Mechanistic analysis of a novel antimicrobial compound, Kaikosin E

（新規抗生物質カイコシンの作用機序の解析）

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Dissertation submitted to The University of Tokyo for the Degree of Doctor of Philosophy

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Su Jie

ABSTRACT

Mechanistic analysis of a novel antimicrobial compound, Kaikosin E

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Su Jie

Introduction

The emergence and spread of multi-resistant pathogenic strains declined the number of clinically available antibiotics and caused a serious public health problem. Therefore, the development of novel antimicrobial agents with effective treatment of the drug-resistant pathogens is needed to combat this situation. Our lab also worked on the discovery of novel antimicrobial compounds and isolated a novel lipopeptide antibiotic named as kaikosin E using silkworm infection model. Kaikosin E exhibits good antibiotic traits such as obvious therapeutic effect in mouse infection model with low toxicity and rapid bactericidal activity compared with other clinically used antibiotics. Cell lysis also occurred after treatment with kaikosin E. Adding kaikosin E to *Staphylococcus aureus* rapidly dissipated membrane potential. Based on these findings, disruption of membrane is one proposed mechanism of action for kaikosin E. However, the details are still unknown. In searching of target for kaikosin E, *fni* or *menA* gene mutation was found in kaikosin E resistant mutant. Both genes are involved in menaquinone biosynthesis pathway. Furthermore mixing kaikosin E with menaquinone caused complex precipitation in water. These results guide me to focus on the importance of menaquinone for uncovering kaikosin E

mechanism. In addition, a recent study showed the antimicrobial activity of kaikosin E against *S.aureus* was enhanced by addition of serum. This finding suggested that an enhancing factor for antimicrobial activity of kaikosin E exists in serum. Therefore, identification of this factor may facilitate knowing the interaction of kaikosin E with its target and provide information for improving the therapeutic activity of kaikosin E. In this study, I intend to further elucidate the antimicrobial mechanism of kaikosin E through the identification of the role of menaquinone in membrane damage and identification of the serum factor responsible for enhancing antimicrobial activity of kaikosin E.

Results

1. Identification of menaquinone as a target

In view menaquinone existed in cell membrane and kaikosin E had membrane damaging activity, identification of the role of menaquinone in membrane damage may facilitate elucidation of kaikosin E mechanism. To test the role of menaquinone on kaikosin E-mediated membrane damage effect，calcein-encapsulated liposome was prepared and used for membrane leakage assay. Kaikosin E significantly caused leakage of liposome containing menaquinone (MK) compared with liposome without menaquinone (**Figure 1**), whereas daptomycin showed no significant difference on leakage of liposome with or without menaquinone. Hence, the existence of menaquinone stimulated membrane damaging activity of kaikosin E. On the other hand, kaikosin E had no hemolytic effect on sheep red blood cells, suggesting that this membrane damaging effect is specific to bacteria. To further confirm the interaction of kaikosin E with menaquinone, isothermal titration calorimetry (ITC) assay was conducted for characterization of binding affinity between them. ITC results showed kaikosin E binds with menaquinone at a molar ratio of 1:1 with an affinity constant (K_a) of 2.2±0.4×10^5M^{-1}. These results suggest kaikosin E binds with menaquinone in cell membrane and induces membrane damage.

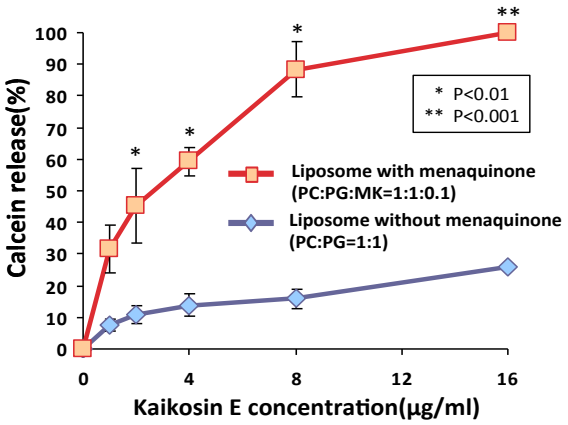

Figure 1. Membrane leakage assay. Values for 100% calcein release were obtained using Triton X-100. ∗ ,P<0.01; ∗ ∗ ,P<0.001.

2. Identification of a serum factor enhancing antimicrobial activity of kaikosin E

Antimicrobial activity of kaikosin E against *S. aureus* was enhanced more than 10-fold in the presence of bovine calf serum. This enhancement effect on antimicrobial activity of kaikosin E was also detected in human plasma and other mammalian serum. Considering the significant enhancement effect of serum on kaikosin E activity, I assume some factors in serum act as enhancers. Thus I tried to purify the enhancing factor from serum. One unit of enhancing activity is defined as the minimum amount of

active factor which can inhibit bacterial growth in the presence of 1 μg/ml kaikosin E. Enhancing factor in serum was purified with ethanol extraction followed by octadecyl silica (ODS) column chromatography with 190-fold increase in specific activity (**Table 1**). ODS column fraction lost 97 % of enhancing activity after trypsin treatment. This result suggested that a protein in serum is responsible for enhancing antimicrobial activity of kaikosin E. To further identify the active protein, fractions from ODS column were analyzed by SDS polyacrylamide gel electrophoresis. Twenty-four kDa protein band was observed in active fractions (**Figure 2**). These fractions were further analyzed by HPLC using size exclusion column, and the band intensity of 24 kDa protein from each fraction correlated with its enhancing activity. Peptide mass fingerprinting analysis revealed the 24 kDa protein was matched with the sequence of apolipoprotein A-I (Apo A-I). Antimicrobial activity of kaikosin E was enhanced 16-fold in the presence of 90 μg/ml of human recombinant apolipoprotein A-I, which is equivalent to 10 % serum. Apolipoprotein A-I itself had no antimicrobial activity against *S. aureus* (**Table 2**). These results suggested apolipoprotein A-I is one serum factor responsible for enhancing antimicrobial activity of kaikosin E. The mechanism of apolipoprotein A-I mediated enhancement effect on antimicrobial activity of kaikosin E needs to be elucidated.

Table 1. Purification of a serum factor enhancing antimicrobial activity of kaikosin E

Fraction	Weight (mg)	Total activity (Units)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Ⅰ.Serum	96	640	7	100	1
Ⅱ.60%EtOH extract	18	2600	140	406	20
Ⅲ.ODS Fr.1	16	<20	–	–	–
Fr.2	0.2	<20	–	–	–
Fr.3	0.8	<20	–	–	–
Fr.4	0.5	640	1300	100	190
Fr.5	0.1	80	800	13	110

Table 2. Effect of serum on antimicrobial activity of kaikosin E

Medium	MIC(μg/ml)
Mueller-Hinton broth	4
+ Serum(10%)	0.25
+ Human recombinant apolipoprotein A-I(90 μg/ml)	0.13

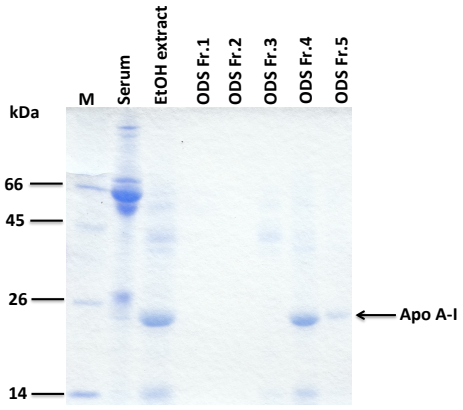

Figure 2. SDS-PAGE analysis of fractions from ODS column.

3. Interaction of apolipoprotein A-I with kaikosin E and menaquinone

Menaquinone was reported to interact with high-density lipoprotein. Apolipoprotein A-I is known as the major component of high-density lipoprotein in plasma. Hence I proposed apolipoprotein A-I enhances antimicrobial activity of kaikosin E through interaction with menaquinone and kaikosin E. To test this hypothesis, interaction assay among apolipoprotein, menaquinone and kaikosin E was performed. After mixing kaikosin E and menaquinone at a molar ratio of 1:1 and centrifugation, yellow pellet was shown in the bottom of the tube. When mixing apolipoprotein A-I, kaikosin E and menaquinone,

organge pellet was shown in the mixing solution whereas apolipoprotein A-I solution itself was transparent. Hence, I speculate that apolipoprotein A-I interacts with the binding complex of kaikosin E with menaquinone and changes the color of pellet. To check this possibility, the precipitate from mixing apolipoprotein A-I, kaikosin E and menaquinone solution was applied into SDS-PAGE analysis. Apolipoprotein A-I band was detected in the precipitate sample from mixing apolipoprotein A-I, kaikosin E and menaquinone solution **(Figure 3)**. These results suggested apolipoprotein A-I interacts with kaikosin E and menaquinone. Apolipoprotein A-I may enhance the antimicrobial activity of kaikosin E through interaction with kaikosin E and menaquinone.

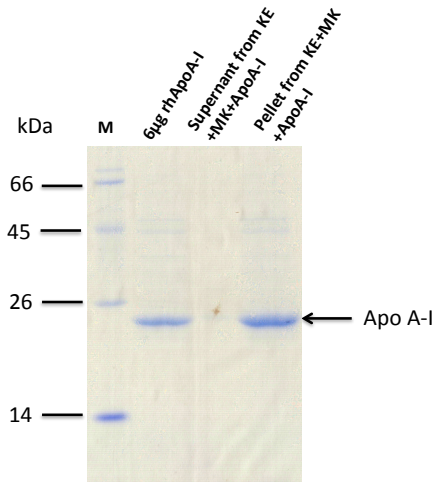

Figure 3. SDS-PAGE analysis on existence of Apo A-I.

