. โสภิต ธรรมอารี, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร. ธนิษฐา ฉัตรสุวรรณ, 71 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านแบคทีเรียของส่วนกึ่งบริสุทธิ์ที่แยกจากพิษงูจงอาง และศึกษากลไกการออกฤทธิ์ของส่วนกึ่งบริสุทธิ์นี้ ส่วนโปรตีน ที่แยกจากพิษงูจงอาง สามารถแยกออกเป็น 7 ส่วนคือ F1-F7 เมื่อทดสอบฤทธิ์ต้านแบคทีเรียแล้วพบว่า F5 มีฤทธิ์ในการต้านเชื้อแบคทีเรียดีที่สุด จากนั้นเพิ่มความเข้มข้นและแยก F5 ต่อด้วยคอลัมน์ Sephadex G-75 พบว่าได้โปรตีนทั้งหมด 6 ส่วนคือ F5.1-F5.6 เมื่อนำโปรตีนทุกส่วนไปศึกษาฤทธิ์ต้านแบคทีเรีย พบว่า F5.1 สามารถยับยั้งการเจริญเติบโตของแบค ทีเรียแกรมบวก *S. aureus* และแบคทีเรียแกรมลบ *E. coli*, *S. aeruginosa* และ *S.* Typhimurium โดยสามารถยับยั้งการเจริญของเชื้อแบคทีเรีย *S. aureus* ได้ดีที่สุด โดยมีค่า MIC เท่ากับ 1.8 ไมโครกรัม/มิลลิลิตร เมื่อนำไปหาน้ำหนักโมเลกุลด้วยเทคนิค SDS-PAGE ได้ค่าเท่ากับ 69 kDa จากการศึกษากลไกการออกฤทธิ์ของ F5.1 ด้วยกล้องจุลทรรศน์อิเลคตรอนแบบส่องกราดพบว่า F5.1 น่าจะออกฤทธิ์ผ่านการทำลายเซลล์เมมเบรน และพบว่า F5.1 ที่ความเข้มข้น MIC มีพิษต่อเซลล์ PBMCs ที่แยกจากเลือดของมนุษย์

ผลการศึกษานี้แสดงให้เห็น ว่า F5.1 ออกฤทธิ์ยับยั้ง การเจริญของแบคทีเรีย *S. aureus* ผ่านการทำลายเซลล์เมมเบรน แต่ พบว่า มีพิษต่อเซลล์ปกติด้วย ดังนั้น การศึกษาครั้งนี้ จึงเป็นข้อมูลพื้นฐานสำหรับการ ศึกษาฤทธิ์ต้านแบคทีเรีย ของส่วนกึ่งบริสุทธิ์ F5.1 จากพิษงูจงอาง และ F5.1 ควรจะศึกษาหาส่วน บริสุทธิ์หรือปรับปรุง โครงสร้างต่ อไป ซึ่งอาจนำไปสู่การพัฒนาพิษงูจงอางให้เป็นสารที่มีฤทธิ์ในการต้านเชื้อแบคทีเรีย ซึ่งมีความปลอดภัยต่อเซลล์ปกติของมนุษย์

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2552 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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USA RERKSIRIKUL : ANTI-PATHOGENIC BACTERIAL ACTIVITIES OF FRACTIONATED VENOM OF KING COBRA (*OPHIOPHAGUS HANNAH* ). THESIS ADVISOR : ASSC. PROF. SOPIT THAMAREE, THESIS CO-ADVISOR : TANITTHA CHATSUWAN, Ph.D., 71 pp.

This study aimed at measuring the antibacterial activity of the semi-purified fractions isolated from King Cobra venom and investigated the mechanism of action. The protein fractions were isolated from King Cobra venom and designated F1-F7. The F5 showed the highest antibacterial activity and was further concentrated and separated by Sephadex G-75 column to obtain six fractions (F5.1-F5.6). The antibacterial assay showed that F5.1 could inhibit the growth of gram-positive *S. aureus* and gram-negative *E. coli, S. aeruginosa* and *S.* Typhimurium bacteria. The F5.1 showed antibacterial activity against *S. aureus* with the MIC of 1.8 µg/ml. Its molecular weight determined by SDS-PAGE was 69 kDa. The mechanism of action may be damaging the cytoplasmic membrane, as shown from the scanning electron microscopy. The MIC concentration of F5.1 also showed potent cytotoxicity on human PBMCs cells. The results obtained from this study indicated that the F5.1 had antibacterial activity against the *S. aureus* through membrane damage, however it was cytotoxic to normal human cells. In conclusion, this study provides basic information on antibacterial effect of the semi-purified F5.1 of King cobra venom. The F5.1 should be further purified or modified to obtain the potential antibacterial compound which will be safe to the normal human cells.

Field of Study : Pharmacology ..... Student's Signature .....

Academic Year : 2009 ..... Advisor's Signature .....

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| | |
|---|---|
| ACE | angiotensin converting enzyme |
| $^{o}C$ | degree Celsius |
| CFU | colony forming unit |
| $CO_2$ | carbondioxide |
| FBS | fetal bovine serum |
| h | hour |
| $H_2O_2$ | hydrogen peroxide |
| LAAO | L-amino acid oxidase |
| MBC | minimum bactericidal concentration |
| MIC | minimum inhibitory concentration |
| min | minute |
| ml | milliliter |
| mm | millimeter |
| mM | millimolar |
| NaCl | sodium chloride |
| $NaH_2PO_4.H_2O$ | monosodium phosphate monohydrate |
| $Na_2HPO_4.7H_2O$ | disodium phosphate heptahydrate |
| OD | Optical density |
| PBMCs | peripheral blood mononuclear cell |
| $PLA_2$ | phospholipase $A_2$ |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| S.E. | standard error |
| SEM | Scanning electron microscope |
| TLC | Thin layer chromatography |
| TBS | Tris buffer saline |
| TSB | Trypticase Soy broth medium |

| | |
|---|---|
| UV | ultraviolet |
| µg/ml | microgram per milliliter |
| µl | microliter |

# CHAPTER I

# INTRODUCTION

## 1.1 Background and Rationale

It has been known that bacteria has been related to humans from birth to death. Some types of bacteria are advantageous and some types are dangerous that can cause infections and diseases. According to the World Health Organization (WHO., 2004), bacterial infection is among the 10 leading causes of death worldwide. Problem in the bacterial infections treatment is increasing, it is due to incidence of drug resistance. The present and current emergence of the multidrug-resistant bacteria is one of the most important problems in the public health. Therefore doctor have to use the newly effective antibacterial agents which are more expensive and most of them are imported from foreign counties. Bacterial resistance is the important factor responsible for the increase of morbidity, mortality and health care cost of bacterial infections. Moreover the cost of health care affects the national economy. Therefore, newly effective antibacterials are necessary for drug research and development. In the past, antibiotics were produced from microorganisms, however antibiotics has also been developed from natural sources such as plants or animals. Snake venoms, examples of rich sources of biological molecules have been developed as therapeutic agents. A good example is the bradykinin potentiating peptides (BBPs), which produce an angiotensin-converting enzyme (ACE) inhibitory activity in *B. jararaca* venom (Ferreira *et al*., 1970). The chemical and pharmacological properties of these peptides were essential for the development of captopril, a ACE inhibitor. The first active site directed inhibitor of ACE is currently used to treat human hypertension (Plosker and McTavish, 1995).

Snake venoms are complex mixtures of major components which are proteins and peptides (90-95%) as well as following minor components nucleotides, amino acids, carbohydrates, free lipids and metallic elements bound to proteins (5%) (Russell, 1980; Tu, 1988; Heise *et al*., 1995; Fry and Wuster, 2004). The snake venom proteins are

biological which are interesting because of their diverse, selective pharmacological and physiological effects through their interaction with various molecular targets. Snake venom protein constituents are partial of toxin and partly of enzymes. Snake mouth and fang are contaminated with a wide variety of potentially pathogenic bacteria. Envenomation is a process associated with a low incidence of bacterial infection (Talan *et al*., 1991; Trabi, Schirra and Craik, 2001). Therefore, some molecules in snake venoms could have antibacterial activity for protecting the snakes from infection during feeding. The first reports about antibacterial activity of snake venoms were in 1948 and 1968 (Glaser, 1948; White, 2000). For instances, cytotoxic substances of *Naja* spp. and *H. haemachatus* venoms were able to disrupt *Staphylococcus aureus* and *Escherichia coli* phospholipid membranes, respectively. Another example is an enzyme, L-amino acid oxidase. L-amino acid oxidase of *Bothrops alternates, Agkistrodon halys pallas* and *Trimeresurus jerdoni* has an inhibitory activity against *Pseudomonas aeruginosa, E. coli* and *S. aureus*, and *Bacillus megaterium*, respectively (Stabeli *et al*., 2004; Yan *et al*., 2000; Lu *et al*., 2002; Skarnes, 1970). The pharmacological activities of snake venoms are interested to investigate and develop the new drugs. However, King Cobra venom, a rich source of proteins and peptides has not been fully investigated for searching such antimicrobial activities. The screening of antibacterial activities of crude venoms from King Cobra was conducted in this study. The preliminary results showed antibacterial activity. Therefore, It was interested to evaluate the antibacterial activities of the fractionated venoms of King Cobra for antibacterial agent. The expected outcome is developing a new agent useful for solving the problem of treatment of multidrug-resistant microorganisms in the future.

## 1.2 Objectives of study

1. To study anti-pathogenic bacterial activities of fractionated venoms of King Cobra.
2. To determine minimum inhibitory and bactericidal concentrations.
3. To investigate the mechanism of fractionated venoms of King Cobra on the cell membrane.

## 1.3 Hypothesis

Fractionated venoms of King Cobra possess the anti-pathogenic bacterial activities against *E. coli*, *S.* Typhimurium, *P. aeruginosa*, *S. aureus* and *S. pyogenes*.

## 1.4 Expected benefit and application

1. To obtain the information on antibacterial activity of fractionated King Cobra venom againt *E. coli*, *S.* Typhimurium, *P. aeruginosa*, *S. aureus* and *S. pyogenes* which will be used as a basic pharmacological data.

2. To obtain the scientific data that confirms the pharmacological activity of the fractionated King Cobra venom. The potentially antibacterial agent from snake venom is likely a leader of compounds for the development of novel therapeutic agents.

# CHAPTER II

# LITERATURE REVIEW

## 2.1 Snake venom

### 2.1.1 Snake venom constituents and its pharmacological activity

Snake venom is a prey-immobilizing substance. All of the known advanced snake species are venomous. Most of the venomous snakes include the families Elapidae (Cobras, Kraits, Coral snakes, sea snakes) and Viperidae (Vipers and Pit vipers). The venom compositions are vary depending on age, feeding and geographic origin (Meier, 1986). Their venoms are complex mixtures of pharmacologically active proteins and polypeptide (90-95%) and amino acids, free lipid, nucleotides, and carbohydrate (5%) (Russell, 1980; Tu, 1988; Heise *et al*., 1995; Fry and Wuster, 2004).

Snake venom proteins have a variety of pharmacological activity. Pharmacological consists of the following major components;

Phospholipase $A_2$ ($PLA_2$) is an enzyme which cleaves phospholipid at the $A_2$ position. The $PLA_2$ can be isolated from several snake venoms. The $PLA_2$ isolated from snake venoms has multiple bioactivities, such as activation and inhibition of collagen, ADP and arachidonic acid-induced platelet aggregation (Huang *et al*., 1997; Takagi *et al*., 1988), anti-coagulation (Kini, 2005), induction of endothelial cell motility (Sa *et al*., 1995; Rizzo *et al*., 2000; Chen *et al*., 2002), and furthermore, antitumor (Roberto *et al*., 2004), myotoxic, cardiotoxic or neurotoxic action (Harris *et al*., 2003; Gutierrez *et al*., 1997). It was reported that the Lys49 and Asp49 mytotoxic $PLA_2$ from *Bothrops asper* snake venom express bactericidal activity (Paramo *et al*., 1998).

Serine protease: serine protease (Batroxobin) from *Bothrops atrox moojeni* venom is a thrombin-like enzyme that converts fibrinogen to fibrin (Bell, 1997).

Bothrojaracin, a c-type lectin-like protein from *Bothrops jararaca,* has antithrombotic activity in rats (Zingali, 2005).

Fibrolase, a metalloproteinase fibrinolytic agent was patented as a novel acting thrombolytic which is useful for lysis of blood clots *in vivo* (Mann, 2007) and directly-acting plasminogen activator agent, which was isolated from copperhead snake venom. Fibrolase rapidly lyses clot and also be able to lyse a carotid artery thrombus *in vivo* (Markland *et al*., 1994).

L-amino acid oxidase is a flavoenzyme enzymes widely distributed in venomous snake families of Viperidae, Crotalidae and Elapidae. The enzyme catalyzes the stereospecific oxidative deamination of amino acid into α-keto acid, ammonia and hydrogen peroxide. L-amino acid oxidase has various biological properties including as apoptosis inducing activity (Ali *et al*., 2000), antivirus activity (Zhang *et al*., 2003), inhibition (Nathan *et al*., 1982) or activation of platelet aggregation (Li *et al*., 1994), antibacterial, antiparasitic (Tempone *et al*., 2001), cytotoxicity (Stabeli *et al*., 2007), and anticoagulant activities (Sakurai *et al*., 2003).

It is possible to observe the presence of other protein compounds with an enzymatic profile in snake venoms. Those include cysteine-rich secretory proteins, which inhibit cyclic nucleotide-gated ion channels and smooth muscle contraction (Nahas *et al*., 1964). There are also other enzymes such as phosphomonoesterase, phosphodiesterase, hyaluronidase, acetylcholine esterases and 5-nucleotidase in snake venoms (Yamazaki and Morita, 2004; Matsui *et al*., 2000). Snake venoms also contain several peptides. However, they may vary from presenting cardiotoxin (Tsetlin *et al*., 2004; Satora *et al*., 2003), neurotoxin (Harvey, 2001), cytotoxic effect (Lu *et al*., 2003).

### 2.1.2 Snake venom with antibacterial activities

Several antimicrobial investigations involving many snake venoms have already been described in the literature. Antibacterial snake venoms contain many proteinaceous components, including neurotoxin (pre and post synaptic), myotoxins, cytotoxins, cardiotoxins, proteases, nucleases (Stiles *et al*., 1991), L-amino acid oxidase, a direct lytic factor, $PLA_2$ and peptide.