Discussion

This study demonstrates kaikosin E binds with menaquinone in cell membrane and induces membrane damage. To my knowledge, this is the first report that menaquinone is identified as a target of antibiotic. Menaquinone is an essential component of bacterial electron transport chain. In contrast, ubiquinone is a coenzyme utilized in human electron transport chain. The finding that kaikosin E specifically binds with menaquinone not ubiquinone explains that membrane damage effect induced by kaikosin E is specific to bacteria. Therefore, menaquinone can be used as a potential target for the development of novel antibacterial drugs.

In this study, I also identified apolipoprotein A-I as a serum factor, which is responsible for enhancing antimicrobial activity of kaikosin E against *S. aureus*. This finding explains the fact that kaikosin E has good therapeutic activity compared with vancomycin in mouse infection model, whereas the antimicrobial activity of kaikosin E detected in culture medium is lower than that of vancomycin. Therefore, the enhanced antimicrobial activity of kaikosin E *in vivo* may be due to the existence of apolipoprotein A-I in serum. This finding provides evidence for kaikosin E to be successful use in future clinical situation. Study on the interaction among apolipoprotein A-I, kaikosin E and menaquinone showed that apolipoprotein A-I interacts with the binding complex between kaikosin E and menaquinone. Therefore, apolipoprotein A-I may enhance antimicrobial activity of kaikosin E through interaction with the binding complex of kaikosin E and menaquinone.

Introduction

1. Emergence of antibiotic resistant pathogen：Threats to human health

Since the clinical success of penicillin open the era of antibiotics, a large number of antibiotics have been developed with effective treatment of infectious diseases and hence contributed to human health (Yoneyama *et al.*, 2006). However, the infectious diseases are still the second cause of leading death (Fauch., 2001). One reason leading to the ineffective control of the infectious diseases is the emergence and spread of drug-resistant bacteria (Demain *et al.*, 2009). For example, penicillin resistance in *Staphylococcus spp* occurred after short time of clinical usage (Demain., 2009). In the UK, half are antibiotic resistant among the 20,000 cases of *Staphylococcus aureus* bacteremia reported every year. And the number of methicillin-resistant *S. aureus* (MRSA) in hospital-acquired infections kept increasing by 10-15 % in recent years in European countries and the USA (García-Lara *et al.*, 2005). Even vancomycin resistant *Staphylococcus aureus* (VRSA) with low occurrence were previously reported in USA, a case of VRSA infection was already confirmed in Portugal in May, 2013 (Melo-Cristino *et al*., 2013). The dangerous multidrug-resistant (MDR) bacterial strain with a novel resistant mechanism, also known as superbugs, has emerged worldwide (Alanis., 2005). The mortality caused by drug-resistant infections is increasing in the United States and around the world (Gollaher *et al*., 2012). The number of clinically available antibiotics has gradually declined due to the emergence of antibiotic-resistant bacteria. To overcome the increasing prevalence of drug-resistance, development of novel antimicrobial agents with treatment of the drug-resistant pathogens is needed.

2. The need for novel antibiotics

Unfortunately, the pharmaceutical industry has been developing slowly and producing too few effective antibiotics after more than 5 decades of success (Coates *et al.*, 2007). The main strategy of pharmaceutical industry for development of new antibiotics has been producing semi-synthetic compounds by modification from the existing antibiotics, with little effort on natural compound research (Yoneyama *et al.*, 2006). Many new antimicrobial agents derived from chemical classes for which are already underlying resistance mechanism, finally failed in clinical usage. Therefore, the development of microbial natural compounds should be emphasized to keep pace with drug resistance. The paucity in understanding the antimicrobial mechanism of antibiotics is another bottleneck in drug development. Most experimental compounds failed entering the clinical phase because of the unclear assessment of their unanticipated biological effects prior to clinical stages. Therefore, the fully understanding of the antimicrobial mechanism of the potent antibiotic candidates is essential to avoid costly and time-consuming clinical setbacks and maximize the number of successful new drugs (Chan *et al.*, 2010).

In addition to shortage in number of new antimicrobial agents, the novel antibiotics with new mechanisms are rare. Success in treatment of the drug resistant bacteria requires the new drugs with the potent advances in treatment of infections compared with the available antibiotics (Gollaher *et al.*, 2012). Therefore, the development of new drugs, particularly with novel mechanisms to overcome the resistance barrier is needed.

3. A novel antibiotic, kaikosin E

Our lab also worked on the discovery of novel antimicrobial compounds and isolated a novel lipopeptide antibiotic from culture supernatant of a lysobacter species using silkworm infection model. To underscore the contribution of Prof. Kazuhisa Sekimizu to the silkworm infection model, this novel antibiotic was named as kaikosin E which originates from the Japanese name of silkworm. Kaikosin E shows a cyclic lipopeptide structure with 12 amino acid residues and a short fatty acid tail with a molecular weight of 1617 Da **(Figure 0)**. Kaikosin E is thermo-stable and keeps the same antimicrobial activity even after autoclaving at 121 ˚C for 20 min. Kaikosin E also exhibits good antibiotic traits such as obvious therapeutic effect in mouse infection model (ED_{50}: 0.6 mg/Kg) with low toxicity and potent antimicrobial activity against gram-positive pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA). Another outstanding feature of kaikosin E is its rapid bactericidal activity against *S. aureus* in short-term (within 1 min). This prominent character of kaikosin E was distinct from other clinically used antibiotics and prompted our interest in elucidation of the mechanism of kaikosin E. The following study showed that kaikosin E has inhibition effect on the macromolecular biosynthesis of DNA, RNA, protein and cell wall. Cell lysis of *S. aureus* was also observed after treatment with kaikosin E. Addition of kaikosin E to culture medium of *S. aureus* rapidly caused dissipation of membrane potential. Based on these findings, the proposed mechanism for kaikosin E is that it disrupts membrane integrity by dissipation of membrane potential, arrests of macromolecular synthesis and causes cell death. However, the details are still unknown.

A recent study by our laboratory showed that the antimicrobial activity of kaikosin E against gram-positive bacteria was greatly enhanced in the presence of bovine serum. This serum-mediated enhancement effect on kaikosin E is contrary to other clinically used antibiotics whose antimicrobial activity are usually inhibited by serum due to protein binding. This finding indicates the novel mechanism of kaikosin E and provides a basis for improving the therapeutic effect of kaikosin E. In this study, I intend to further elucidate the antimicrobial mechanism of kaikosin E in view of its desirable antibiotic feature.

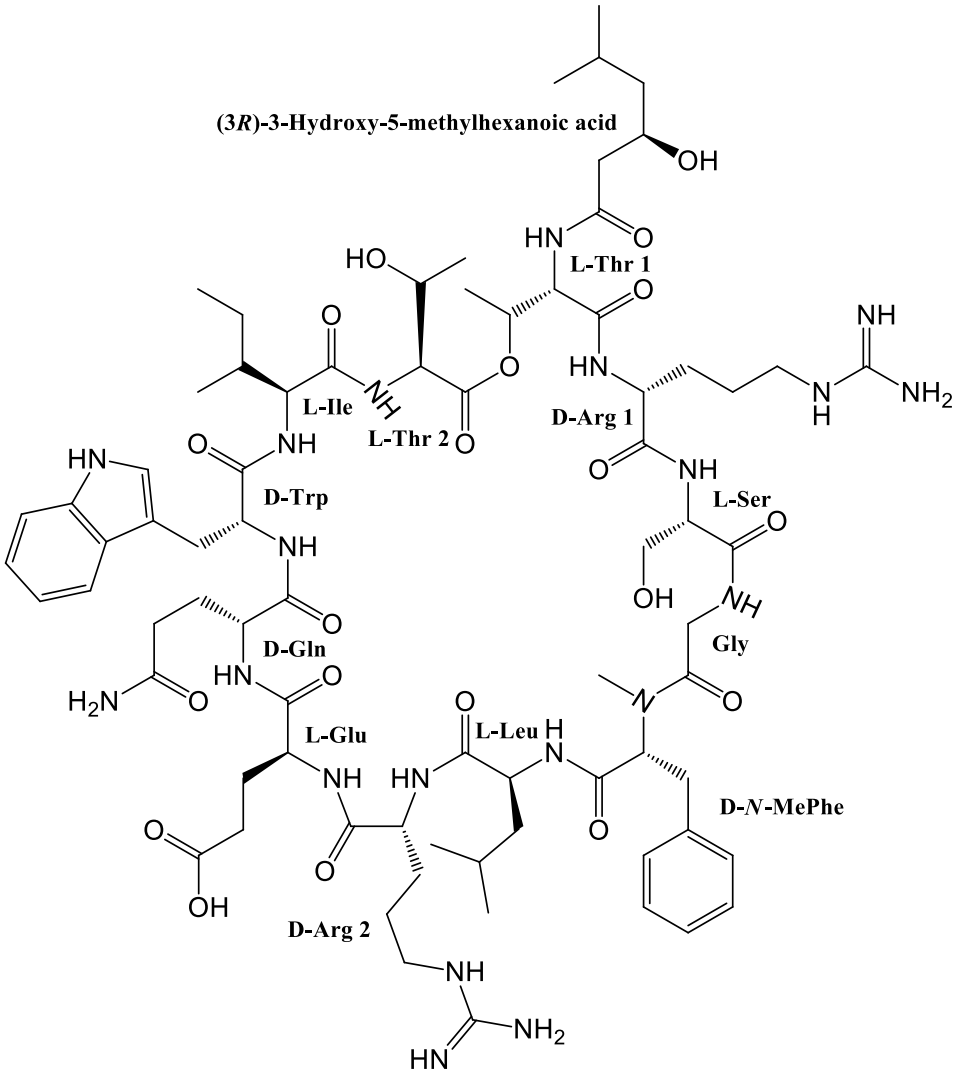

Figure 0. Structure of kaikosin E

Chapter 1. Target identification of kaikosin E

Background

In the process of searching the target for kaikosin E, *fni* or *menA* gene mutation was found in kaikosin E resistant mutants (Paudel A. 2013). The *fni* gene encoding isopentenyl diphosphate (IPP) isomerase is involved in mevalonate pathway and essential for biosynthesis of isoprenoids which is required for menaquinone biosynthesis. The *menA* gene encoding 1,4-dihydroxy-2-naphthoate (DHNA) prenyl transferase is responsible for synthesis of dimethyl menaquinone (DMK) which finally converted to menaquinone (MK) after methylation (Bentley *et al.*, 1982). Both *fni* gene and *menA* gene are involved in menaquinone biosynthesis pathway **(Figure 1-1)**. Menaquinone is an essential component of bacterial electron transport chain. The addition of menaquinone inhibited antimicrobial activity of kaikosin E, whereas the addition of ubiquinone which is a coenzyme utilized by mammalian electron transport chain had no effect on antimicrobial activity of kaikosin E. Furthermore, mixing kaikosin E with menaquinone caused complex precipitation in water.

These results guided me to focus on the importance of menaquinone for eliciting kaikosin E mechanism. In view menaquinone existed in cell membrane and kaikosin E had membrane damaging effect, therefore, identification of the role of menaquinone in kaikosin E-mediated membrane damage may facilitate elucidation of kaikosin E mechanism.

1.1 Membrane damaging activity of kaikosin E

1.1.1 Potassium leakage

Kaikosin E damages membrane of *S. aureus* by dissipation of membrane potential. Bacteria maintain membrane potential by establishing multiple ion gradients across the cytoplasmic membrane. The proper maintenance of K^+ gradient is important for bacterial viability (Silverman *et al.*, 2003). Dissipation of membrane potential is an indicative of ion movement across the cytoplasmic membrane. The effect of kaikosin E on potassium ion leakage from *S. aureus* membrane was determined by Prof. Katsu of Okayama University. Adding kaikosin E into *S. aureus* caused potassium release from cell membrane, accompanied with decrease of cell viability. The potassium release also correlated with viable cell number of *S. aureus* **(Figure 1-2)**. This result suggested that kaikosin E triggers the potassium release from *S. aureus* and causes cell death.

1.1.2 Hemolytic activity of kaikosin E

Even treatment with more than 100 μg/ml of kaikosin E had no lysis effect on red blood cell, suggesting the lysis ability of kaikosin E is specific to bacteria **(Table 1-1)**.

1.2 Identification of menaquinone as a target for kaikosin E

1.2.1 Correspondence between menaquinone amount and cell susceptibility to kaikosin E

Menaquinone was extracted from *S. aureus* using quinone extraction method. The relative amounts of menaquinone in wild type strain RN4220 was defined as 1, the amounts of menaquinone amounts in kaikosin E resistant mutant (1-#9 *fni* mutant or 2-#5 *menA* mutant) is half lower than in wild type strain RN4220, whereas minimum inhibitory concentration (MIC) of kaikosin E against resistant mutant is two-fold higher than wide type *S. aureus* RN4220 **(Table 1-2)**. Therefore, amounts of menaquinone in *S. aureus* correlate with bacterial susceptibility to kaikosin E. These results suggest menaquinone level may be responsible for antimicrobial activity of kaikosin E.

1.2.2 Membrane damage caused by kaikosin E correlates with menaquinone amounts in *S. aureus*

Membrane depolarization was determined by using a fluorescent assay with a membrane potential sensitive dye DiS-C_3(5). The fluorescence of the dye DiS-C_3(5) decreases when it partitions into the cell, the dye will be released from the cell and increases the fluorescence in the medium once the membrane was dissipated. The pore-forming antimicrobial nisin (1×, 2×MIC) was used as positive control. As shown in **Figure 1-3(A)**, kaikosin E dissipated membrane potential even at low concentration. Also, a significant decrease of membrane depolarization triggered by kaikosin E was observed in kaikosin E resistant *menA* mutant compared with wild type RN4220, whereas no significant difference was observed in nisin-treated strains

(Figure 1-3 (B)). These data demonstrated kaikosin E has strong ability to dissipate cell membrane potential of wild type *S. aureus* compared with #5 *menA* mutant. And #5 *menA* mutants showed kaikosin E resistance with low production of menaquinone compared with wild type *S. aureus*. These results suggest menaquinone may be in charge of kaikosin E-mediated membrane damage. To test this possibility, artificial membrane was used to examine the role of menaquinone on membrane damage induced by kaikosin E.

1.2.3 Role of menaquinone on kaikosin E-mediated membrane damage

Calcein-encapsulated liposome was prepared by a method of Zhang *et al.* Membrane leakage was assessed by dequenching of calcein fluorescence after release from liposome. As shown in **Figure 1-4(A)**, kaikosin E significantly caused calcein release from liposome containing menaquinone compared with the liposome without menaquinone. Therefore, the existence of menaquinone stimulated the membrane damaging activity of kaikosin E. Daptomycin as a clinically used antibiotic is highly active against *S. aureus* and owns a cyclic lipopeptide structure similar to kaikosin E. It was also reported that the mechanism of action for daptomycin is disruption of cell membrane (Strauss *et al.*, 2006). Therefore, daptomycin was used as a control in this experiment. However, daptomycin showed no significant difference on calcein release from liposome with or without menaquinone **(Figure 1-4(B))**. Thus, membrane damage depending on menaquinone was specific for kaikosin E. The antimicrobial mechanism of kaikosin E is different from daptomycin. This artificial membrane study indicated that kaikosin E acts on membrane with menaquinone. To further know the interaction of kaikosin E with menaquinone, isothermal titration calorimetry (ITC)

method will facilitate us to achieve this aim.

1.2.4 Interaction of kaikosin E with menaquinone

Kaikosin E-menaquinone interaction was measured with isothermal titration calorimetry (ITC) instruments. Dr. Urai also contributes to this part of experiments. ITC measures heat released or absorbed upon binding and provides the value of reaction molar ratio (n). In **Figure 1-5(A)** top panel, heat release was observed from the reaction solution of kaikosin E and menaquinone. The peaks from the top panel are integrated to present in bottom panel. As shown in sigmoidal curve in bottom panel, released heat gradually decreased and approached to zero, which means the binding of menaquinone was almost saturated. ITC results also showed the molar ratio (n) of kaikosin E : menaquinone is 1:1. However, there is only background of released heat detected in solution of kaikosin E with ubiquinone **(Figure 1-5(B))**. Hence, there is no binding between kaikosin E and ubiquinone. The above data demonstrated kaikosin E binds with menaquinone at a molar ratio of 1:1 with an affinity constant (K_a) of $2.2 \pm 0.4 \times 10^5\ M^{-1}$.

Menaquinone is an essential component of bacterial electron transport chain. In contrast, ubiquinone is a coenzyme utilized in human electron transport chain (Kurosu *et al.* 2010). The finding that kaikosin E specifically binds with menaquinone but not ubiquinone explains that membrane damaging effect induced by kaikosin E is specific to bacteria. The membrane damaging activity of kaikosin E which is specific to bacterial membrane also explains the fact that kaikosin E has no hemolytic activity on red blood cell and shows the low toxicity of kaikosin E to mammalians.

Most importantly, human does not utilize menaquinone in the electron transport chain, which is a central component in the production of ATP and the sequent growth of bacteria. Therefore, menaquinone can be used as a potential target for the development of novel antibacterial drugs.

1.3 Conclusion

Adding kaikosin E into *S. aureus* triggers potassium leakage from cell membrane. Hemolytic assay reveals that the membrane damaging activity of kaikosin E is specific to bacteria. The amounts of menaquinone in *S. aureus* correlate with bacterial susceptibility to kaikosin E. The existence of menaquinone in liposome stimulates the membrane damaging activity of kaikosin E. Isothermal titration calorimetry study also revealed kaikosin E binds with menaquinone at a molar ratio of 1:1 with an affinity constant (K_a) of $2.2\pm0.4\times10^5 M^{-1}$. These results suggest kaikosin E binds with menaquinone in cell membrane and induces membrane damage.

Menaquinone is an essential component of bacterial electron transport chain. In contrast, ubiquinone is a coenzyme utilized in human electron transport chain (Kurosu *et al.* 2010). Both menaquinone **(Figure 1-6(A))** and ubiquinone **(Figure 1-6(B))** consist of a polar head group and a hydrophobic side chain in their molecular structures. The hydrophobic side chain provides the molecules with a lipid-soluble character to allow them to perform vital functions in membrane lipid bilayers, whereas the polar head group enables interaction with membrane proteins (Fujimoto *et al*., 2012). In view kaikosin E specifically binds with menaquinone not ubiquinone, kaikosin E may interact with the polar head of menaquinone followed by structural

change. Based on these findings, I proposed the model of antimicrobial action for kaikosin E as shown **in Figure 1-7.** First, kaikosin E binds with menaquinone in cell membrane and forms in an aggregate structure. Next, the binding complex with the aggregate structure disrupts the membrane integrity and causes cell death. Further confirmation needs to be performed.

To my knowledge, this is the first report that menaquinone is identified as a target of antibiotics. The novel mechanism of kaikosin E makes it a potential drug candidate in treatment of drug-resistant bacteria.