The first reported bactericidal effects elicited by L-amino acid oxidase from *Crotalus adamanteus* venom (Skarnes, 1970). Also found that two L-amino acid oxidases from the venom of *Pseudechis australis* have powerful effects against Gram-positive and Gram-negative bacteria (Stiles *et al*., 1991). L-amino acid oxidase from *Bothrops moojeni* venom has been reported to kills *Leishmania spp*. promastigotes in vitro (Tempone *et al*., 2001). The $H_2O_2$ is thought to play the major role since this killing effect is inhibited by catalase ( Skarnes, 1970; Stiles *et al*., 1991; Tempone *et al*., 2001). L-amino acid oxidase purified from *Pseudechis autralis* was 70 times more effective than tetracycline against *Aeromonas* (Lu *et al*., 2002) and from *Bothrops alternates* against *E. coli* and *S. aureus* (Stabeli *et al*., 2004) and from *Bothrops moojeni* against *E. coli, S.* Typhimurium*, P. aeruginosa* and *S. aureus* (Stabeli *et al*., 2007). The purified L-amino acid oxidase from *Crotalus durissus cascavella* venom inhibited the bacterial growth of Gram-negative (*Xanthomonas axonopodis pv passiflorae*) and Gram-positive (*S. mutans*) strains. It is believed that the antibacterial effect of L-amino acid oxidase is due to the liberated $H_2O_2$, as addition of catalase completely suppressed the antibacterial activity. Electron microscopy studies suggested that the $H_2O_2$ generated in the oxidation process induced bacterial membrane rupture and then loss of cytoplasmic content (Toyama *et al*., 2006).

A direct lytic factor or cytotoxin, low molecular weight protein from *Naja spp*. and *H. haemachatus* has been reported to disrupt *S. aureus* and *E. coli* phospholipid membranes, respectively (White, 2000; Glaser, 1948).

The venom of the snake *Bothrops asper* was found to contain two bactericidal $PLA_2$s (Lys49 and Aps49). $PLA_2$ myotoxins purified from *Bothrops asper* snake venoms presented bactericidal activity, both against S.Typhimurium and *S. aureus* (Paramo *et al*., 1998; Santamaría *et al*., 2005). A number of snake venom $PLA_2$s were found to be bactericidal activity against the Gram-negative bacterium *Burkholderia pseudomallei* (Perumal Samy *et al*., 2006). The $PLA_2$ myotoxins of *Bothrops asper* snake venoms towards Gram-negative and Gram-positive bacteria is based on their general membrane perturbing effects mediated by collection of hydrophobic amino acids in the C-terminal region of the molecules. The bactericidal mechanism is assumed to be

similar to that of cationic peptides, such as lactoferin and defensins, i.e. displacement of the metal ions $Ca^{2+}$ amd $Mg^{2+}$ from the bacterial cell surface. The secretory phospholipase $A_2$ ($sPLA_2$) purified from *B. fasciatus* snake venoms had activity against *S. aureus* and *E. coli.* The MIC was 0.4 and 0.1 μM, respectively (Chunhua, Dongying and Haining, 2007).

Snake venom metalloproteinases comprise a family of zinc-dependent enzymes, which display antibacterial activitiy. A 23.1 kDa protein was isolated from *Agkistrodon halys* (pallas, Chinese viper) snake venom. The *Agkistrodon halys* metalloproteinases have been shown to exhibit antibacterial properties and were more active against *S. aureus, P. vulgaris, P. mirabilis* and multidrug-resistant *B. pseudomallei* bacteria (Perumal Samy *et al*., 2008).

Omwaprin, a 50- amino acid cationic protein from the venom of *Oxyuranus microlepidotus* has been shown to possess the selective and dose-dependent antibacterial activity against Gram positive bacteria. The mechanism of action is via membrane disruption, as shown by scanning electron microscopy (Nair *et al*., 2007).

Cationic peptide derived from $PLA_2$ myotoxins of *Bothrops asper* snake venoms showed bactericidal activity against both Gram-negative and Gram-positive bacteria by interacting with lipopolysaccharide (LPS) and lipid A from different Gram-negative bacteria or with lipoteichoic acid from *S. aureus* and the mechanism relies on a membrane-permeabilizing to exert its bactericidal effects (Paramo *et al*., 1998). Small peptides isolated from *Naja atra* venom were investigated against multidrug-resistant *Mycobacterium tuberculosis*. Small peptide from *Naja atra* venom had *in vitro* activities against multidrug-resistant *M. tuberculosis*. The MIC was 8.5 mg/l (Xie *et al*., 2003) and Cathelicidin-BF separated from *B. fasciatus* venom against Gram-negetive bacteria. No hemolytic and cytotoxic activities were observed at the dose of up to 400 μg/ml (Wang *et al*., 2008). Also three snake venoms (*Naja naja soutratrix, Vipera russelli* and *C. adamanteus*) had antibacterial activity against *E. coli* (Stocker and Traynor, 1986) and Crotarid venom against Gram negative and Gram positive bacteria (Talan *et al*., 1991).

### 2.1.3 Descriptions of King Cobra

King Cobra (*Ophiophagus Hannah*) belonging to the Elapidae family is the largest venomous snake in the world. This snake is broadly found in Thailand, India, Myanmar, Malaysia, Indochina, Southern China, Indonesia, Japan and Philippines (Tin *et al*., 1991). In Thailand it is found mostly in the south. The King Cobra is the largest and the most venomous snake. Little investigation has been carried out about the venom compared with those from other snakes. Most studies involved major snake venom constituents and toxicity with body systems, but the biological activities of snake venoms remain poorly characterized despite being a rich source of biological active proteins with therapeutic potential particularly the antibacterial activity. Thus there is a need to study antibacterial activities and the possibility of King Cobra venoms for developing a new source of antibacterial agents.

Hannalgesin is a neurotoxin and isolated from King Cobra venom. The neurotoxin was purified by using Sephadex-C25, G50 and HPLC columns, orderly. Hannalgesin was tested for nociceptive activity in mice using the hot plate and rota rod. After i.p. administration, Hannalgesin caused significant antinociceptive effects at dose range of 16-32 ng/g body weight (Pu *et al*., 1995). Moreover CM-11 isolated from King Cobra venom was tested for analgesic, sedative and anticonvulsant activities in mice. These results demonstrated that neurotoxin CM-11 caused antinociceptive activity.

The purified phospholipase $A_2$ (OHV A-$PLA_2$) from the King Cobra venom has some effects on hemostasis. OHV A-$PLA_2$, an acidic protein was applied to a CM-Sephadex and Sephadex G-50 column. In *in vitro* tests, OHV A-$PLA_2$ showed an inhibitory on platelet aggregation effect induced by ADP, collagen and arachinodic acid in both human whole blood and platelet-rich plasma (Huang *et al*., 1997).

L-amino acid oxidase from King Cobra venom has cytotoxicity. The enzyme was purified by a combination of gel-filtration, ion-exchange and reverse-phase chromatographic steps. The cytotoxicity of L-amino acid oxidase was observed in stomach cancer, murine melanoma, fibrosarcoma, colorectal cancer and Chinese hamster ovary cell lines. These resulted in the loss of ability in attachment and inhibition

of cell proliferation. The mechanism of L-amino acid oxidase action may be related to the inhibition of thymidine incorporation and an interaction with DNA (Anh *et al*., 1997).

Hannahpep from King Cobra venom affected hemostasis. It is a peptide with molecular weight 610 Da which was purified by a combination of TLC and HPLC. Hannahpep had defibrinogenating activity in male albino mice and exhibited fibrinolytic and fibrinogenolytic activity *in vitro* (Gomes and Pallai De, 1999)

KC-MMTx showed effect on CNS and anticonvulsant activity. It was isolated and purified from King Cobra venom by TLC and column chromatography (HPLC). KC-MMTx was tested for anticonvulsant effects in male albino mice by significant protection against drug (strychnine, pentylenetetrazole and yohimbine) induced convulsions (Saha *et al*., 2006).

L-amino acid oxidase (Oh-LAAO) affect on hemostasis. It was isolated and purified from King Cobra venom by gel-filtration, ion-exchange and heparin chromatographic. It showed an inhibitory effect on platelet aggregation induced by ADP and U46619 but not by thrombin, mucetin, ristocetin and stejnulxin (Yang *et al*., 2007). This result was opposite to the studied by Li and co-workers (1994) which demonstrated that LAAO from King Cobra venom induced platelet aggregation.

## 2.2 Protein Purification

Protein purification is a series of process intended to separate the desired protein from the sample or complex mixture.

*Gel filtration*

Gel filtration is a chromatographic technique for the separation of molecules according to size and shape. The matrix consists of tiny porous beads. Protein molecules are small enough to pass through the holes in the beads. They are delayed and travel more slowly through the column. The large molecules cannot enter the beads and are eluted first (Robert, 1994) (see Fig 2.1).

*Ion exchange chromatography*

Ion exchange chromatography technique is used for separation of the molecules according to charge-charge interactions between the proteins in the sample and the immobilized-charge resin. Ion exchange chromatography can be divided into two major types according to charge immobilized on the resin. Cation exchange, in which positively charged ions bind to a negatively charged resin and anion exchange, in which the binding ions are negative (see Fig 2.2).

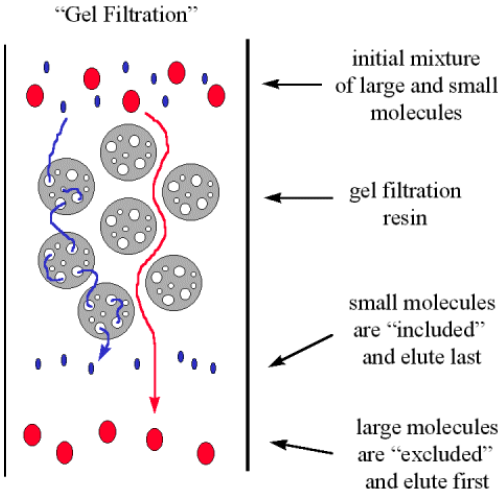



**Figure 2.1** Gel filtration chromatography

(Available from http://slohs.slcusd.org/pages/teachers/rhamley/Biology/Basis%20of%20Life/gelfiltr.gif) [2008, 12 February]

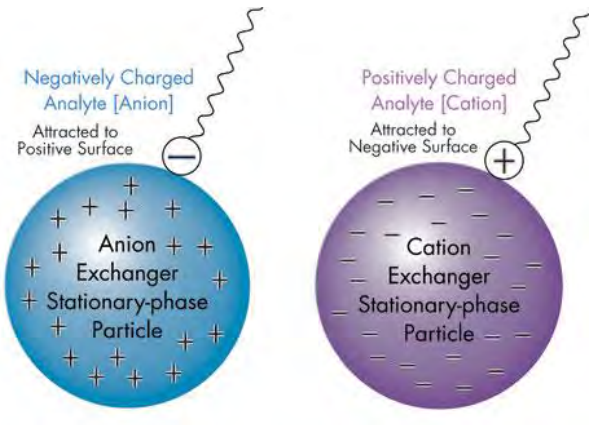



**Figure 2.2** Ion exchange chromatography

(Available from www.waters.com/waters/nav.htm?locale=en_us&cid=10049076) [2008, 12 February]

## 2.3 Microorganisms

### 2.3.1 Bacterial structure

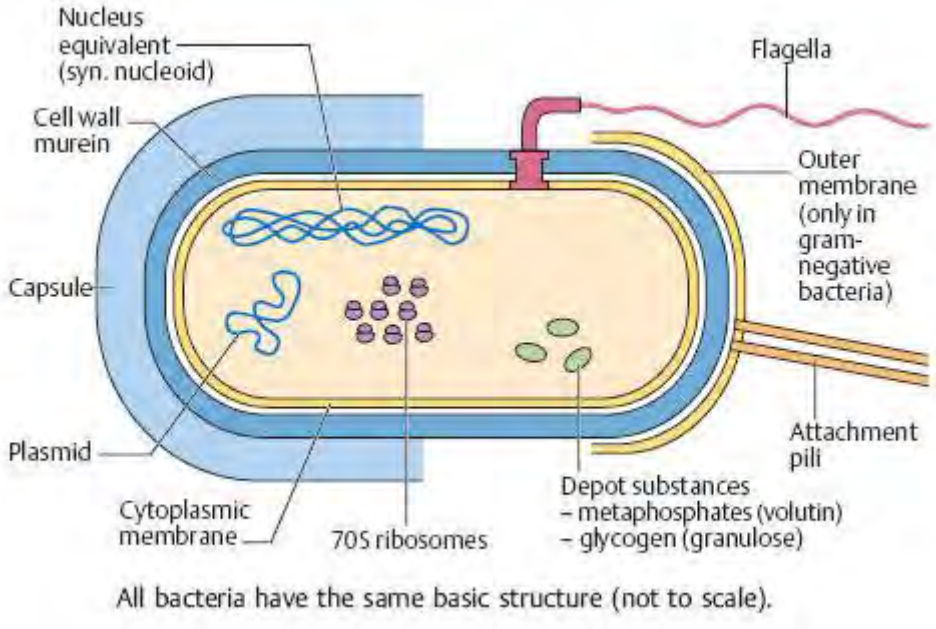



**Figure 2.3** Bacterial structure

(Available from http://www.arabslab.com/myimages/bacteria/bacteria_cell_wall_structure.jpg) [2008, 12 November]

**2.3.1.1 Gram-negative** (Marie, 2005)

**Cell wall**

• The **outer membrane** serves as the primary permeability barrier of the cell and helps to retain proteins in the periplasmic space. (Some authors do not consider this membrane to be part of the cell wall.)

• **Lipopolysaccharides** are found on the surface of the cell and are the major harmful component of endotoxin. They contribute to the bacterium's ability to cause disease and they give Gram-negative bacteria to their net negative charge.

• **Lipoproteins** are attach the outer membrane to the murein layer.

• The **peptidoglycan layer** of Gram-negative bacteria is a relatively thin polymer layer consisting of cross-linked N-acetylmuramic acid and N-acetylglucosamine. It is often referred to as the murein layer or cell wall and is responsible for maintaining the shape of the organism. It is located in the outer layer of outer membrane.

• The **periplasmic space** lies between the outer membrane and the cytoplasmic membrane. Periplasmic proteins include binding proteins for specific substrates, hydrolytic enzymes and detoxifying enzymes.

**Cytoplasmic membrane**

The cytoplasmic membrane surrounds the cytoplasm of the cell and contains proteins and phospholipids. The cytoplasmic membrane also serves as a permeability barrier and a permeability link for substances entering the cell.

**Cytoplasm and other internal components**

The cell cytoplasm contains the chromosome, ribosomes and other internal structures. The vast majority of bacteria have a single chromosome but a few, such as *Vibrio cholera,* have two chromosomes.

**2.3.1.2 Gram-positive**

**Cell wall**

Since the Gram-positive cell wall contains only two major components it is much less complicated than the Gram-negative cell wall (see Fig 2.4).

• **Teichoic acids** are polymers that are interwoven in the peptidoglycan layer and extend as hair-like projections beyond the surface of the gram-positive cell. They also are major surface antigens in those organisms that possess them.