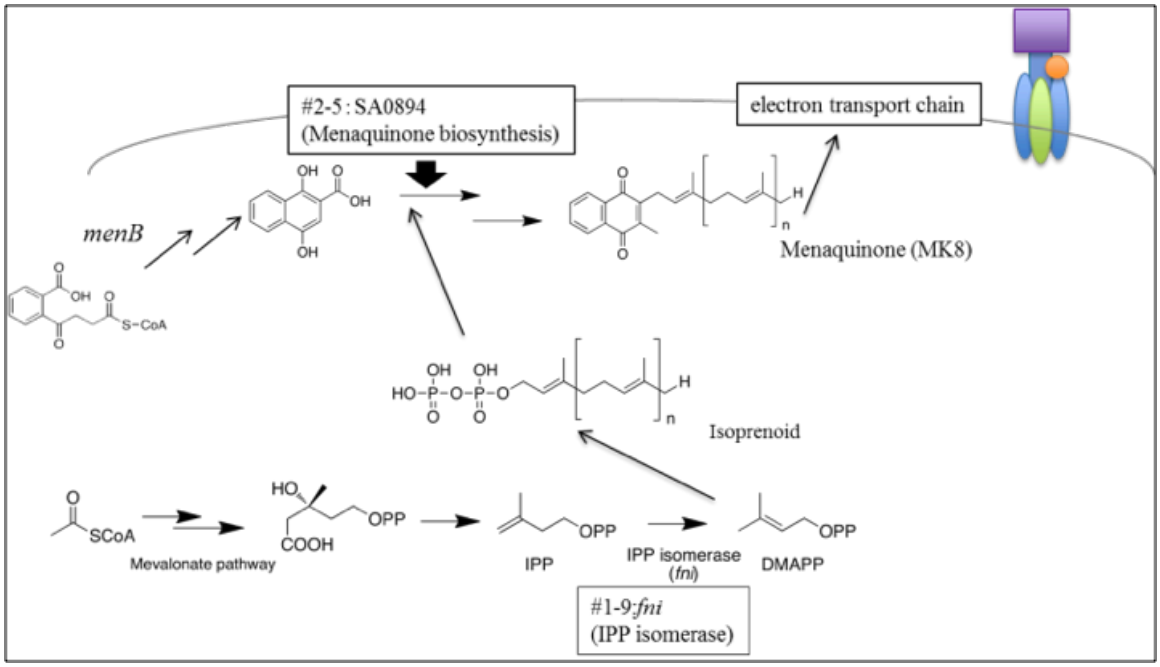

Figure 1-1. Involvement of *fni* and *menA* genes in menaquinone biosynthetic pathway.

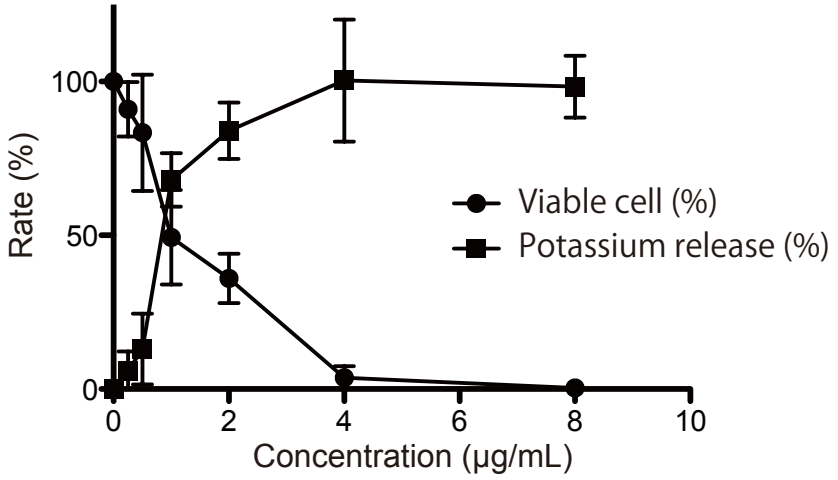

Figure 1-2. Potassium leakage assay.

Drug (μg/mL)		8	16	32	63	125
Hemolytic activity (%)	Kaikosin E	0	0	0	0.2	0.3
	Daptomycin	0.1	0	0	0	0.1

Table 1-1. Hemolytic activity assay. Antibiotics were incubated with sheep red blood cell for 1 hour at 37 ˚C. Treated with 1 % Triton X-100 as 100 % hemolytic.

Strain	Mutated gene	Relative amounts of menaquinone	MIC (μg/ml)
RN4220		1	3.1
#1-9	*fni*	0.4	6.3
#2-5	*menA*	0.5	6.3

Table 1-2. Amounts of menaquinone in *S. aureus*.

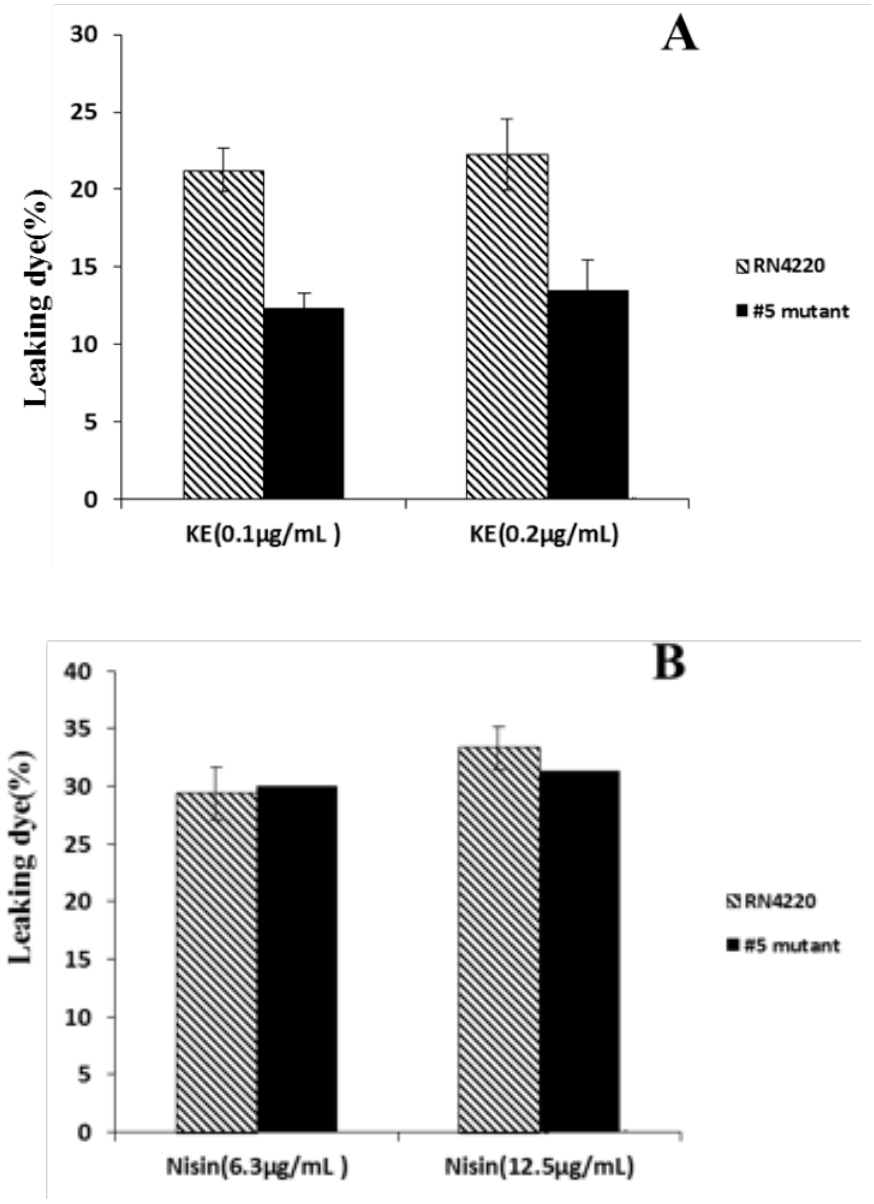

Figure 1-3. Dissipation of membrane potential in *S. aureus*. (A) Kaikosin E treatment. (B) Nisin as a positive control. Symbols: striped bars, RN4220; black bars, #5 mutant.

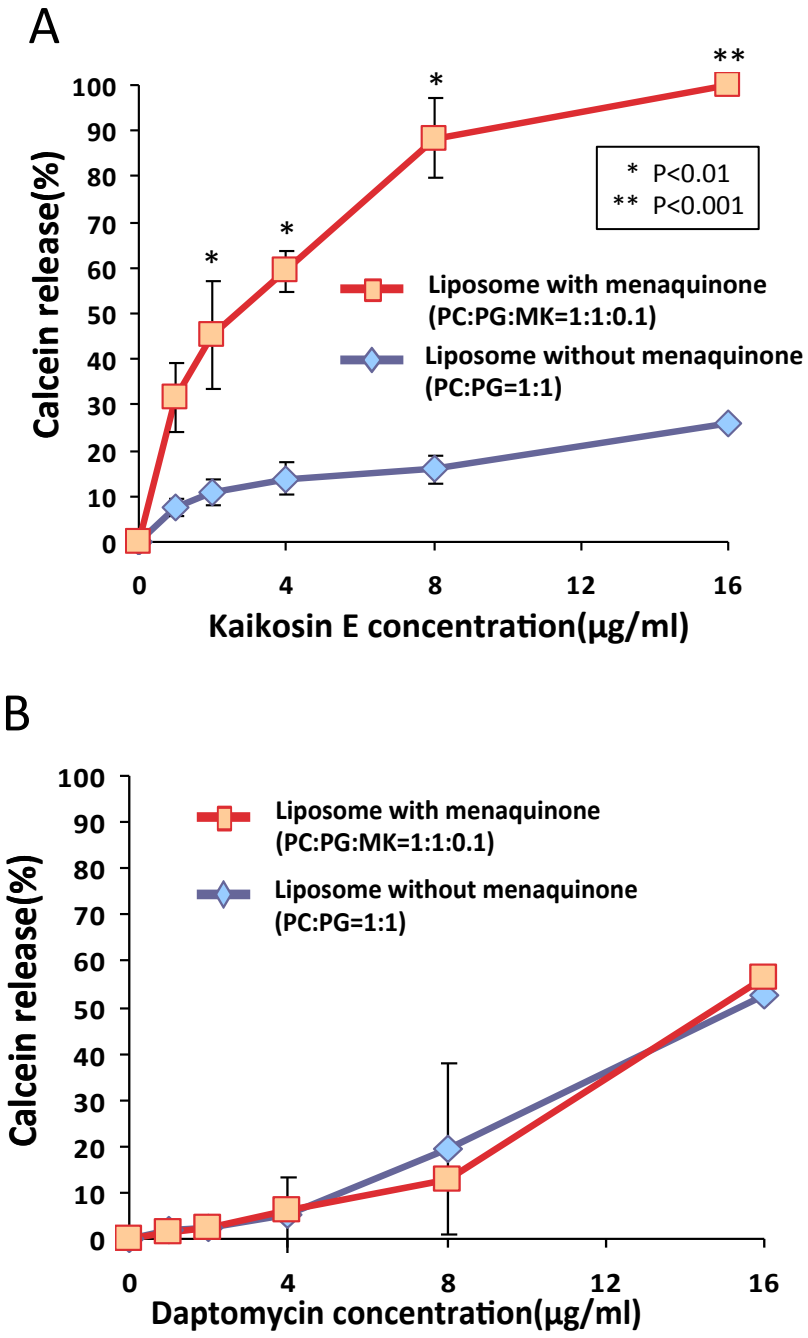

Figure 1-4. Liposome leakage assay. Values for 100 % calcein release were obtained using Triton X-100. (A) Kaikosin E treatment. (B) Daptomycin treatment. Symbols: squares, liposome with menaquinone; diamond, liposome without menaquinone.

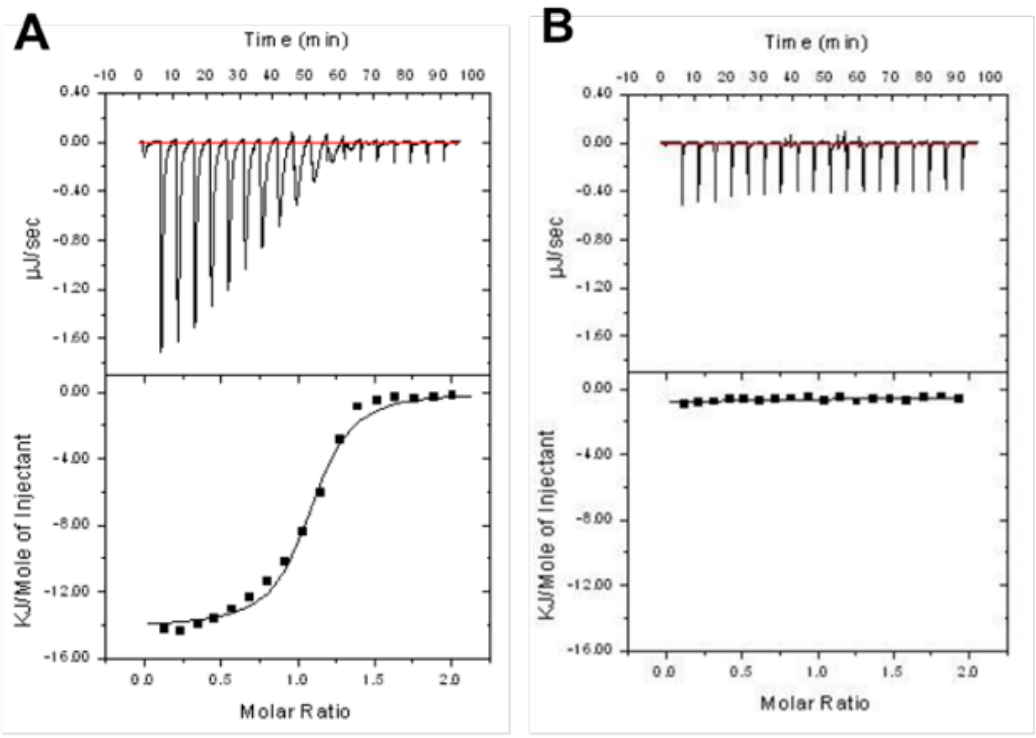

Figure 1-5. Raw ITC data (top panel) and binding isotherms for titration of kaikosin E (bottom panel) with (A) menaquinone (B) ubiquinone.

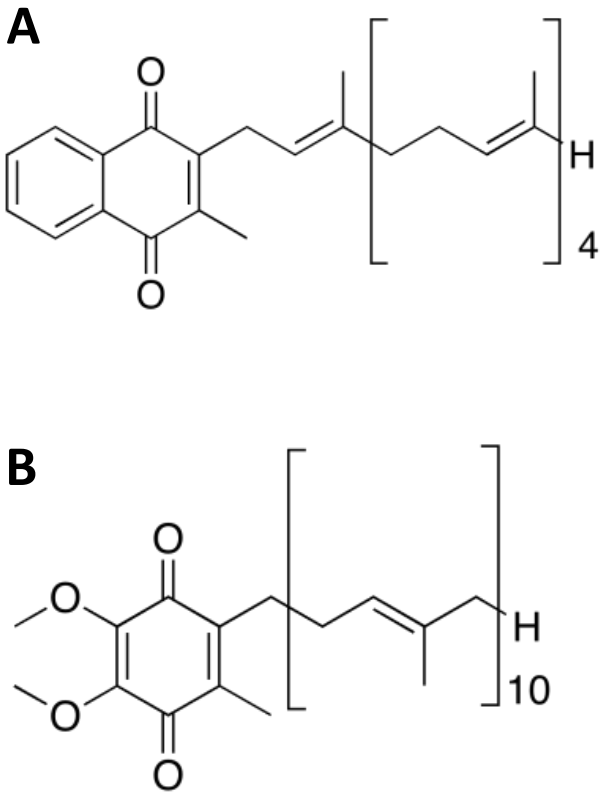

Figure 1-6. Structure of menaquinone and ubiquinone. (A) menaquinone, (B) ubiquinone.

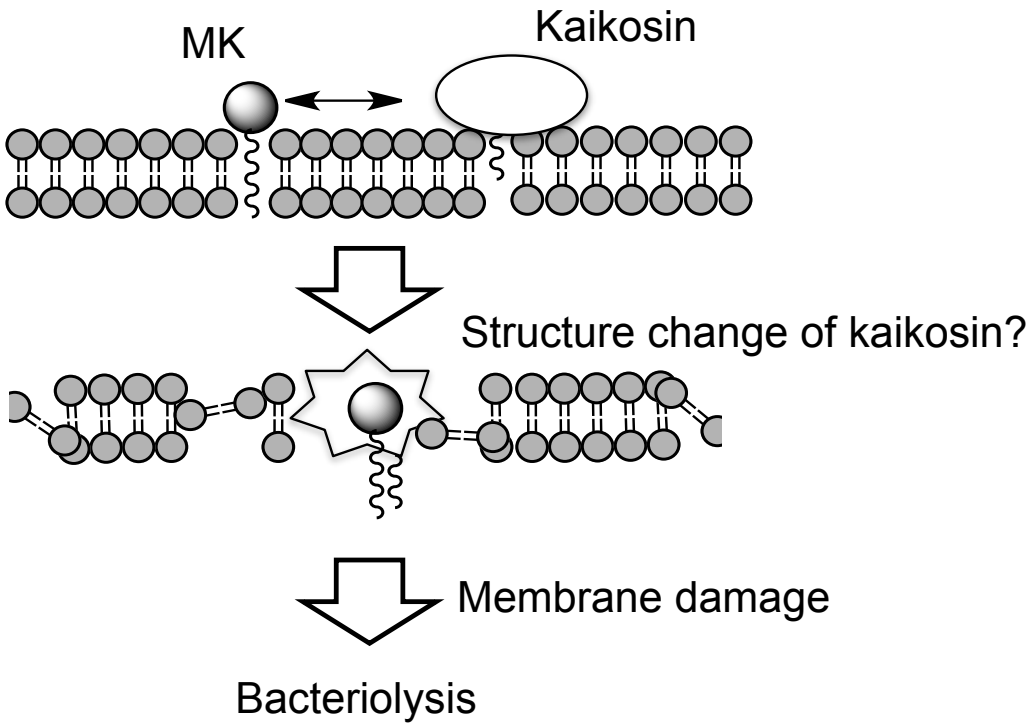

Figure 1-7. The proposed model of antimicrobial action for kaikosin E.

Chapter 2. Identification of a serum factor enhancing antimicrobial activity of kaikosin E

Background

Many antimicrobial drugs, effective against microorganisms *in vitro* study, are found ineffective in animal infection model *in vivo* study due to the protein binding in serum (Zeitlinger *et al.*, 2011). Therefore, the influence of serum on antimicrobial activity of antibiotics may provide the basis for evaluating the antimicrobial efficacy of antibiotics for their further clinical usage. A recent study showed the antimicrobial activity of kaikosin E was greatly enhanced by addition of serum. Previous study showed ED_{50} value of kaikosin E is lower than that of vancomycin, MIC value is higher than that of vancomycin, which means kaikosin E has good therapeutic effect compared with vancomycin in mouse infection model, whereas the antimicrobial activity of kaikosin E detected in culture medium is lower than that of vancomycin. This result suggests kaikosin E is more effective in animal body fluid such as serum than in culture medium. Therefore, the enhancement effect of serum on antimicrobial activity of kaikosin E explains the good therapeutic activity of kaikosin E.

Considering the significant enhancement effect of serum on antimicrobial activity of kaikosin E, I assume some factors in serum act as enhancers. Therefore, identification of these factors may facilitate knowing the interaction of kaikosin E with its target and provide information for improving the therapeutic effect of kaikosin E. In this study, I will identify the serum factors responsible for enhancing antimicrobial activity of kaikosin E.

2.1 Enhancement effect of serum on antimicrobial activity of kaikosin E

2.1.1 Effect of bovine calf serum on antimicrobial activity of kaikosin E

MIC of kaikosin E against different gram-positive bacteria was decreased more than 4-fold in the presence of 10 % bovine calf serum **(Table 2-1)**, which means the antimicrobial activity of kaikosin E was enhanced by addition of serum.