• The **peptidoglycan layer**, or murein layer, of gram-positive bacteria is much thicker than that of gram-negative bacteria. It is responsible for maintaining the shape of the organism and often is referred to as the cell wall.

**The cytoplasmic membrane, cytoplasm, and other internal components**

These structures are very similar in both Gram-positive and Gram-negative bacteria.

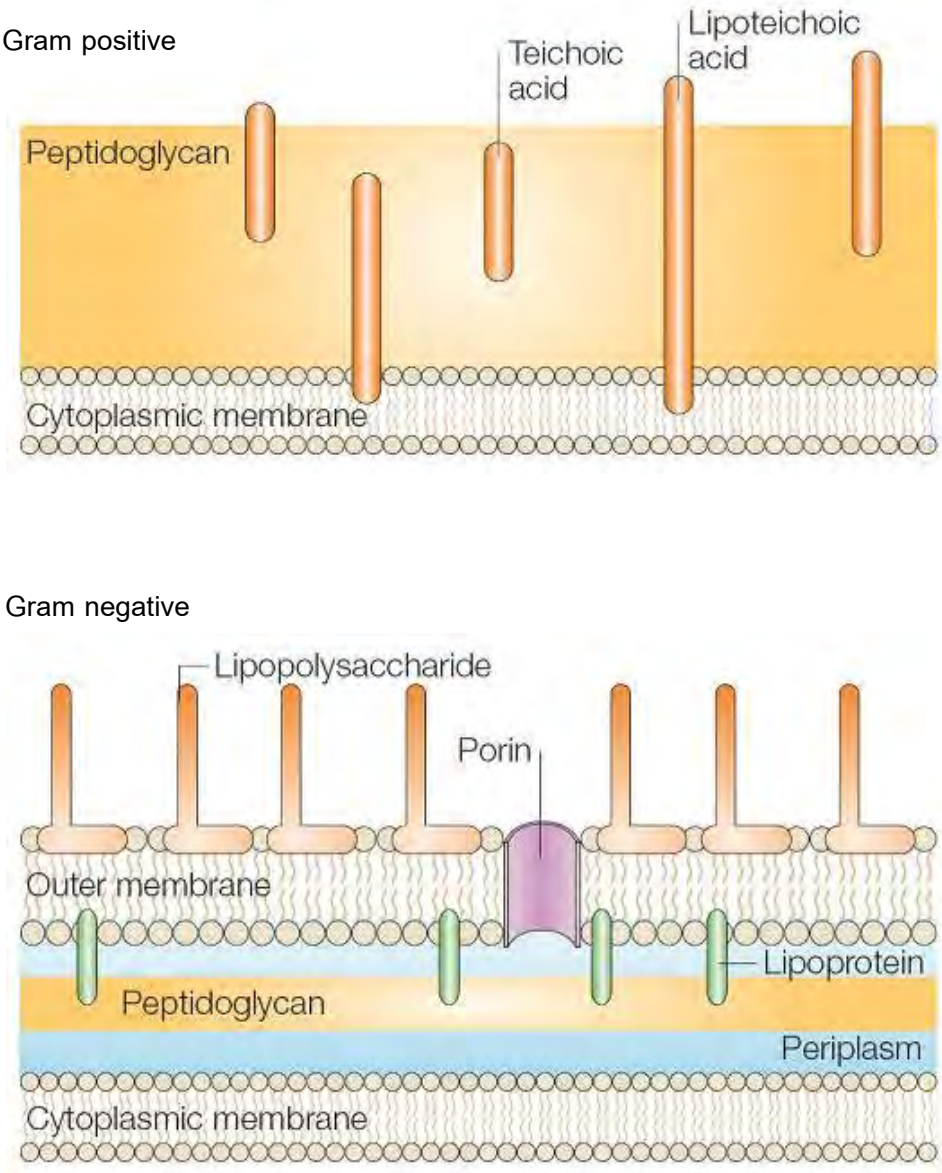



**Figure 2.4** Gram-positive and Gram-negative cell walls (Mathew and Chistine, 2005

**2.3.2 Pathogenic bacteria** (Betty *et al*., 2007; William *et al*., 2001)

***P. aeruginosa***

*P. aeruginosa* is the most commonly encountered gram-negative species. This organism widely distributed in nature (soil, water, plants, animals). It may colonize in healthy humans without causing disease, but it is also a significant opportunistic pathogen, and a major cause of nosocomial infections. *P. aeruginosa* is regularly a cause of nosocomial pneumonia, nosocomial infections of the urinary tract, severe burns, surgical sites, and of infections of patients with cystic fibrosis. *P. aeruginosa* can grow in distilled water, hot water baths, hot tubs, whirlpools, sinks, showers and other water-containing vessels. *P. aeruginosa* is a very successful opportunistic pathogen. Factors that contribute to pathogenicity include pili may mediate attachment to host cells, production of several proteolytic enzymes and hemolysins that destroy cells and tissue, and pyocyanin promote tissue damage and local invasion and dissemination of the organism. Systemic disease is promoted by an antiphagocytic capsule, endotoxins and exotoxins A, which kills host cells by inhibiting protein synthesis.

- Localized infections:

Localized *P. aeruginosa* infections may occur in the skin (folliculitis), eye (following trauma), ear (otitis externa), urinary tract, respiratory tract, gastrointestinal tract, and central nervous system. In most cases of localized infections have the potential of leading to disseminated infection.

- Systemic infection:

The gastrointestinal tract is a particularly common site for perforation. The resulting systemic infections may include bacteremia, secondary pneumonia, joint and bone infections, endocarditis, and skin/soft tissue or central nervous system infections.

***S. aureus***

*S. aureus* is a gram-positive species. *S. aureus* is part of normal flora of human anterior nares, nasopharynx, perineal area, and skin. Infection occurs during penetration of the skin (for example, due to a wound, or during surgery), typically resulting in a pustule. Subsequent diseases can be caused by the actual infection, by the toxins in the

absence of infection (toxinosis), or by a combination of intoxication and infection. Important *S. aureus* virulence factors include: 1) cell wall virulence factors that can promote binding to mucosal cell and exert antiopsonic (and therefore antiphagocytic) effects; 2) cytolytic exotoxin (includeing hemolysins); 3) superantigen exotoxins, including enterotoxins (which causes food poisoning), toxic shock syndrome toxin, and exfoliative toxin (which causes scale skin syndrome in children, and also bullous impetigo). The most common diseases caused by *S. aureus* are the followings.

- Localized skin infections

These infections include 1) small, superficial abscesses involving sweat or sebaceous glands, or hair follicles; 2) subcutaneous abscesses (furuncles or boils) that form around foreign bodies such as splinters; and 3) larger, deeper infections (carbuncles) that can lead to bacteremia.

- Diffuse skin infection –impetigo (pyoderma)

This is a superficial, spreading, crusty skin lesion generally seen in children.

- Deep localized infections

*S. aureus* is the most common cause of acute and chronic infection of the bone marrow (osteomyelitis), and also the most common cause of arthritis resulting from acute infection of the joint space (septic joint).

- Other infections

*S. aureus* can cause acute endocarditis, septicemia and severe necrotizing pneumonia.

- Toxinoses

Toxic shock syndrome is causes by strains of *S. aureus* that produce a specific, absorbable toxin. The syndrome results in high fever, rash, vomiting, diarrhea, hypotension and multiorgan involvement (especially GI, renal and hepatic damage). Staphylococcal gastroenteritis is caused by ingestion of food contaminated with toxin produced by *S. aureus.*

Scaled skin syndrome (mild cases are sometimes called bullous impetigo) involves the appearance of superficial bullae resulting from the action of an exfoliative

toxin that attacks the intercellular adhesion of the stratum granulosum, causing marked epithelial desquamation.

*S. pyogenes*

*S. pyogenes* is a Gram positive species. Its habitat is skin and upper respiratory tract of humans. Most common sources of contagion are through breaks in skin after direct contact with infected person or person to person by contaminated droplets produced by coughs or sneezes. *S. pyogenes* causes some of the most rapidly progressive infections known, causing cellutitis anywhere in the body. Other complications that result from *S. pyogenes* infection are the poststreptococcal disease including rheumatic fever and acute glomerulonephritis.

- Acute pharyngitis

*S. pyogenes* is the most common bacterial cause of sore throats, especially in patients two to twenty years old. Pharyngitis (for example, strep throat) is the most common type of *S. pyogenes* infection. Pyrogenic exotoxin may be released and produce scarlet fever, which occurs in association with streptococcal pharyngitis and is manifested by a rash of the face and upper chest.

- Acute rheumatic fever

Rheumatic fever is characterized by inflammatory changes involving the heart, joints, blood vessel and subcutaneous tissues. This disease is causes by cross-reactions between antibodies produced against *S. pyogenes* antigen and human heart and joint tissues.

- Impetigo

This is a localized skin infection with vesicles progressing to pustules and no evidence of systemic disease that primarily affects exposed areas (face, arm and legs) It usually affects young children.

- Erysipelas

Erysipelas is an acute infection of the localized skin. Patients experience localized pain, inflammation, lymph node enlargement and systemic symptoms. It affects all age groups.

- Acute glomerulonephritis

Acute glomerulonephritis is characterized by inflammation of the renal glomeruli with edema, hypertension, hematuria and proteinuria. This disease is mediated by antibody- *S. pyogenes* antigen complexes that deposit in kidney resulting in damage to glomeruli.

***E. coli***

*E. coli,* a Gram-negative species, is the normal flora habitant of human and other animal intestine but can cause the diseases of both inside and outside the gastrointestinal tract. *E. coli* is composed of three type antigens, O, K and H. Pili encouraging the attachment of bacteria with human epithelial surfaces. Three general clinical diseases can result from infection of *E. coli*.

1. Urinary tract infections (UTI)

*E. coli* is the most common cause of UTIs, especially in women. Symptoms include dysuria, hematuria, polyuria and pyuria.

2. Diarrhea

Several different *E. coli* strains cause several categories of diarrhea. Among the most prevalent are the following:

Enterotoxigenic *E. coli* (ETEC): This organism is an important cause of traveller's diarrhea in developing countries. The transmission is transmitted by contaminated food and water. ETEC colonizes in the small intestine and in a process mediated by an enterotoxin that stimulates increased cAMP production. This leads to hypersecretion of chloride ions and water while simultaneously inhibit the resorption of sodium resulting in significant watery diarrhea.

Enteropathogenic *E. coli* (EPEC): This organism is an important cause of potentially infant diarrhea. EPEC attaches to mucosal cells of the small intestine resulting in destruction of microvilli and in watery diarrhea which may become chronic.

Enterohemorrhagic *E. coli* (EHEC): EHEC causes bloody diarrhea (haemorrhagic colitis) and haemolytic uremic syndrome (HUS). EHEC binds to cells in the large intestine and produces an exotoxin (verotoxin) that destroys microvilli. Serotype O157:H7 is the most

common strain of *E. coli* that produces verotoxin. EHEC transmitted by ingestion of undercooked ground beef or raw milk.

- Meningitis in infants

*E. coli* is common cause of neonatal meningitis. Newborns lack IgM and therefore are particularly susceptible to *E. coli* sepsis. These bacteria translocate from the blood to the central nervous system, which can result in the organisms being carried to the brain.

### *S.* Typhimurium

*S.* Typhimurium is a Gram-negative bacteria predominantly found in the gastrointestinal tracts of human and other animals. It is transmitted through contaminated food products or via the oral and fecal route. *S.* Typhimurium causes enterocolitis (gastroenteritis, food poisoning) in human and other mammals. Within 6 to 48 hours after ingestion of contaminated food or water, the symptoms include nausea, vomiting and diarrhea ensue.

## 2.4 Antibacterial agents

### 2.4.1 Mode of antibacterial action

Antibacterial agents can be classified into five major groups according to mode of action. These agents may cause inhibition of cell wall synthesis, cytoplasmic membrane functions, protein synthesis, nucleic acid synthesis and a metabolic pathway (see Fig 2.5).

1. Inhibition of cell wall synthesis

Cell wall synthesis inhibited by β-lactams such as penicillins, cephalosporins and carbapenems which inhibit peptidoglycan polymerization by inhibiting enzyme transpeptidase are that required for cell wall synthesis. This causes weakened or defective cell walls and leads to cell lysis and death. Vancomycin binds to peptidoglycan precursors and causes large size complex. This large complex cannot

crosslink with peptidoglycan resulting in inhibition of cell wall synthesis. Antibacterial agents that inhibit cell wall synthesis are bactericidal.

2. Inhibition of cytoplasmic membrane function

Polymyxin molecules diffuse through outer membrane and cell wall to cytoplasmic membrane. They bind to the cytoplasmic membrane and disrupt it. This causes leak out of cytoplasm resulting in cell death.

3. Inhibition of protein synthesis

3.1 Inhibition of protein synthesis by binding to the 30S ribosomal subunit

- Aminoglycosides (streptomycin, neomycin, netilmicin, tobramycin, gentamicin, amikacin) bind to the 30S subunit of bacterial ribosomes and block protein synthesis in 2 different ways. Firstly, They can bind to the 30S subunit of bacterial ribosomes and prevent the 30S subunit from attaching to mRNA. Secondly, aminoglycosides may cause misreading of the RNA. This leads to the insertion of the wrong amino acid into the protein or interference with the ability of amino acid to connect with one another. These activities often occur simultaneously and the overall effect is bactericidal.

-Tetracyclins (tetracycline, doxycycline, demeclocycline, minocycline) bind to the 30S subunit of ribosomes and inhibit the attachment of tRNA. Since new amino acids cannot be added to the growing protein chain, synthesis of protein is inhibited. The action of tetracyclines is transient, so these agents are bacteriostatic.

3.2. Inhibition of protein synthesis by binding to the 50S ribosomal subunit

- Macrolides (e.g. erythromycin, azithromycin and clarithromycin) and lincosamides (e.g. clindamycin) attach reversibly to the 50S ribosomal subunit causing termination of the growing protein chain and inhibition of protein synthesis.

- Chloramphenicol also binds to peptidyltransferase on the 50S subunit of the ribosome and interferes with binding of amino acids to the growing protein. Antimicrobial agents that inhibit protein synthesis both Gram-positive and Gram-negative bacteria in this manner are bacteriostatic.

4. Interference with nucleic acid synthesis

- Rifampin blocks transcription by inhibiting RNA polymerase, the enzyme needed for synthesis of mRNA from DNA template.

- Fluoroquinolones (e.g. norfloxacin, lomefloxacin, ciprofloxacin, enoxacin) work by inhibiting DNA gyrase and topoisomerase IV, DNA gyrase is for relaxing supercoiled DNA to circular DNA and topoisomerase IV is for cutting DNA strands and separate daughter chromosome in cell division process.

5. Inhibition of the metabolic pathway for folic acid synthesis

For many organisms para-aminobenzoic acid (PABA) and pteridin are important in folic acid synthesis. Nucleic acid synthesis has to use dihydropteroate synthetase for combind PABA and pteridin to dihydropteroic acid that is an important intermediate. Sulfonamide will compete with PABA for enzyme dihydropteroate synthetase and trimethoprim inhibits dihydroforate reductase.

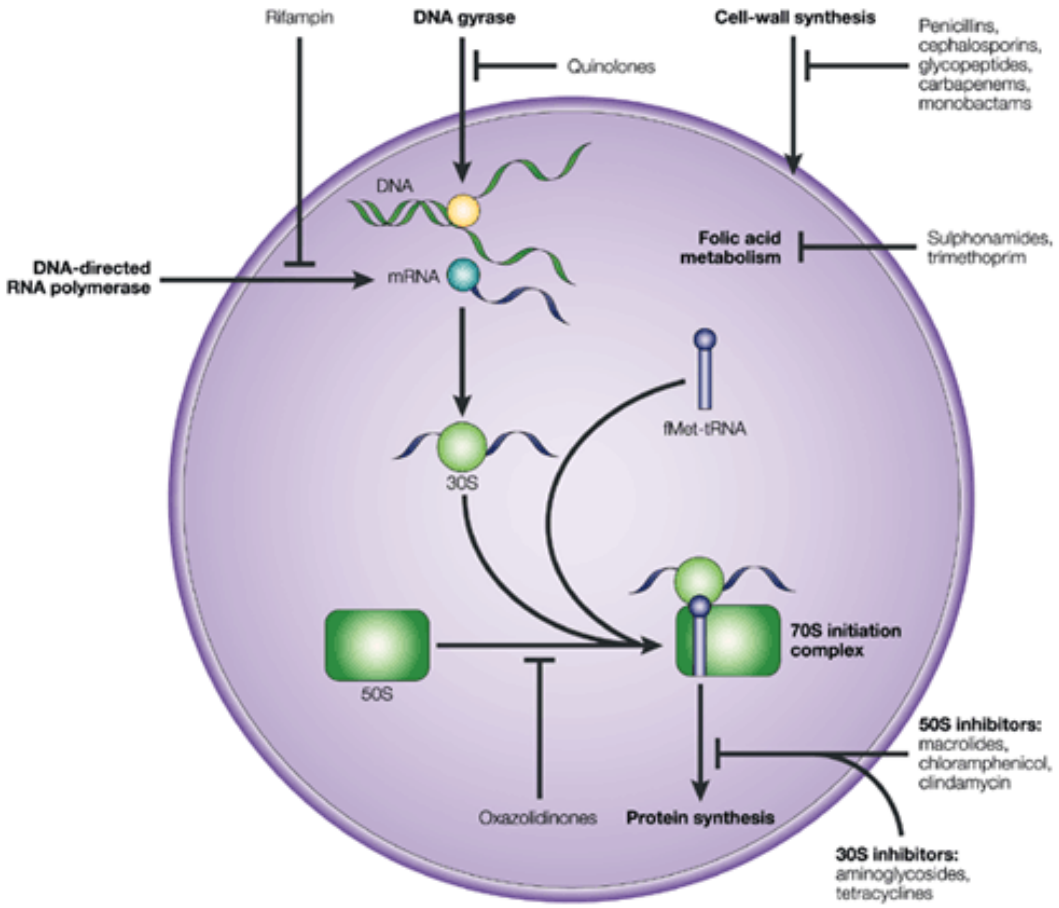



**Figure 2.5** Antibacterial drug targets (Anthony, 2002)

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Instruments and equipments

The list of instruments and equipments used in this study were as the following; lyophilizer (Dura-dry), centrifuge machine (Hettich), $CO_2$ incubator (Thermo), spectrophotometer (UV-1601 UV/VIS Shimadzu, Japan), microplate reader (Labsystem), hematocytometer (Fisher Scientific), light microscope (Nikon), analytical balance (GMPH, Satorius and UMT2, Mettler Toledo), gel electrophoresis (Bio-Rad), scanning electron microscopy (SEM)(JSM-5410LV,GEOL), pH meter (Mettler toledo, Switzerland), 96-flat well plate (Corning)

## 3.2 Chemical reagents

Mueller Hinton agar (MHA), Mueller Hinton broth (MHB), Trypticase Soy broth (TSB) medium and ciprofloxacin 5 µg/disc were from BBL, Becton Dickinson Inc. Monosodium phosphate monohydrate, di-sodium phosphate anhydrous, ciprofloxacin, sodium chloride, rezasurin, trypan blue dye, RPMI 1640 medium and histopaque were purchased from Sigma-Aldrich Inc. Molecular weight standards and reagent for sodium dodecyl sulphate-polyacrylamine gel electrophoresis (SDS-PAGE) were from Bio-Rad laboratories Inc. Fetal bovine serum (FBS) was from Gibco.

## 3.3 Snake venom sample collection

King Cobra crude venom was collected from several snakes from Queen Saovabha Memorial Institute, Bangkok, Thailand. The venom was freeze-dried and kept at 4 $^{o}$C.

### 3.4 Bacterial strains

The test microorganisms used in this study included the Gram-positive *S. aureus* (ATCC 25923), *S. pyogenes* (ATCC 19615) and Gram-negative species *E. coli* (ATCC 25922), *S.* Typhimurium (ATCC 14028) and *P. aeruginosa* (ATCC 27853).

### 3.5 Estimation of protein

Protein contents in venom fractions were estimated from absorbance at 280 nm in a 1 cm cell, based upon the assumption that the absorbance of 1 mg/ml of fractionated venom was 1.113, the same as that of the crude venom (Tamotsu, 1995).

### 3.6 Selection of antibacterial properties of King Cobra crude venom

#### 3.6.1 Preparation for tested bacterial inoculums

Tested bacteria were grown on Mueller Hinton agar (MHA) 37°C for 24 hours. At least two to three identical colonies were selected from agar plate culture and transfered into a tube containing 2 ml of TSB medium. The broth culture was incubated at 37°C for 2-6 hours and adjusted turbidity to the 0.5 McFarland with sterile normal saline.

#### 3.6.2 Screening for antibacterial activity of King Cobra crude venom

Disc diffusion assay

The swab was rotated several times and pressed firmly onto the inside wall of the tube above the fluid level. The swab was spread onto MHA plates and let to dry. The discs were placed onto the agar surface and 10 µl of various concentrations of King Cobra crude venom in the range of 0.3125-20 mg/ml and solvent without any sample served as negative control was added to each disc. Ciprofloxacin (5 µg/disc) was used as positive control to confirm that all the microorganisms tested were inhibited by the antibiotic. The plates were incubated at 37 °C for 24 hours, antibacterial activity was observed as an inhibition zone and recorded in mm. Experiments were replicated five times.

### 3.7 Antibacterial activity of the first fractionation fractions from King Cobra crude venom

Crude venom was first fractionated at the Snake Venom Research Division, Queen Saovabha Memorial Institute, Bangkok, Thailand, using Q-sepharose chromatography resulted in seven fractions (F1, F2, F3, F4, F5, F6 and F7). All fractions were screened for antibacterial activities.

### 3.8 Selection of antibacterial properties of the F1-F7

#### 3.8.1 Examination of antibacterial properties

Fraction 1 to fraction 7 (F1-F7) from the mentioned above were tested for antibacterial activities, including *S. aureus* (ATCC 25923), and Gram-negative species *E. coli* (ATCC 25922), *S.* Typhimurium (ATCC 14028) and *P. aeruginosa* (ATCC 27853).

#### 3.8.2 Preparation for tested bacterial inoculums

The bacterial inoculums were prepared in the same manner as described in section 3.6.1 but the broths were replaced by Mueller Hinton broth (MHB).

#### 3.8.3 Preparation of the tested fractions (F1-F7)

F1-F7 were dissolved in 10 mM sodium phosphate buffer (pH 7.4). The final dilutions range from 0.97, 1.95, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000 µg/ml.

#### 3.8.4 Microbroth dilution assay

The tested compounds and antibiotic were diluted in 96 well plates. Solvent without any sample served as negative control and ciprofloxacin was used as positive control. Bacterial inocula at 0.5 McFarland were diluted and inoculated into each well to a final density of approximately $5 \times 10^5$ CFU/ml (or $5 \times 10^4$ CFU/well). The plates were incubated at $37^{\circ}C$ for 24 hours. The minimum inhibitory concentration (MIC) was determined as the lowest concentration at which there was no visible growth. All experiments were performed in triplicate.

### 3.9 Secondary fractionation of antibacterial activity fraction

The fraction presenting the highest antibacterial activity was collected and dialyzed. The dialyzed fraction was applied to a gel filtration Sephadex G-75 column (2.6 cm x 35 cm) equilibrated and eluted with 20 mM Tris buffer saline (TBS), pH 7.4 at a flow rate of 2.25 ml/min. and 1.5 ml fractions were collected. Protein elution was monitored at UV absorption of 280 nm.

### 3.10 Selection of antibacterial properties of the secondary fractions

#### 3.10.1 Preparation for tested bacterial inoculums

The bacterial inoculums were prepared in the same manner as described in section 3.6.2

#### 3.10.2 Preparation for tested fractions

The tested compounds were dissolved in 10 mM sodium phosphate buffer (pH 7.4). The final dilutions range from 0.97, 1.95, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500 µg/ml.

#### 3.10.3 Microbroth dilution assay

The microbroth dilution assay were prepared in the same manner as described in section 3.8.4

Minimum bactericidal concentration (MBC)

The minimum bactericidal concentrations (MBC) were determined by plating out the contents of the first 3 wells showing no visible bacterial growth onto Mueller Hinton agar plates and incubate at 37$^{o}$C for 24 hours. The highest concentration yielding no growth after incubation will be considered to be the MBC.

### 3.11 Antibacterial properties and mechanism of the final semi-purified fraction

#### 3.11.1 Determination of the minimum inhibitory concentration (MIC)

##### 3.11.1.1 Preparation for tested bacterial inoculums

The bacterial inoculums were prepared in the same manner as described in section 3.6.2

##### 3.11.1.2 Preparation for tested fractions

The tested compounds were dissolved in 10 mM sodium phosphate buffer (pH 7.4). The final dilutions range from 0.61-62.5 µg/ml.

##### 3.11.1.3 Microbroth dilution assay

MIC were determined by the microliter broth method in 96 well plates. The groups of microbroth test are described below.

Negative controls – (cells + TSB)

Positive controls – (cells + TSB + ciprofloxacin)

Vehicle controls – (cells + TSB +10 mM sodium phosphate buffer)

Media controls – (TSB)

Bacterial inocula at 0.5 McFarland standard were diluted and inoculated into each well to a final density of approximately 5 x $10^5$ CFU/ml (or 5 x $10^4$ CFU/well). The microlitter plate was incubated at 37$^{o}$C for 24 hours, and then measured the turbidity of the cultured medium at 600 nm. The lowest concentration of the final semi-purified fraction that inhibits the growth of bacteria was considered as the MIC.

#### 3.11.2 Determination of 50 % inhibitory concentration against bacterial growth ($IC_{50}$)

After measurement of the turbidity of the cultured medium at 600 nm, the percent inhibition was calculated by using the formula below

$$\% \text{ inhibition} = \left[ 1- \left[ \frac{OD t_{24} - OD t_0}{OD gc_{24} - OD gc_0} \right] \right] \times 100$$

$OD t_{24}$ = optical density (600 nm) of the test well at 24 hours post-inoculation

$OD t_{0}$ = optical density (600 nm) of the test well at 0 hours post-inoculation

$OD gc_{24}$ = optical density (600 nm) of the growth control well at 24 hours post-inoculation

$OD gc_{0}$ = optical density (600 nm) of the growth control well at 0 hours post-inoculation

All data were analysed using Graphpad Prism version 5.0 (www.graphpad.com). Nonlinear regression was performed on dose response data and a sigmoidal curve with variable slope was fitted to each of data sets. The equation used for the sigmoidal curve with variable slope was:

$$Y = \text{bottom} + (\text{top-bottom})/(1+10^{\log IC50 \times \text{Hill slope}})$$

The 50 % inhibitory concentration was calculated from *X*-value of the response halfway between top and bottom plateau (Toit, 2000).

### 3.11.3 Scanning electron microscopy (SEM)

Approximately $10^8$ CFU/ml of *S. aureus* resuspended in 10 mM sodium phosphate buffer (pH 7.4) were treated with the F5.1 at a concentration of 1.8 µg/ml and incubated at 37 °C for 3-6 hours. After incubation, the bacteria were washed and fixed with an equal volume of 2.5% glutaraldehyde. The fixed samples were stored over-night to several days at 4 °C in the fixative solution. Samples were sent to be examined on a scanning electron microscope at the Scientific and Technology Research Equipment Centre, Chulalongkorn University.

### 3.11.4 Determination of membrane integrity

Bacterial culture into TSB was performed and incubated at 37 °C for 24 hours. After incubation, the bacterial cells were separated by centrifuge x10,000 g for 10 min, wash and resuspended in 0.9 % sodium chloride solution. The final cell suspensions were adjusted to an absorbance at 420 nm of 0.7. The 0.5 ml of sample or 10 mM sodium phosphate buffer (control) was mixed with 0.5 ml of each bacterial cell suspension. Samples were removed from tubes every 20 min. The samples were then immediately filtered with 0.2 µm syringe filters to remove the bacteria. The levels of

materials to leak from cells were determined by measuring the absorbance at 260 nm (Chen and Cooper, 2002).

### 3.12 SDS-PAGE analysis

SDS-PAGE was performed on a 12.5 % acrylamide gel according to Laemmli (1970).For determination of protein, the protein bands were stained with 0.2% Coomassie brilliant blue dye solution. Protein standards were myosin (210 kDa), $\beta$-galactosidase (131 kDa), bovine serum albumin (78 kDa), carbonic anhydrase (41.3 kDa), soybean trypsin inhibitor (31.8 kDa), lysozyme (18.1 kDa) and aprotinin (7.1 kDa). Molecular weight markers were used for calibration.

### 3.13 Determination of L-amino acid oxidase activity

**Preparation for tested fractions**

The tested compounds were dissolved in 0.9% normal saline. The final dilution was 1.0 mg/ml.