2.1.2 Effect of different species of serum on antimicrobial activity of kaikosin E

Antimicrobial activity of kaikosin E was greatly enhanced in the presence of human plasma or other mammalian serum. However, the antimicrobial activity of vancomycin or daptomycin was inhibited in the presence of human plasma, bovine serum, sheep serum and mouse serum **(Table2-2)**.

2.1.3 Effect of serum concentration on antimicrobial activity of kaikosin E

To determine whether serum-mediated enhancement effect on antimicrobial activity of kaikosin E is dose-dependent，MIC of kaikosin E against *S. aureus* was measured in the Mueller-Hinton broth (MHB) medium with serum from 0-20 %. MIC value of kaikosin E and serum concentration are displayed in **Figure 2-1**. Serum-mediated enhancement effect on antimicrobial activity of kaikosin E is dose-dependent.

2.2 Identification of apolipoprotein A-I as an enhancing factor in serum

2.2.1 Establishment of enhancing activity assay system

Enhancing activity measurement was modified from MIC assay. Full growth of *S.aureus* was adjusted to 10^4-10^5 CFU/ml with MHB medium and added into each well for 100 μl aliquots with 1 μg/ml kaikosin E in a 96-well microtiter plate. Then serum sample was added into the first well and serially diluted at 2-fold. The plate was incubated at 37 ˚C for 18-20 hr **(Figure 2-2)**. Enhancing activity was evaluated by visible inhibition of bacterial growth. One unit of activity is defined as the minimum amount of enhancing factor which can inhibit bacterial growth in the presence of 1 μg/ml kaikosin E. Total activity was calculated as followed:

$$\text{Total activity } (\text{U}) = 1\,\text{unit} \times \frac{\text{Sample volume } (\text{mL})}{\text{Minimum amount } (\text{mL})}$$

2.2.2 Purification of the enhancing factor from serum using ethanol extraction

As shown in **Table 2-3**, supernatant fraction from 50 % ethanol extraction showed 400 % recovery, whereas 25 % and 75 % ethanol extraction showed 50 % recovery. This result suggests that an optimal ethanol concentration is needed to achieve high recovery. Hence, I set ethanol concentration around 50 % in narrow range and tested their purification effects. As shown in **Table 2-4**, supernatant fraction from 60 % ethanol extraction showed 400 % recovery with highest specific activity. The 400 % recovery achieved by ethanol extraction may be due to the remove of the contaminants such as the inhibitors of serum enhancing factors. Hence, ethanol extraction is suitable for purification of the enhancing factor from serum. Next, column chromatography using organic solvent will be employed for further purification.

2.2.3 Purification of the enhancing factor by an ODS open column

The 60 % ethanol extract was further applied into ODS open column using step-wise elution method. As shown in **Table 2-5**, 75 % ethanol elute fraction from ODS open column showed 203 % recovery with more than 100-fold increase in specific activity compared with serum. Hence, ODS column can be used for purification of serum factors responsible for enhancing antimicrobial activity of kaikosin E.

2.2.4 Purification of the enhancing factor by HPLC

The 75 % ethanol elute fraction was further applied into HPLC using size exclusion column (SEC) for fractionation. Purification of the enhancing factor by HPLC using SEC was shown in **Table 2-6**. Fr.5 showed highest activity with 100 % recovery among active fractions. Moreover, the enhancing activity and protein amount of each fraction were plotted in HPLC profiles and shown in **Figure 2-3**. Total retention time is 10 minutes; each fraction was collected per 1 min and named as Fr.1 to Fr.10 in order. Striped bar represents activity of each fraction and a round dash line represents protein amount of each fraction. Active fractions were eluted from 3 min to 8 min with total recovery of 154 %. Fr.5 with retention time ranging from 4 to 5 min showed highest activity with 100 % recovery among active fractions. In view Fr.5 from HPLC showed similar specific activity with ODS fraction, the specific activity seemed saturated after ODS open column. The active peak pattern showed the maximum absorbance at wavelength of 280 nm, which is the absorbance for peptide measurement. Then I speculate that peptide is the enhancing factor in serum. Protease treatment can be employed for testing this possibility.

2.2.5 Effect of trypsin treatment on the enhancing activity in serum

In view of kaikosin E as a lipopeptide, effect of trypsin on antimicrobial activity of kaikosin E will be firstly determined. **Table 2-7** showed effect of trypsin on antimicrobial activity of kaikosin E. MIC of kaikosin E against *S. aureus* is same (6.3 μg/ml) with or without trypsin treatment. Therefore, trypsin treatment has no effect on antimicrobial activity of kaikosin E. Trypsin usually cleaves the peptide chain at the carboxyl side of amino acid lysine or arginine in L-form. Kaikosin E only contains D-form arginine without lysine. This explains the fact that antimicrobial activity of kaikosin E is not affected by trypsin. Hence, trypsin can be employed to test the role of serum protein in serum-mediated enhancement effect on antimicrobial activity of kaikosin E.

Effect of trypsin on enhancing activity of serum sample was shown in **Table 2-8**. Fraction from ODS column lost 97 % of enhancing activity after trypsin treatment. The above results suggest protein in the serum is responsible for enhancing antimicrobial activity of kaikosin E.

2.2.6 SDS-PAGE analysis of ODS column fractions

To further identify the active protein, fractions from ODS column were analyzed by SDS polyacrylamide gel electrophoresis. Fr.4 from ODS column with the highest specific activity showed major band with molecular weight of 24 kDa. This 24 kDa protein band was also observed in other active fractions and absent in non-active fractions **(Figure 2-4)**. This result suggested the 24 kDa protein is one candidate of

enhancing factor in serum.

2.2.7 SDS-tricine gel electrophoresis analysis of Size Exclusion Column (SEC) fractions

Figure 2-5 showed SDS-tricine gel electrophoresis analysis of SEC fractions. Fr.4, Fr.5 and fraction from ODS column with high enhancing activity all contained one band with molecular weight of 24 kDa, which was absent in other fractions with no detectable enhancing activity. The 24 kDa band intensity also correlated with its enhancing activity from the above 3 fractions. This result suggested that the 24 kDa protein is responsible for enhancing antimicrobial activity of kaikosin E.

2.2.8 Identification of 24 kDa protein by peptide mass fingerprinting

Peptide mass fingerprinting analysis revealed the 24 kDa protein was matched with the sequence of apolipoprotein A-I (ApoA-1) **(Figure 2-6)**. Apolipoprotein A-I is a single polypeptide and the major protein component of high-density lipoprotein (HDL) in plasma (Irshad *et al.*, 2005).

2.2.9 Effect of apolipoprotein A-I on antimicrobial activity of kaikosin E

Human recombinant apolipoprotein A-I showed enhanced specific activity compared with serum **(Table 2-9)**, whereas itself had no antimicrobial activity against *S. aureus*. These results suggested apolipoprotein A-I is one enhancing factor. Fr.5 from SEC as a semi-purified fraction showed higher specific activity compared with human recombinant apolipoprotein A-I. This result suggested that there are other enhancing factors existing in serum except apolipoprotein A-I.

MIC of kaikosin E in the presence of 90 μg/ml of human recombinant apolipoprotein A-I, which is equivalent to 10 % serum was 16-fold lower in MHB, even two-fold lower than in the presence of 10 % serum **(Table 2-10)**. It means that antimicrobial activity of kaikosin E is enhanced by addition of human recombinant apolipoprotein A-I. These results suggest apolipoprotein A-I is one serum factor responsible for enhancing antimicrobial activity of kaikosin E.

2.3 Conclusion

Antimicrobial activity of kaikosin E is enhanced in the presence of serum. The serum-mediated enhancement effect is also detected in human plasma and other mammalians. Antimicrobial activity of kaikosin E is dose-dependent with serum concentration. In searching of the enhancing factors in serum, apolipoprotein A-I was found to be responsible for enhancing antimicrobial activity of kaikosin E. This is the first report that the protein in serum enhances antimicrobial activity of antibiotics. These results suggest that the enhanced antimicrobial activity of kaikosin E *in vivo* is due to the apolipoprotein A-I in serum. Antimicrobial activity of kaikosin E is enhanced by addition of human apolipoprotein A-I, this finding provides an evidence for kaikosin E to be useful in future clinical application. In view of apolipoprotein A-I absent in skin and the potent antimicrobial activity of kaikosin E against *S. aureus* which usually caused skin infection, apolipoprotein A-1 is useful for combination with kaikosin E to enhance its therapeutic effect for treatment of skin infection caused by *S.aureus* as topical application.

Microorganism	MIC (μg/ml)		Reducing
	without serum	with 10 % serum	fold
S. aureus MSSA1	4	0.13	30
S. aureus MRSA4	4	0.25	16
S. aureus Smith ATCC 13709	2	0.25	8
S. aureus JCM2424	4	0.5	8
S.haemolyticus JCM2416	2	0.008	250
S.pseudintermedius JCM17571	4	0.13	30
Bacillus subtilis JCM2499	2	0.25	8
Bacillus cereus JCM20037	2	0.5	4

Table 2-1. The effect of serum on antimicrobial activity of kaikosin E against different microorganism. Antimicrobial activity of kaikosin E against different microorganism was determined with broth dilution method.

Medium	MIC (μg/ml)		
	Kaikosin E	**Daptomycin**	**Vancomycin**
Mueller-Hinton broth	2	0.5	0.5
+Human plasma	<0.1	1	1
+Bovine serum	<0.1	1	1
+Rabbit serum	<0.1	0.5	0.5
+Sheep serum	<0.1	1	1
+Mouse serum	<0.1	1	1

Table 2-2. Effect of different species of serum on antimicrobial activity of kaikosin E. MIC of antibiotics against MSSA1 was performed in MHB with 10% serum following the broth dilution assay.