**L-amino acid oxidase assay** (Tan *et al*., 1986)

Add 0.05 ml of 0.007% peroxidase into 1 ml of 0.2 M Triethanolamine buffer,pH 7.6. The 0.2 M Triethanolamine buffer contained 1% L-leucine and 0.0065 % o-dianisidine. Add 0.1 ml of tested compounds. The initial rate was measured by following the increase of absorbance at 426 nm every 15 sec for 180 min. One unit of the enzyme activity was defined as the oxidation of 1 µM L-leucine per minute. The L-amino acid oxidase activity of tested compounds were calculated by using the formula below

$$\text{L-amino acid oxidase activity of sample (unit)} = \frac{\text{absorbance rate per minute}}{0.001}$$

### 3.14 Cytotoxic assay

**Isolation of peripheral blood mononuclear cells (PBMCs)**

Heparinized blood was taken from healthy and 20-35 year-old male donors at National Blood bank, Thai Red Cross Society for Blood Donation with informed consent. The whole blood was centrifuged at 3200 g for 10 min at room temperature. The plasma was removed and buffy coat was transfered to a 15 ml centrifugation tube that contains about 5 ml of RPMI 1640 medium (serum free). It was mixed well, then transfered buffy coat and RPMI 1640 medium (serum free) mixture to a new 15 ml centrifugation tube containing about 5 ml of Histopaque (Care was taken not to mix the layers). The tube was centrifuged at 2200 rpm for 30 min. The lymphocyte bands were removed from each samples of each subject and placed into 15 ml centrifugation tubes, 10 ml of RPMI 1640 medium (serum free) was added. The tube was centrifuged at 1,200 rpm for 10 min at room temperature. The supernatant was discarded and cell pellet was resuspended in 10 ml RPMI medium (serum free) and centrifuged again at 1,200 rpm for 10 min. Discard supernatant and resuspended cell pellet in RPMI 1640 with 10 % fetal bovine serum medium.

**Rezasurin assay**

90 µl of the cell pellet in RPMI 1640 with 10 % fetal bovine serum medium at a concentration of $1.0 \times 10^6$ cells/ml was added to each well. Cells were cultured in 96 well plates at 37 $^{\circ}$C in a $CO_2$ incubator and allowed to adhere overnight. The cells were then treated with 10 µl of the snake venom fractions at the final dilution of MIC concentration or 10 mM sodium phosphate buffer, pH 7.4 (negative control) and 5 µl rezasurin (1 mg/ml) was added to each wells. The cells were exposed to the venom fractions for 24 hours. The absorbances were measured at 570 nm and 600 nm with a microplate reader. To determine cell viability, percent viability was calculated by the following formula.

$$\% \text{ cell viability} = \frac{(\text{delta absorbance of treated cells}) \times 100}{(\text{delta absorbance of controls})}$$

### 3.15 Statistical analysis

Data was presented as mean plus or minus standard error (mean ± S.E.). Statistical comparisons were made by one-way ANOVA followed by Tukey's post hoc test. All statistical analysis was performed according to the statistic program, SPSS version 17. Any *P*-value < 0.05 was considered statistically significant.

# CHAPTER IV

# RESULTS

## 4.1 Antibacterial activity of King Cobra crude venom

### Disc diffusion technique

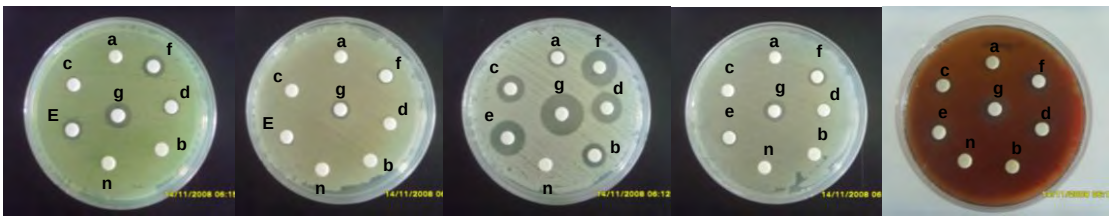
The results of antibacterial activity of the crude venom was tested by the disc diffusion method against *S. aureus*, *S. pyogenes*, *E. coli*, *S.* Typhimurium and *P. aeruginosa* are shown in Table 4.1. The control, 20 mM sodium phosphate buffer without crude venom gave no clear zone (Figure 4.1 A). The crude venom showed concentration-dependent antibacterial activity against gram positive strains such as *S. aureus* and Gram negative strains such as *E. coli*, *S.* Typhimurium and *P. aeruginosa*. The order of the susceptibility of bacteria tested againts crude venoms is as follows: *S. aureus* more than *P. aeruginosa, S.* Typhimurium and *E. coli*. However, crude venom did not show antibacterial effect on the *S. pyogenes* (Figure 4.1 B).

**Table 4.1** Antibacterial activity (Zone of inhibition) of crude venom of King cobra

| Microorganism | Mean values ± S.E. of inhibition zone (mm)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Crude venom (µg/disk) | | | | | | |
| | 3.125 | 6.25 | 12.5 | 25 | 50 | 100 | 200 |
| *E. coli* | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 6.9 ± 0.1 | 8.2 ± 0.1 | 9.4 ± 0.2 | 10.0 ± 0.0 |
| *P. aeruginosa* | 0.0 ± 0.0 | 7.4 ± 0.1 | 8.4 ± 0.2 | 10.0 ± 0.0 | 10.9 ± 0.1 | 11.9 ± 0.1 | 13.0 ± 0.0 |
| *S.*Typhimurium | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 7.7 ± 0.2 | 9.1 ± 0.1 | 10.0 ± 0.0 | 10.7 ± 0.1 |
| *S. aureus* | 9.1 ± 0.1 | 11.7 ± 0.2 | 13.9 ± 0.1 | 15.6 ± 0.2 | 17.8 ± 0.1 | 18.8 ± 0.2 | 20.7 ± 0.2 |
| *S. pyogenes* | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |

[a] The values are average of inhibition zone from five replicate.

A




B

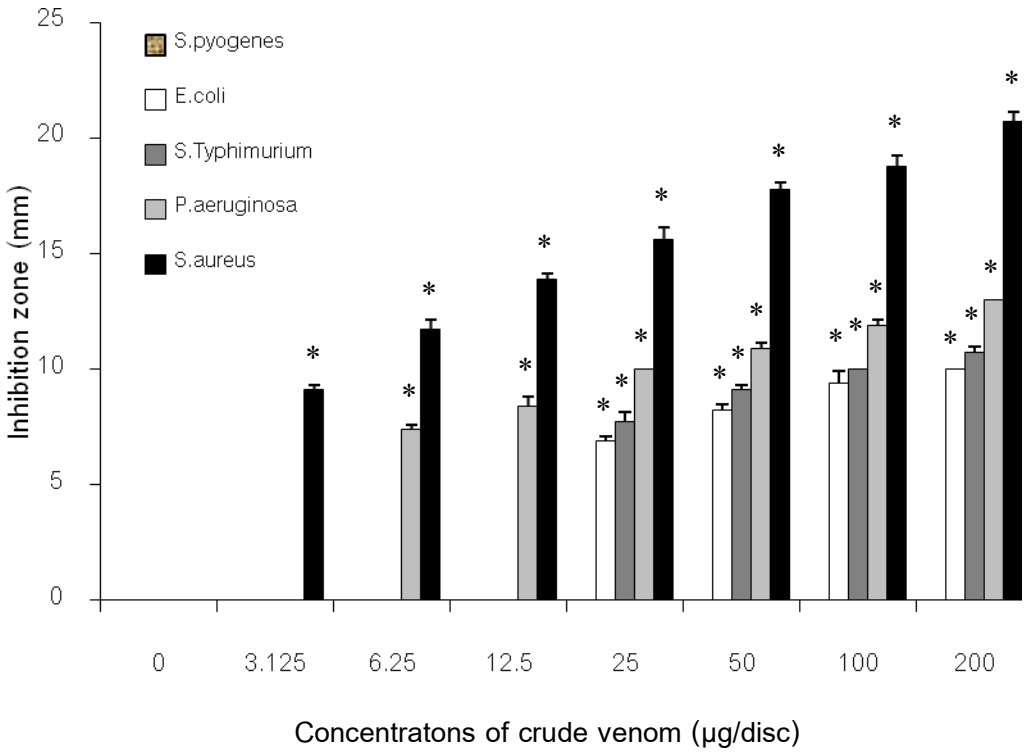



**Figure 4.1 Antibacterial activity of King Cobra crude venom**

**(A)** Disc diffusion assay. The negative control (buffer only, n), crude venom (3.125 µg/disc, a), crude venom (6.25 µg/disc, b), crude venom (12.5 µg/disc, c), crude venom (25 µg/disc, d), crude venom (50 µg/disc, e), crude venom (100 µg/disc, f), crude venom (200 µg/disc, g). **(B)** Antibacterial activity of King Cobra crude venom. Bars represent mean ± S.E. of five replication. Data were analyzed by one way ANOVA, Tukey's test and $P < 0.05$ was considered sigfinicantly. * Significantly different from untreated control cultures, ($P < 0.05$).

**4.2 Antibacterial activity of the first fractionation of the crude venom (F1-F7)**

The antibacterial activities of F1-F7 were evaluated by the broth dilution method. The F1-F7 were examined at the concentration of 0.97-1000 µg/ml. The results were shown that F5 had the strongest inhibition against *S. aureus* and *P. aeruginosa*. The MIC was 3.90 µg/ml and 62.5 µg/ml respectively.

The F6 had the strongest inhibition against *E. coli* and *S.* Typhimurium. The MIC was 125 µg/ml and 62.5 µg/ml respectively.

The F1 and F3 were not antibacterial against all of tested organisms. The antibacterial activities of F1-F7 were showed in Table 4.2

**Table 4.2** Antibacterial activities of Q-Sepharose fractionated fractions determined by microbroth dilution method

| Microorganism | MIC (µg/ml) | | MIC (µg/ml)[a] Q-Sepharose | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Cipro-floxacin | Crude venom | F1 | F2 | F3 | F4 | F5 | F6 | F7 |
| *E. coli* | 0.015 | > 1,000 | > 1,000 | > 1,000 | > 1,000 | 500 | 250 | 125 | 250 |
| *P. aeruginosa* | 0.5 | > 1,000 | > 1,000 | > 1,000 | > 1,000 | 1,000 | 62.5 | 250 | 250 |
| *S.* Typhimurium | 2.0 | > 1,000 | > 1,000 | > 1,000 | > 1,000 | > 1,000 | 1,000 | 62.5 | 250 |
| *S. aureus* | 0.5 | 15.62 | > 1,000 | 1,000 | > 1,000 | 62.5 | 3.90 | 250 | 500 |

MIC, Minimum inhibitory concentration

a, These concentrations obtained from three independent experiments (n=3) performed in triplicate.

### 4.3 Purification of the fraction showing antibacterial activity

After the screening of antibacterial activities of seven protein fractions (F1-F7) obtained from the Snake Venom Research Division, Queen Saovabha Memorial Institute, Bangkok, Thailand, the F5 showed the highest antibacterial activity. The F5 was further concentrated and separated by Sephadex G-75 column (2.6 cm x 35 cm), equilibrated and eluted with 20 mM potassium phosphate buffer, pH 7.4 at a flow rate 2.25 ml/min. and 1.5 ml fractions were collected. Protein elution was detected at UV absorption of 280 nm, and six fractions (F5.1, F5.2, F5.3, F5.4, F5.5, F5.6) were collected (Fig. 4.2).

The isolated F5.1 accounted approximately 39 % of the total protein of F5 (Table 4.3)

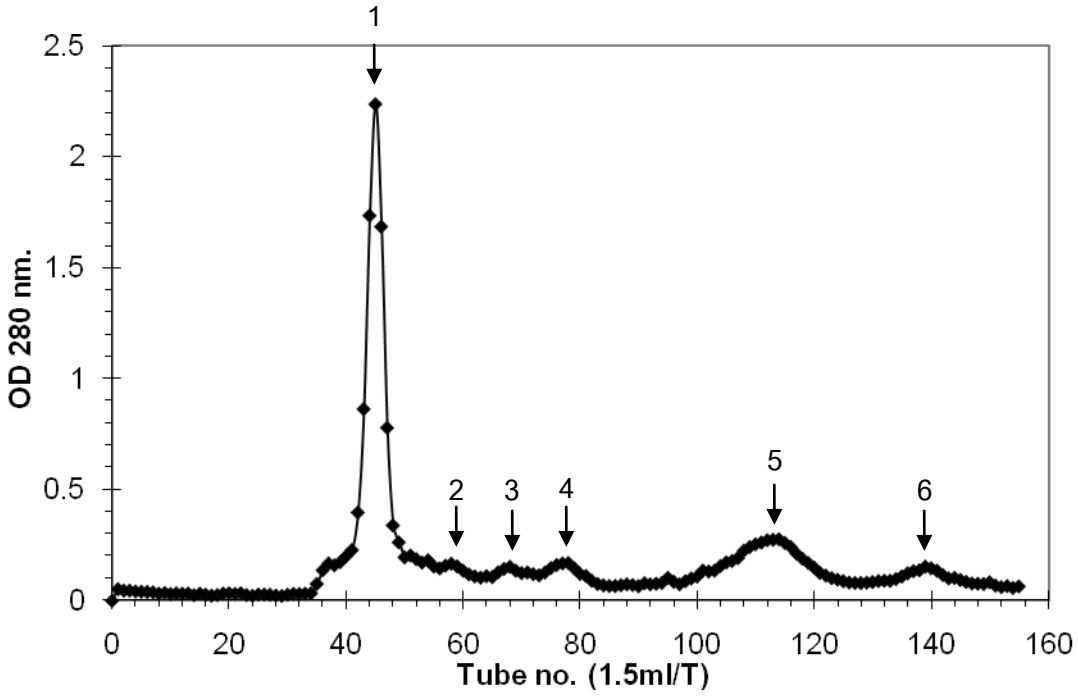



**Figure 4.2** Sephadex G-75 gel filtration of fraction 5 from Q- sepharose
Sephadex G-75 column (2.6 cm x 35 cm) eluted with 0.02 M Tris buffer saline, pH 7.4, at flow rate of 2.25 ml/min and 1.5 ml fractions were collected

**Table 4.3** Fractionation of the F5 showing antibacterial activity

| Fraction No. | Protein (mg) | Yield (%) |
|---|---|---|
| Q-Sepharose | | |
| F5 | 60 | 100 |
| Sephadex G-75 | | |
| F5.1 | 23.4 | 39 |
| F5.2 | 4.9 | 8.2 |
| F5.3 | 2.8 | 4.7 |
| F5.4 | 3.2 | 5.3 |
| F5.5 | 14.4 | 24 |
| F5.6 | 2.4 | 4 |

### 4.4 SDS-PAGE analysis

The molecular weight of final protein product, F5.1 determined by SDS-PAGE in reduced condition was approximately 69 kDa (Fig.4.3).