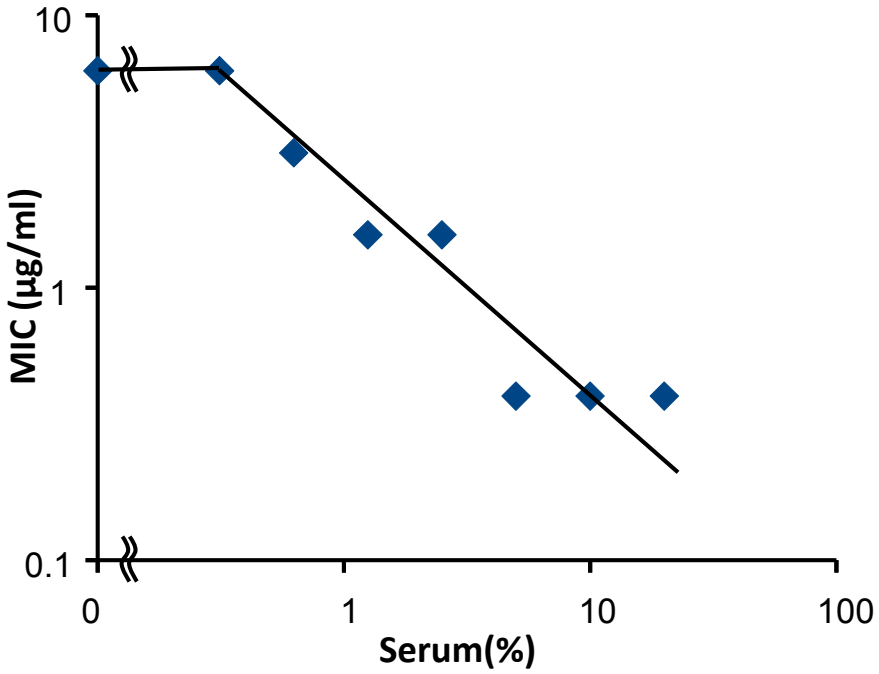

Figure 2-1. Effect of serum concentration on antimicrobial activity of kaikosin E. MIC of kaikosin E against *S. aureus* RN4220 was measured in the MHB medium with serum from 0-20 %.

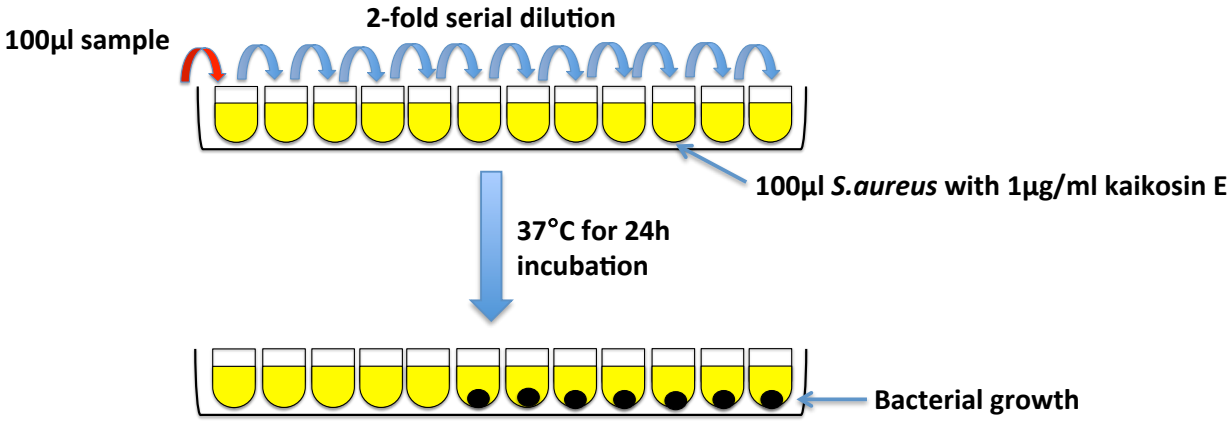

Figure 2-2. Assay system for serum-mediated enhancement effect on antimicrobial activity of kaikosin E.

Fraction	Total activity (Units)	Recovery (%)
Serum	640	100
25 % EtOH supernatant	<20	-
Pellet	320	50
50 % EtOH supernatant	2560	400
Pellet	<20	-
75 % EtOH supernatant	320	50
Pellet	<20	-
90 % EtOH supernatant	80	12.5
Pellet	<20	-

Table 2-3. Purification of a serum factor enhancing kaikosin E activity using ethanol extraction. Note: - means not detected.

Fraction	Weight (mg)	Total activity (Units)	Specific activity (U/mg)	Yield (%)
Serum	61	160	3	100
40 % EtOH supernatant	15	160	11	100
pellet	47	<20	–	–
50 % EtOH supernatant	9	640	71	400
pellet	69	<20	–	–
60 % EtOH supernatant	6	640	107	400
pellet	77	<20	–	–

Table 2-4. Identification of the optimal ethanol extraction method for purification of a serum factor enhancing kaikosin E activity. Note: - means not detected.

Fraction	Weight (mg)	Total activity (Units)	Specific activity (U/mg)	Yield (%)	Purification (fold)
I.Serum	96	640	7	100	1
II.60 % EtOH extract	18	2600	140	406	20
III.ODS flow through (Fr.1)	16	<20	-	-	-
25 % EtOH elute (Fr.2)	0.2	<20	-	-	-
50 % EtOH elute (Fr.3)	0.8	<20	-	-	-
75 % EtOH elute (Fr.4)	0.5	640	1300	100	190
100 % EtOH elute (Fr.5)	0.1	80	800	13	110

Table 2-5. Fractionation of a serum factor enhancing antimicrobial activity of kaikosin E using ODS open column. Note: - means not detected.

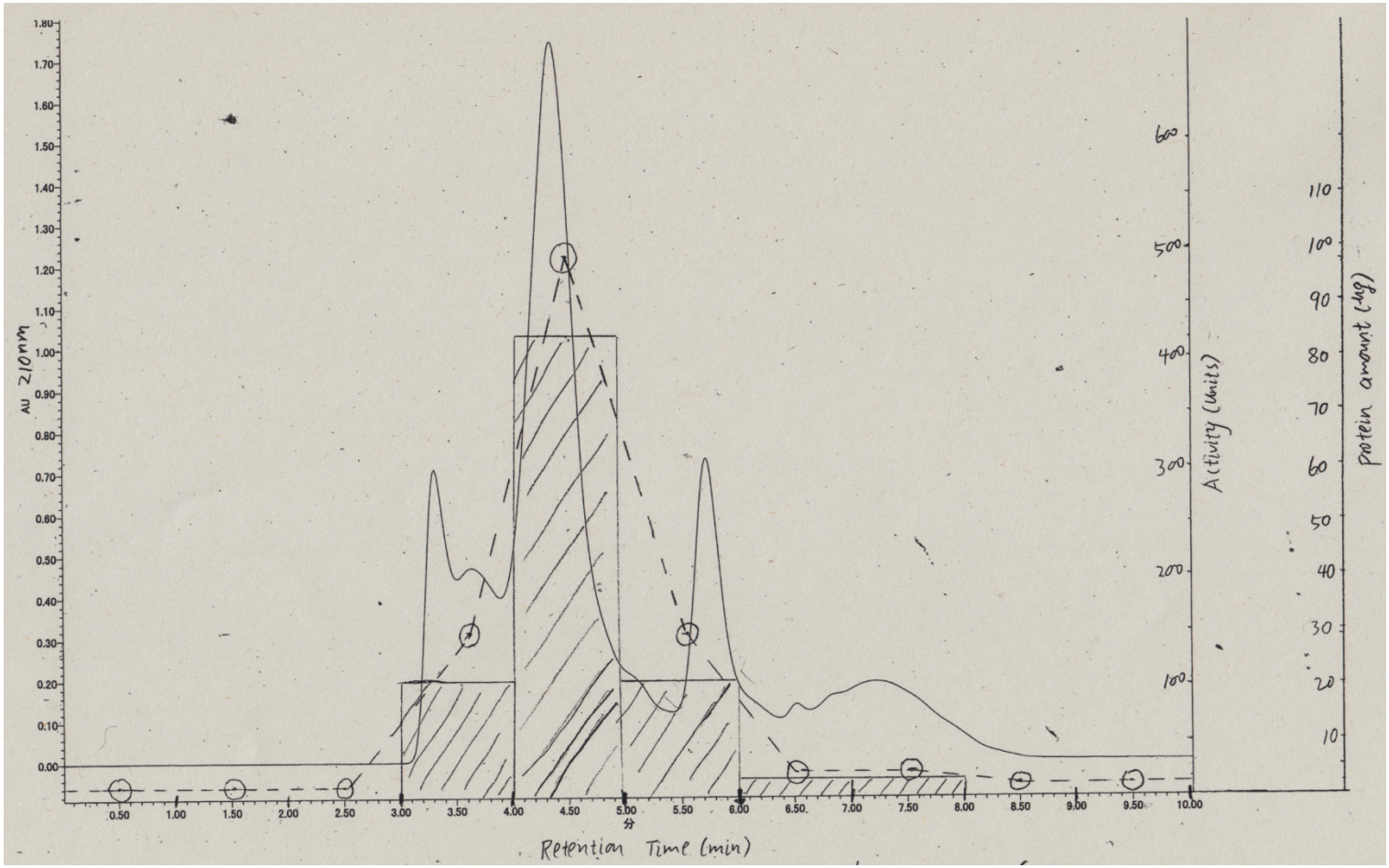

Figure 2-3. HPLC profiles of ODS column fraction using SEC.

Striped bar represents activity of each fraction. Round dash line represents mounts of each fraction.