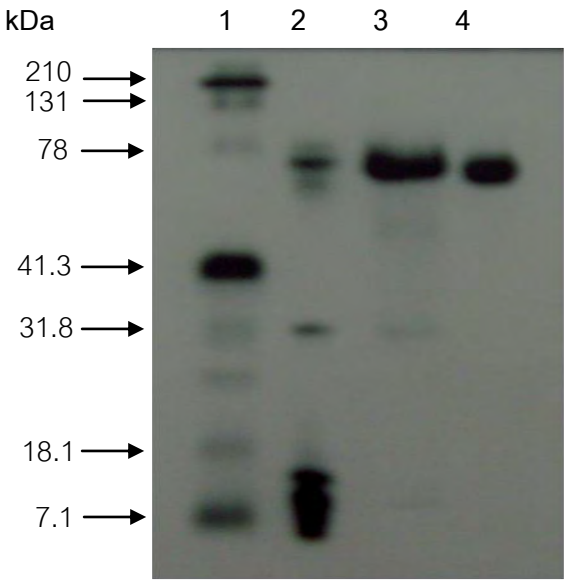



**Figure 4.3** Molecular weight of F5.1 analyzed by 12.5% SDS-PAGE: Lane 1: protein markers including myosin (210 kDa), $\beta$-galactosidase (131 kDa), bovine serum albumin (78 kDa), carbonic anhydrase (41.3 kDa), soybean trypsin inhibitor (31.8 kDa), lysozyme (18.1 kDa) and aprotinin (7.1 kDa); Lane 2: King Cobra crude venom; Lane 3: F5; Lane 4: F5.1. The molecular masses (kDa) of the markers are indicated on the left.

### 4.5 Antibacterial activity of the secondary fractions obtained from F5 (F5.1-F5.6)

The antibacterial activities of F5.1-F5.6 were evaluated by the broth dilution method. The F5.1-F5.6 were examined at the concentrations of 0.97-500 µg/ml. The results were presented in table 4.4. The F5.3, F5.4 and F5.6 were inactive against all the tested bacteria. The F5.5 was active against only *P. aeruginosa* with the MIC value of 500 µg/ml. The F5.1 and the F5.2 were active against all the tested bacteria. The F5.1 was found to be the strongest inhibitor against all the tested bacteria with MIC value of 1.95-7.81 µg/ml, however the F5.2 had MIC value of 125-500 µg/ml

### 4.6 Antibacterial properties of the final semi-purified fraction (F5.1)

The antibacterial activity of F5.1 was determined by measurement of the turbidity of each well in the microplate at 600 nm. The antibacterial activity of F5.1 against *E. coli*, *P. aeruginosa*, *S.* Typhimurium and *S. aureus* were shown in Fig. 4.4 and Table 4.5. While, increasing concentration of the F5.1 against *E. coli* varied from 5.0 to 7.0 µg/ml, the OD values decreased with the MIC value of 7.0 µg/ml (Fig. 4.4a). Increasing of concentration of the F5.1 against *P. aeruginosa* varied from 1.95 to 6.0 µg/ml, the OD values decreased with the MIC value of 6.0 µg/ml (Fig. 4.4b). Increasing concentration of F5.1 against *S.* Typhimurium varied from 3.9 to 6.0 µg/ml, the OD values decreased with the MIC value of 6.0 µg/ml (Fig.4.4 c). For *S. aureus*, increasing of concentration of the F5.1 against *S. aureus* varied from 1.4 to 1.8 µg/ml, the OD values decreased with the MIC value of 1.8 µg/ml (Fig. 4.4 d). Sigmoidal dose-response curves were obtained after analysis of the results from microbroth dilution method assay. From these curves the $IC_{50}$ values were shown in Fig. 4.4 and table 4.5. The $IC_{50}$ values of *E. coli*, *P. aeruginosa*, *S.* Typhimurium and *S. aureus* were 6.0, 4.6, 4.8 and 1.6 µg/ml respectively (Fig. 4.4 e, f, g, h).

**Table 4.4** Antibacterial activity of Sephadex G-75 fractionated fractions determined by microbroth dilution method

| Microorganism | Sephadex G-75 fractions of F5 (μg/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F5.1 | | F5.2 | | F5.3 | | F5.4 | | F5.5 | | F5.6 | |
| | MIC[a] | MBC[a] | MIC[a] | MBC[a] | MIC[a] | MBC[a] | MIC[a] | MBC[a] | MIC[a] | MBC[a] | MIC[a] | MBC[a] |
| *E. coli* | 7.81 | 7.81 | 500 | 500 | > 500 | > 500 | > 500 | > 500 | > 500 | > 500 | > 500 | > 500 |
| *P. aeruginosa* | 7.81 | 7.81 | 125 | 250 | > 500 | > 500 | > 500 | > 500 | 500 | 500 | > 500 | > 500 |
| *S.* Typhimurium | 7.81 | 62.5 | 500 | 500 | > 500 | > 500 | > 500 | > 500 | > 500 | > 500 | > 500 | > 500 |
| *S. aureus* | 1.95 | 1.95 | 125 | 125 | > 500 | > 500 | > 500 | > 500 | > 500 | > 500 | > 500 | > 500 |

MIC, Minimum inhibitory concentration

MBC, Minimum bactericidal concentration

a, These concentrations obtained from three independent experiments (n=3) performed in triplicate.

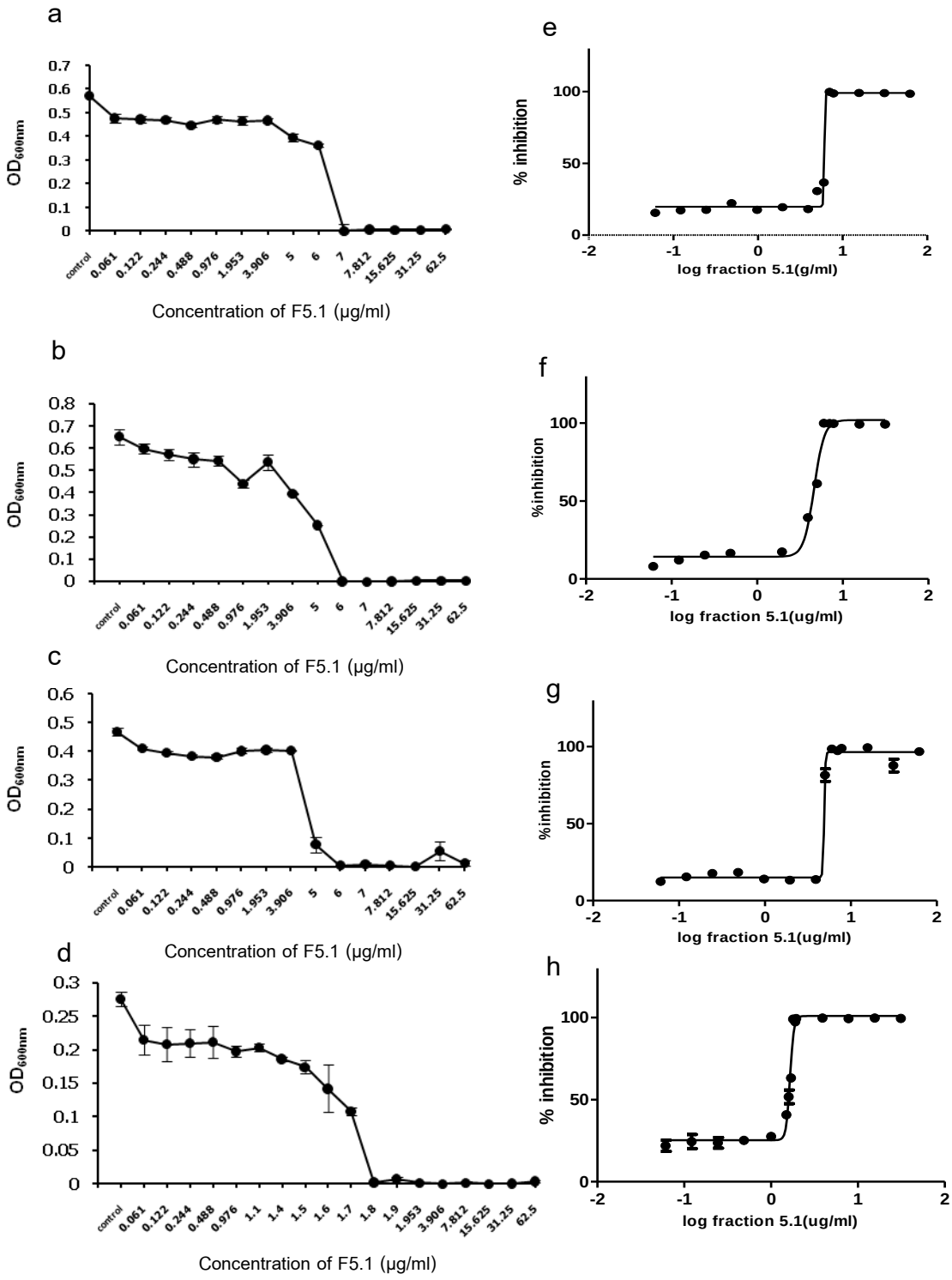



**Figure 4.4** Effect of concentration to the antibacterial activity of F5.1 against *E. coli* (a), *P. aeruginosa* (b), *S.* Typhimurium (c), *S. aureus* (d). Sigmoidal dose-response curve from microbroth dilution assay of *E. coli* (e), *P. aeruginosa* (f), *S.* Typhimurium (g), *S. aureus* (h). The $IC_{50}$ values were calculated from the x-values. The OD values and % inhibition represent mean± S.E. values of three independent experiments (n=3).

**Table 4.5** Minimum inhibitory concentration (MIC) and $IC_{50}$ values of the F5.1 against *S. aureus* determined by microbroth dilution method

| Microorganism | F5.1 | |
|---|---|---|
| | MIC (μg/ml) | $IC_{50}$ (μg/ml)[a] |
| *E. coli* | 7.0 | 6.0 |
| *P. aeruginosa* | 6.0 | 4.6 |
| *S.* Typhimurium | 6.0 | 4.8 |
| *S. aureus* | 1.8 | 1.6 |

$IC_{50}$, 50% Inhibitory concentration against bacterial growth

a, These concentrations obtained from three independent experiments performed in triplicate.

### 4.7 Integrity of bacterial cell membranes

The release of 260 nm absorbing material upon addition of the F5.1 to *S. aureus* suspension was show in Fig. 4.5. The $A_{260}$ of *S. aureus* suspension with the F5.1 to a bacteria suspension without the F5.1 was plotted versus time. When *S. aureus* suspensions were exposed to the F5.1 at the MIC concentration (1.8 μg/ml) and at two times the MIC concentration (3.6 μg/ml), the absorbance of the suspensions at 260 nm was more than that observed in the untreated control suspensions and increased up to 50 min but there was a decreased rate after 50 min. Furthermore, $A_{260}$ values were lesser in suspensions treated with 1.8 μg/ml of the F5.1 than treated with 3.6 μg/ml of the F5.1. Thus, the release rate of intracellular components caused by the F5.1 was concentration-dependent.

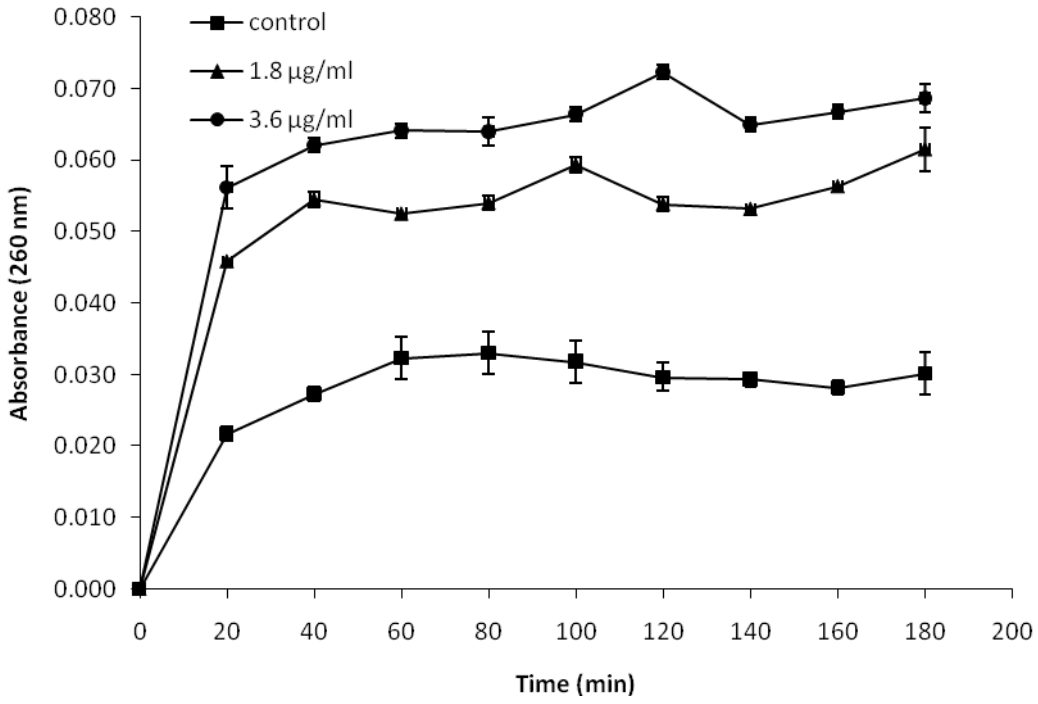



**Figure 4.5** Effect of the F5.1 on the release of intracellular components

Release of 260 nm absorbing material from *S. aureus* suspensions treated with 1.8 µg/ml (-▲-), 3.6 µg/ml (-●-) of the F5.1 and 10 mM sodium phosphate buffer (control) (-■-). The OD values represent mean ± S.E. values of three independent experiments performed in duplicate (n=3).

### 4.8 Scanning electron microscopy (SEM)

In order to investigate the mechanism of action of F5.1, morphological changes induced by the F5.1 on *S. aureus* were examined by SEM. Fig. 4.6 showed the SEM micrographs of the untreated and the F5.1 treated cells of *S. aureus* at 3-6 hours time of exposure. Untreated *S. aureus* had smooth and normal surface morphology (Fig. 4.6 A, 4.6 C). *S. aureus* treated with MIC (1.8 µg/ml) concentration of the F5.1 showed large globular surface protrusions on the bacterial cell surface (Fig.4.6 B, 4.6D).

### 4.9 Determination of L-amino acid oxidase activity

L-amino acid oxidase activities were showed in table 4.6. The L-amino acid oxidase activity of F5.1 was 13,244 U/mg proteins.

**Table 4.6** L-amino acid oxidase activitiy of King Cobra venom (n=2)

| Sample | Mean values ± S.E. of L-amino acid oxidase activity (U/mg protein) |
|---|---|
| Crude venom | 3,324 ± 0.001 |
| F5 | 13,064 ± 0.006 |
| F5.1 | 13,244 ± 0.003 |

### 4.10 Cytotoxic assay

The in vitro activity of F5.1 was tested against Human PBMCs. The result showed cytotoxic activity at the MIC (1.8 µg/ml) concentration.

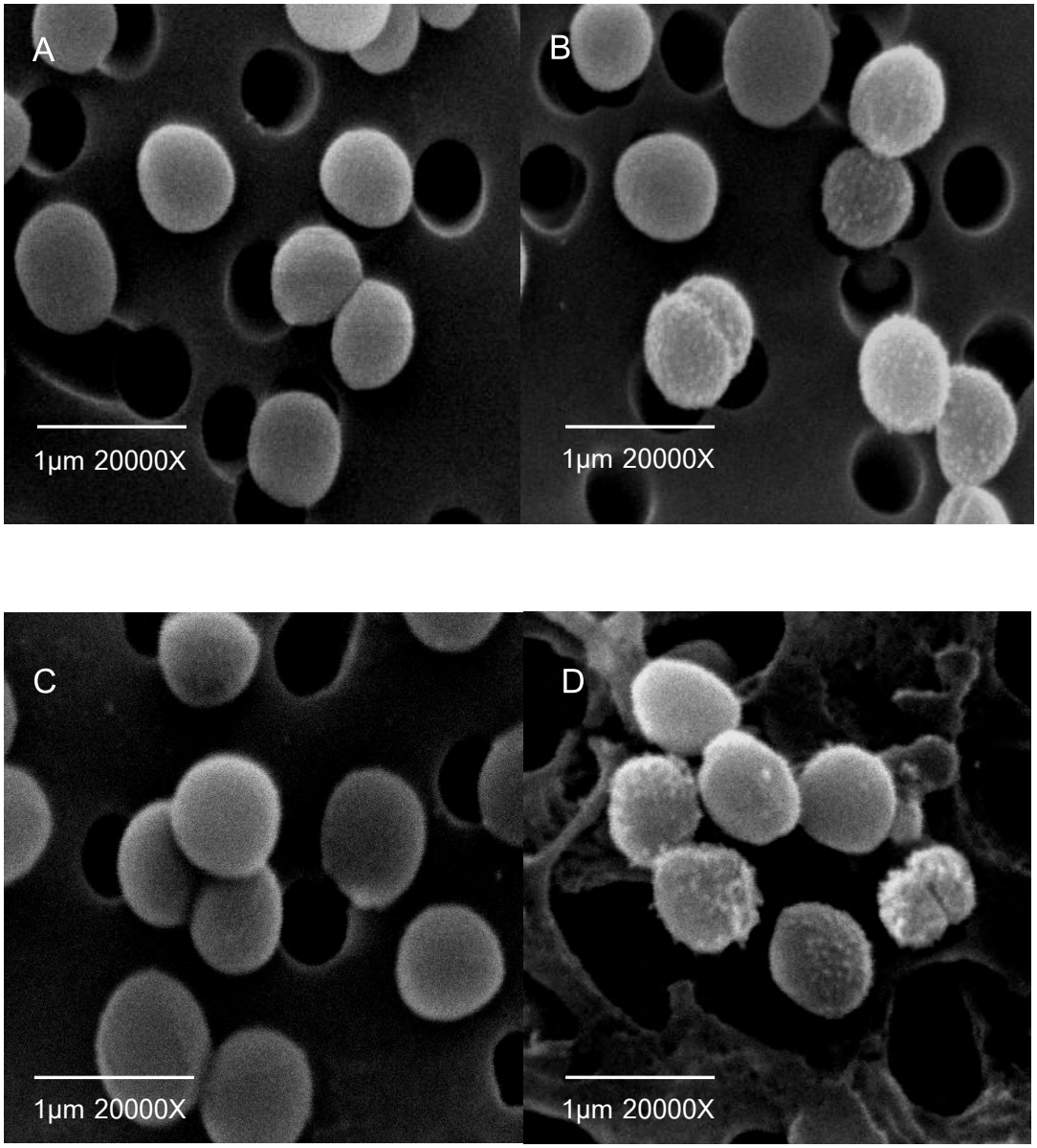



**Figure 4.6** Scanning electron micrographs of *S. aureus* treated with the F5.1 Incubated *S. aureus* at 37 °C for 3 h, without the F5.1 (A) and with 1.8 μg/ml of the F5.1(B) and for 6 h, without the F5.1 (C) and with 1.8 μg/ml of the F5.1(D).

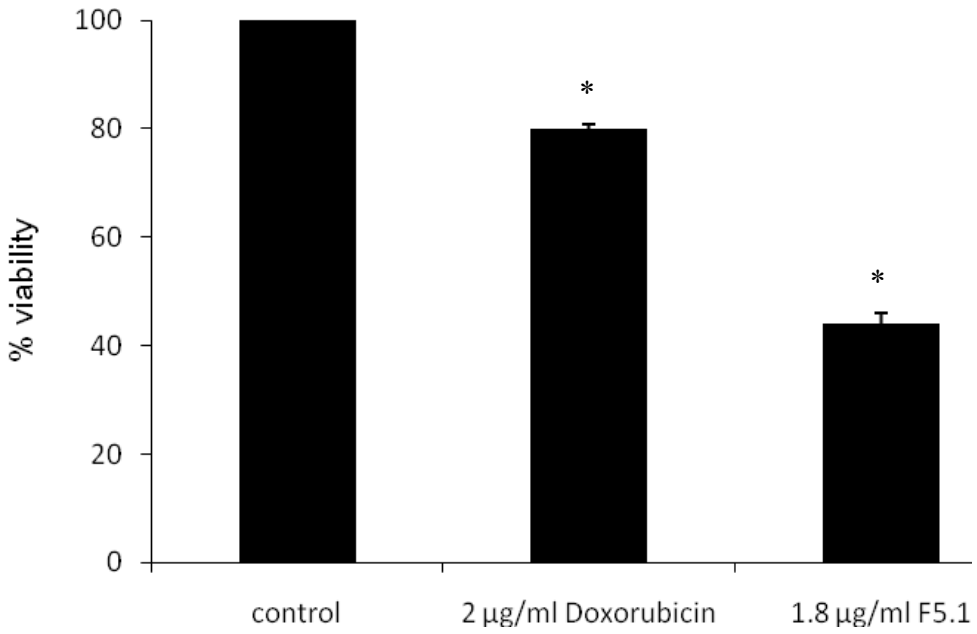



**Figure 4.7** Cytotoxic effect of the F5.1 in Human PBMCs

Human PBMCs cells were treated with the MIC concentrations (1.8 µg/ml) of the F5.1 for 24 hours. Cell viability (Human PBMCs cells) was determined by rezasurin assay to evaluate the cytotoxic effect of the F5.1. The data were expressed as mean ± S.E. values of three independent experiments performed in triplicate (n=3). *Significantly different when compared with the untreated control cultures, ($P < 0.05$).

# CHAPTER V

# DISCUSSION AND CONCLUSION

It have been previously reports demonstrated biological properties of King Cobra venom including, antinociceptive effects, anticonvulsant, inhibition or induction of platelet aggregation and cytotoxicity (Pu *et al*., 1995; Anh *et al*., 1997; Saha *et al*., 2000; Li *et al*., 1994). Many protein and peptide components from King Cobra venom have been identified and characterized including $PLA_2$ (Huang *et al*., 1997; Pung *et al*., 2005), neurotoxin (He *et al*., 2004), protease inhibitor (Chang *et al*., 2001), haemotoxin (Lee *et al*., 1995; Du *et al*., 2002; Tan and Saifuddin, 1990) and also LAAO (Anh *et al*., 1997; Yang *et al*., 2007). Recently proteins and peptides isolated from other snake venoms have been reported to exhibited antibacterial activities (Perumal Samy *et al*., 2008; Nair *et al*., 2007). It has been suggested that these activities of many proteins inhibit the growth of bacteria by interacting with cell membrane. However, the antibacterial activities and mechanism of fractionated of King Cobra venom has not been reported. This study intended to evaluate the antibacterial effects and mechanism on cell membrane of *S. aureus* (ATCC 25923), *S. pyogenes* (ATCC 19615), *E. coli* (ATCC 25922), *S.* Typhimurium (ATCC 14028) and *P. aeruginosa* (ATCC 27853) cells of fractionated King Cobra venom.

In this report, a antibacterial semi-purified protein fraction, F5.1 was isolated from the venom of King Cobra to a high degree of molecular homogeneity after two chromatographic steps. This protein fraction presents yellow color and in SDS-PAGE profile presents a molecular mass of 69 kDa. The semi-purified F5.1 has been exhibited LAAO activity.

Antibacterial activity of the F5.1 was determined to identify the MIC values by using microbroth dilution method and measurement the turbidity of cultured medium at 600 nm. If the absorbance of the F5.1 is lower than that of the blank it is indicated the concentration can inhibit the test organisms. The results in this study demonstrated that the F5.1, the proteins from King Cobra venom, showed dose-dependent antibacterial

activity against both Gram-positive and Gram-negative bacteria (Fig. 4.4). The activity of antibacterial proteins is normally affected by several factors, including their bacterial membrane composition, tree-dimensional structure, amino acid sequence, net charge of the protein and salinity of the environment (Nagoaka *et al*., 2000). The F5.1 shows stronger antibacterial activity against Gram-positive than Gram-negetive bacteria and the strongest activity is against the Gram-positive bacterium *S. aureus* (MIC= 1.8 µg/ml). This selective antibacterial may be due to various factors such as lipid composition of the cytoplasmic membrane and electrostatic potential across this membrane, only an outer peptidoglycan layer in Gram-positive bacteria, or structure and charge density of lipopolysaccharides in case of Gram-negative bacteria. Also, proteins transport and efflux mechanisms may lead to species-selectivity of antibacterial proteins (Devine and Hancock., 2002).

The antibacterial mechanism of protein is complicated and working in different pathways. The cellular target for most antibacterial proteins is the bacterial cell membrane. Lysozyme could kill the target cell by lysis the cell wall (Nilson *et al*., 1999). Membrane targeted proteins could insert into cell membrane, leading to cell death by forming the ion channels or disrupting the membrane (Lear *et al*., 1988; Ebran *et al*., 1999). Recently, a new member of antibacterial proteins have been found in inland taipan venom (Nair *et al*., 2007). Moreover, some peptides with variety of mechanism of antibacterial action have recently been demonstrated including, inhibition of protein synthesis, nucleic acid synthesis, enzymatic activity and cell wall synthesis (Broden, 2005). The mechanism of antibacterial action were by affecting the cytoplasmic release studies and membrane integrity, as indicated by our SEM analyses of bacteria treated with MIC (1.8 µg/ml) concentration of the F5.1 for 3-6 hours. The results in this study showed that the F5.1 induced globular surface protrusions on cell surface of bacteria and the release studies indicate that the action of the F5.1 is probably towards the cell envelope. Similar effects were observed for antibacterial proteins, for example antibacterial protein P2 (Mackintosh *et al*., 1998) and temporin (Wade *et al*., 2000). In the case of snake venom, Cationic protein from the venom of inland taipan (*Oxyuranus microlepidotus*) shows selective against Gram-positive bacteria is via membrane

disruption. The $H_2O_2$ produced by LAAO from *Crotalus durissus cascarella* venom induced bacterial membrane rupture and consequently loss of cytoplasmic content (Toyama *et al*., 2006). These results suggest that the bacterial cellmembrane is an important target of the F5.1 molecule.

The cytotoxicity of F1-F7 were screened in several cancer cell lines including, Human Gastric carcinoma (KATO-III), Human Ductal carcinoma (BT 474), Human Colon adenocarcinoma (SW620), Human Liver hepatoblastoma (Hep-$G_2$), Human Lung undifferentiated (Chago) cell lines, the F5 showed cytotoxic activity against all tested cancer cell lines (โสภิต ธรรมอารี, 2552). This study evaluated the cytotoxic activity of the F5.1 protein from King Cobra venom. This protein showed cytotoxicity towards Human PBMCs. The results suggested that the cytotoxicity of the F5.1 may be useful as anticancer and should be further characterized for the mechanism or modification of this protein in order to increase the selectivity of antimicrobial protein against the pathogenic bacteria, with minimal toxicity towards eukaryotic cells. The success of structured modification seen from the pEM-2, a modified synthetic peptide derived from the snake venom Lys49 phospholipase $A_2$ which showed reduced toxicity towards muscle cells, while retaining high bactericidal potency (Santamaria *et al*., 2005). While the original native snake venom compounds are usually unsuitable as therapeutics, modifications by medical chemists as well as clinicians and scientists in pharmaceutical R&D have made it possible to use the snake venom proteins as therapeutic means for various diseases based on the available structural and functional information.

In summary, This study evaluated the antibacterial and cytotoxic activity of the semipurified protein fraction, F5.1 from King Cobra venom. This protein showed strongest antibacterial activity against Gram-positive *S. aureus* bacteria. The mechanism involved membrane damage. This mechanism may also be responsible for its cytotoxicity. Therefore, the F5.1 should be further studied modify the amino acid sequence of this protein for the purpose of decreasing toxicity and reta
antibacterial activity or investigations with cytotoxic activity against cancer cell

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# APPENDICES

# APPENDIX A

## Buffer and Reagents

1. 10 mM sodium phosphate buffer (pH 7.4), 500 ml

| | |
|---|---|
| $NaH_2PO_4.H_2O$ | 0.16 g |
| $Na_2HPO_4.7H_2O$ | 1.04 g |
| Distilled water | 450 ml |
| adjust pH to 7.4 with 1 M HCl | |
| add Distilled water to 500 ml | |

2. 0.2 M Tris-HCl, 1L

| | |
|---|---|
| Tris-HCl | 24.2 g |
| add Distilled water to 1 L | |

3. 0.02 M Tris buffered saline (TBS) (pH 7.4), 1L

| | |
|---|---|
| 0.2 M Tris-HCl | 100 ml |
| NaCl | 8.77 g |
| Distilled water | 850 ml |
| adjust pH to 7.4 with 1 M HCl | |
| add Distilled water to 1L | |

SDS-PAGE

1. Stock solutions

A. 30% monomer

| | |
|---|---|
| Acrylamide | 30 g |
| Bis | 0.8 g |
| Distilled water | to 100 ml |

B. 3.0 M Tris-HCl (pH 8.8), 100 ml

| | |
|---|---|
| Tris-base | 36.3 g |
| Distilled water | 50 ml |
| adjust pH to 8.8 with 1 M HCl | |
| add Distilled water to 100 ml | |

C. 0.5 M Tris-HCl (pH 6.8), 100 ml

| | |
|---|---|
| Tris-base | 6.05 g |
| Distilled water | 50 ml |
| adjust pH to 6.8 with 1 M HCl | |
| add Distilled water to 100 ml | |

D. 1% SDS (w/v), 100 ml

| | |
|---|---|
| SDS | 1 g |
| add $ddH_2O$ to 100 ml | |

E. APS 2.5%

| | |
|---|---|
| APS | 0.5 g |
| Distilled water | 2 ml |

2. Running buffer

| | |
|---|---|
| Tris-base | 3.03 g |
| Glycine | 14.4 g |
| SDS | 1 g |
| add Distilled water to 1000 ml | |

3. Staining solution

| | |
|---|---|
| Coomassie blue R-250 | 0.2 g |
| Methanol | 46.5 ml |
| Glacial acetic acid | 7 ml |
| Distilled water | 46.5 ml |

4. Destain solution

| | | |
|---|---|---|
| Distilled water | 625 | ml |
| Glacial acetic acid | 125 | ml |
| Methanol | 125 | ml |

5. Gel preparation

| | Separating gel (ml) | Stacking gel (ml) |
|---|---|---|
| 30% monomer | 2.9 | 0.5 |
| 3.0 M Tris-HCl pH 8.8 | 1.75 | - |
| 0.5 M Tris-HCl pH 6.8 | - | 1.0 |
| 1% SDS | 0.7 | 0.4 |
| APS (2.5%) | 0.35 | 0.2 |
| TEMED | 0.01 | 0.005 |
| Distill water | 1.3 | 1.9 |

**Cytotoxic assay**

1. RPMI 1640 stock solution, 1 liter

| | | |
|---|---|---|
| RPMI powder | 10.4 | g |
| $NaHCO_3$ | 1.5 | g |
| Glucose | 4.5 | g |
| Sodium pyruvate | 0.1 | g |
| HEPES (1M) | 10 | ml |
| Penicillin/Streptomycin | 10 | ml |
| Distilled water | 900 | ml |

Adjust pH to 7.2 with HCl

Add distilled water to 1 l and filtering through 0.45 µm membrane filter

2. Complete RPMI 1640 medium, 100 ml

| | | |
|---|---|---|
| RPMI stock | 90 | ml |
| Fetal Bovine Serum | 10 | ml |

# APPENDIX B

## Results

**Table A** Antibacterial activity (Zone of inhibition) against *E.coli* of crude venom of King cobra

| Conc. (µg/disc) | Inhibition zone (mm) | | | | | Mean | SE |
|---|---|---|---|---|---|---|---|
| | N1 | N2 | N3 | N4 | N5 | | |
| 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 |
| 3.12 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 |
| 6.25 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 |
| 12.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 |
| 25 | 7.0 | 7.0 | 6.5 | 7.0 | 7.0 | 6.9 | 0.10 |
| 50 | 8.0 | 8.0 | 8.5 | 8.5 | 8 | 8.2 | 0.12 |
| 100 | 9.0 | 10.0 | 10.0 | 9.0 | 9.0 | 9.4 | 0.24 |
| 200 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 0.70 |

**Table B** Antibacterial activity (Zone of inhibition) against *P.aeruginosa* of crude venom of King cobra

| Conc. (µg/disc) | Inhibition zone (mm) | | | | | Mean | SE |
|---|---|---|---|---|---|---|---|
| | N1 | N2 | N3 | N4 | N5 | | |
| 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 |
| 3.12 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.00 |
| 6.25 | 7.5 | 7.5 | 7.5 | 7.0 | 7.5 | 7.4 | 0.10 |
| 12.5 | 8.0 | 8.0 | 8.5 | 9.0 | 8.5 | 8.4 | 0.19 |
| 25 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 0.00 |
| 50 | 11.0 | 10.5 | 11.0 | 11.0 | 11.0 | 10.9 | 0.10 |
| 100 | 12.0 | 12.0 | 11.5 | 12.0 | 12.0 | 11.9 | 0.10 |
| 200 | 13.0 | 13.0 | 13.0 | 13.0 | 13.0 | 13.0 | 0.00 |

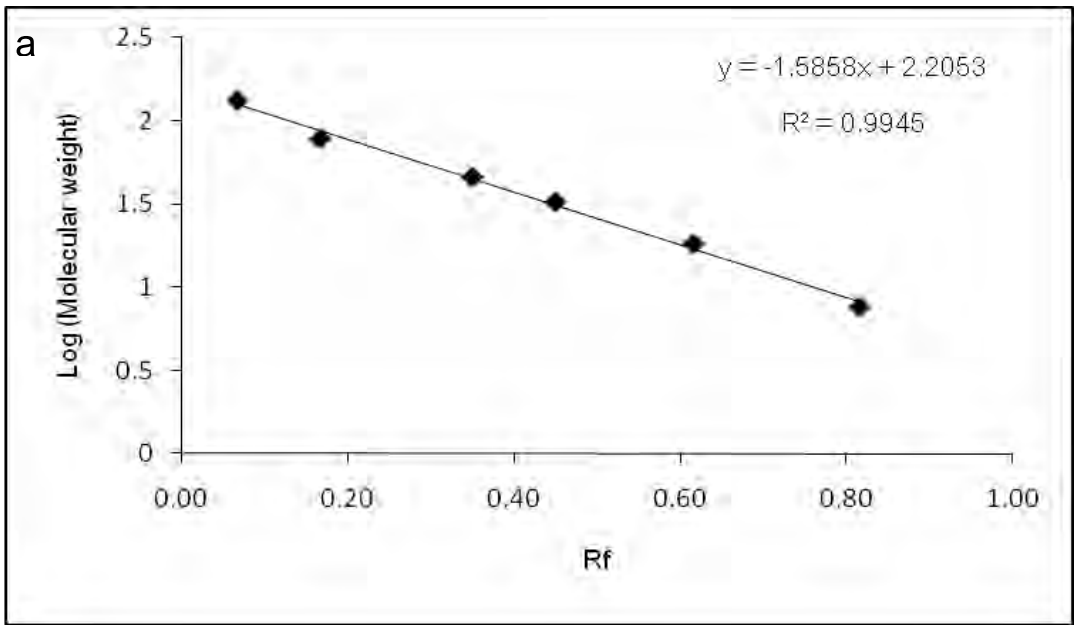
**Table C** Antibacterial activity (Zone of inhibition) against *S.*Typhimurium of crude venom of King cobra

| Conc. (µg/disc) | Inhibition zone (mm) | | | | | Mean | SE |
|---|---|---|---|---|---|---|---|
| | N1 | N2 | N3 | N4 | N5 | | |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3.12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6.25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 25 | 7 | 8 | 8 | 8 | 8.5 | 7.7 | 0.20 |
| 50 | 9 | 9 | 9 | 9.5 | 9 | 9.1 | 0.10 |
| 100 | 10 | 10 | 10 | 10 | 10 | 10 | 0 |
| 200 | 10.5 | 10.5 | 11 | 11 | 10.5 | 10.7 | 0.12 |

**Table D** Antibacterial activity (Zone of inhibition) against *S.aureus* of crude venom of King cobra

| Conc. (µg/disc) | Inhibition zone (mm) | | | | | Mean | SE |
|---|---|---|---|---|---|---|---|
| | N1 | N2 | N3 | N4 | N5 | | |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3.12 | 9 | 9 | 9.5 | 9 | 9 | 9.1 | 0.10 |
| 6.25 | 11 | 12 | 12 | 12 | 11.5 | 11.7 | 0.20 |
| 12.5 | 14 | 13.5 | 14 | 14 | 14 | 13.9 | 0.10 |
| 25 | 16 | 15 | 16 | 15 | 16 | 15.6 | 0.24 |
| 50 | 18 | 17.5 | 17.5 | 18 | 18 | 17.8 | 0.12 |
| 100 | 19 | 19 | 19 | 18 | 19 | 18.8 | 0.20 |
| 200 | 21 | 20 | 21 | 21 | 20.5 | 20.7 | 0.20 |

a




b

| Std. protein | Molecular weight (kDa) | Rf | Molecular weight from graph (kDa) |
|---|---|---|---|
| $\beta$-galactosidase | 131 | 0.07 | - |
| bovine serum albumin | 78 | 0.17 | - |
| carbonic anhydrase | 41.3 | 0.35 | - |
| soybean trypsininhibitor | 31.8 | 0.45 | - |
| lysozyme | 18.1 | 0.62 | - |
| aprotinin | 7.1 | 0.82 | - |
| F5.1 | | 0.23 | 69 |

**Figure A** Creating standard curves. The Bio-Rad Kaleidoscope prestained standards (7 µl) was electrophoresed on 12.5% SDS-PAGE at 30 mA until the dye front was 0.1 cm from the bottom of a 9x6-cm gel. Gels were stained with COOMASSIE BRILLIANT BLUE® 250 and Rf measured for each band (a). Standard curves were generated by plotting the log of molecular weight versus the distance migrated. The distance migrated was then plugged back into the equation for the best-fit line to generate the calculated molecular weights (b).

**Table E** Release of 260 absorbing material from S.aureus treated with F5.1

| Time (min) | control | F5.1 (1.8 µg/ml) | F5.1 (3.6) µg/ml) |
|---|---|---|---|
| 0 | 0.000±0.000 | 0.000±0.000 | 0.000±0.000 |
| 20 | 0.022±0.001 | 0.046±0.000 | 0.056±0.003 |
| 40 | 0.028±0.001 | 0.055±0.001 | 0.062±0.001 |
| 60 | 0.032±0.003 | 0.053±0.000 | 0.064±0.001 |
| 80 | 0.033±0.003 | 0.054±0.001 | 0.064±0.001 |
| 100 | 0.032±0.003 | 0.060±0.001 | 0.067±0.001 |
| 120 | 0.030±0.002 | 0.054±0.001 | 0.072±0.001 |
| 140 | 0.029±0.001 | 0.053±0.000 | 0.065±0.001 |
| 160 | 0.028±0.001 | 0.057±0.000 | 0.067±0.001 |
| 180 | 0.030±0.003 | 0.062±0.001 | 0.069±0.002 |

**Table F** Effect of F5.1 on Human PBMCs by rezasurin assay (n=3)

| Sample | concentration (µg/ml) | % viability |
|---|---|---|
| F5.1 | 1.8 | 44.16±1.11 |
| Doxorubicin | 2.0 | 79.88±0.57 |

**Table G** L-amino acid oxidase activity

| Time (min) | Absorbance | | | | | |
|---|---|---|---|---|---|---|
| | Crude venom | | F5 | | F5.1 | |
| | N1 | N2 | N1 | N2 | N1 | N2 |
| 0 | 0.0870 | 0.1107 | 0.2143 | 0.1847 | 0.2154 | 0.2316 |
| 15 | 0.1419 | 0.1577 | 0.3361 | 0.3131 | 0.3401 | 0.3552 |
| 30 | 0.1923 | 0.2051 | 0.4474 | 0.4315 | 0.4549 | 0.4689 |
| 45 | 0.2353 | 0.2522 | 0.5506 | 0.5401 | 0.5612 | 0.5737 |
| 60 | 0.2809 | 0.2960 | 0.6458 | 0.6399 | 0.6592 | 0.6703 |
| 75 | 0.3251 | 0.3390 | 0.7346 | 0.7315 | 0.7532 | 0.7603 |
| 90 | 0.3679 | 0.3812 | 0.8179 | 0.8156 | 0.8424 | 0.8432 |
| 105 | 0.4103 | 0.4221 | 0.8898 | 0.8932 | 0.9200 | 0.9204 |
| 120 | 0.4479 | 0.4619 | 0.9591 | 0.9643 | 0.9872 | 0.9923 |
| 135 | 0.4862 | 0.5015 | 1.0232 | 1.0296 | 1.0552 | 1.0593 |
| 150 | 0.5232 | 0.5366 | 1.0816 | 1.0899 | 1.1177 | 1.1192 |
| 165 | 0.5589 | 0.5722 | 1.1367 | 1.1478 | 1.1752 | 1.1752 |
| 180 | 0.5934 | 0.6068 | 1.1873 | 1.1955 | 1.2274 | 1.2255 |
| dA/min | 0.1671 | 0.1653 | 0.3203 | 0.3329 | 0.3341 | 0.3280 |
| Mean | 0.1662 | | 0.3266 | | 0.3311 | |
| SE | 0.001 | | 0.006 | | 0.003 | |

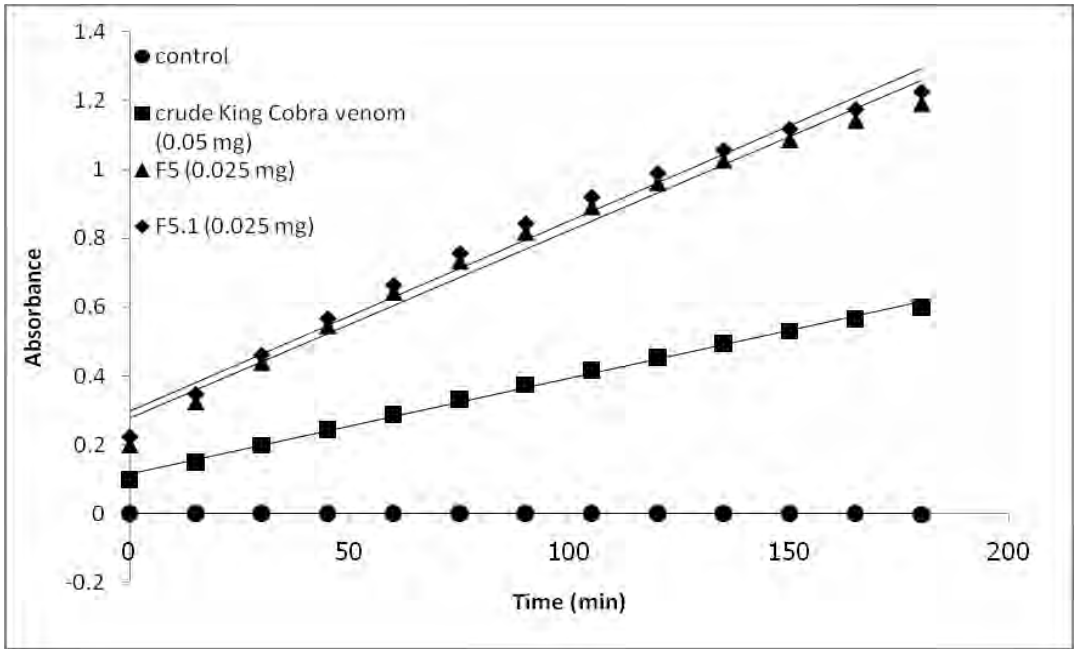



**Figure B** Graph shown correlations between time and absorbance for dertemination LAAO activity

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