Fraction	Protein amounts (μg)	Total activity (Units)	Specific activity ($X10^3U/\mu g$)	Yield (%)	Purification (fold)
I.Serum	60000	420	7	100	1
II.60%EtOH extract	650	1700	2600	405	370
III.ODS column	210	830	4000	198	570
IV.SEC chromatography Fr.1	2	<13	-	-	-
Fr.2	2	<13	-	-	-
Fr.3	2	<13	-	-	-
Fr.4	30	100	3300	24	470
Fr.5	100	420	4200	100	600
Fr.6	30	100	3300	24	470
Fr.7	5	13	2600	3	370
Fr.8	5	13	2600	3	370
Fr.9	2	<13	-	-	-
Fr.10	2	<13	-	-	-

Table 2-6. Purification of a serum factor by HPLC using size exclusion column.

ODS column: octadecyl silica; SEC: Size exclusion column. Each fraction from SEC was collected per 1 min and named as Fr.1 to Fr.10 in order.

Kaikosin E	MIC (μg/mL)
No treatment	6.3
Trypsin treatment	6.3

Table2-7. Effect of trypsin on antimicrobial activity of kaikosin E. Kaikosin E was treated with 2.5 % trypsin and incubated at 37 °C for 30 min. MIC of kaikosin E was determined by broth dilution method.

Sample	Enhancing activity (Units)	Ratio (%)
No treatment	64	100
Trypsin treatment	2	3

Table 2-8. Effect of trypsin on enhancing activity of serum sample. Semi-purified sample (Fr.4 from ODS open column) was treated with 2.5 % trypsin and incubated at 37 °C for 30 min. Then serum sample was applied into enhancing activity assay.

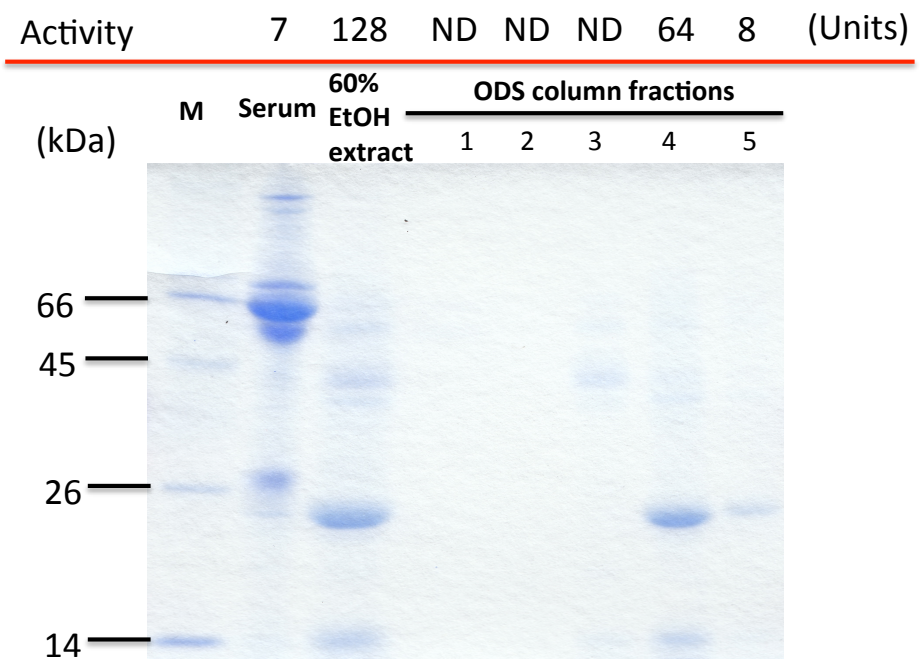

Figure 2-4. SDS-PAGE analysis on fractions from ODS column. Loading activity of each fraction was shown in the upper red line of the gel. ND: Not detected.

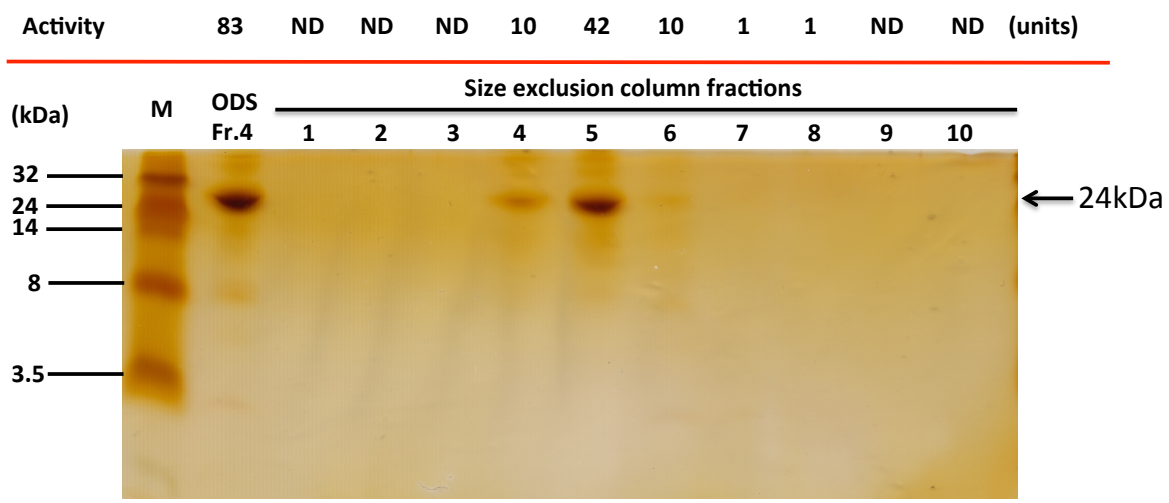

Figure 2-5. SDS-tricine gel electrophoresis of fractions from size exclusion column. Loading activity of each fraction was shown in the upper red line of the gel. ND: Not detected.

Apolipoprotein A-I, apoA-1 [Bos=cattle, Peptide, 247 aa]

Protein sequence coverage: 63%
Matched peptides shown in ***bold red***.

1 RHFWQQDDPQ SSWDR**VKDFA TVYVEAIKDS GRDYVAQFEA SALGK**QLNLK
51 **LLDNWDTLAS TLSKVREQLG PVTQEFWDNL EKETASLR**QE MHKDLEEVKQ
101 K**VQPYLDEFQ KKWHEEVEIY RQKVAPLGEE FREGAR**QK**VQ ELQDKLSPLA**
151 **QELR**DRAR**AH VETLRQQLAP YSDDLR**QRLT ARLEALKEGG GSLAEYHAKA
201 SEQLKALGEK **AKPVLEDLRQ GLLPVLESLK** VSILAAIDEA SKKLNAQ

Figure 2-6. Peptide mass fingerprinting analysis of the 24 kDa protein.

Fraction	Specific activity (U/mg)
Serum	7
Fr.5 from size exclusion column	4300
Human recombinant apolipoprotein A-I	1100

Table 2-9. Enhancing activity of apolipoprotein A-I.

Medium	MIC (μg/ml) of kaikosin E
Mueller-Hinton broth	4
+ Serum (10 %)	0.25
+ Human recombinant Apo A-I (90 μg/ml)	0.13

Table 2-10. Effect of apolipoprotein A-I on antimicrobial activity of kaikosin E. MIC of kaikosin E in the presence of 90 μg/ml of human recombinant apolipoprotein A-I (human recombinant Apo A-I), which is equivalent to 10 % serum.

Chapter 3. Interaction of apolipoprotein A-I with the binding complex of menaquinone and kaikosin E

Background

Apolipoprotein A-I, the major protein constituent of high-density lipoprotein (HDL) in plasma, was identified as a serum factor responsible for enhancing antimicrobial activity of kaikosin E. One proposed mechanism of action for kaikosin E is that kaikosin E binds with menaquinone in cell membrane and induces membrane damage. However, the enhancing mechanism of apolipoprotein A-I on antimicrobial activity of kaikosin E remains unclear. Previous study showed the amounts of menaquinone in *S. aureus* correlates with bacterial susceptibility to kaikosin E. Hence, one hypothesis is that apolipoprotein A-I stimulates menaquinone production of *S. aureus* and facilitates kaikosin E to disrupt membrane through binding with large amounts of menaquinone. To test this hypothesis, the amounts of menaquinone from *S. aureus* with and without addition of serum should be determined.

3.1 Amounts of menaquinone in *S. aureus* growing in the presence of serum

Menaquinone was extracted from *S. aureus* using quinone extraction method. The relative amounts of menaquinone in wild type strain RN4220 were defined as 1. The menaquinone amounts of RN4220 in the presence of 10 % serum or 10 % ODS column fraction is 1 and 0.8 respectively **(Table 3-1)**. Therefore, the addition of serum had no effect on the menaquinone production of *S. aureus*. These results suggested serum-mediated enhancement effect on antimicrobial activity on kaikosin E is not through stimulating menaquinone production of *S. aureus*. Hence, this result overthrew the hypothesis that apolipoprotein A-I enhances antimicrobial activity of kaikosin E through stimulating menaquinone amounts in *S. aureus*.

Menaquinone was reported to interact with high-density lipoprotein (Schurgers *et al.*, 2002). Apolipoprotein A-I is known as the major component of high-density lipoprotein in plasma. Hence, I proposed the second hypothesis that apolipoprotein A-I enhances antimicrobial activity of kaikosin E through interaction with menaquinone and kaikosin E. To test this hypothesis, interaction assay between apolipoprotein A-I, menaquinone and kaikosin E will be performed.

3.2 Interaction assay between serum, menaquinone and kaikosin E

To determine the interaction between serum, menaquinone and kaikosin E, different combination group among serum, ODS column fraction, menaquinone and kaikosin E was prepared. After mixing each combination group and centrifugation, there is no precipitate observed in the combination groups from menaquinone with serum or ODS column fraction and kaikosin E with serum or ODS column fraction. And kaikosin E, menaquinone, serum or ODS fraction solution alone had no pellet **(Table 3-2)**. Mixing solution from kaikosin E and menaquinone showed yellowed pellet in the bottom of the tube. When mixing 10 % ODS fraction, kaikosin E and menaquinone together and centrifugation, orange precipitate was shown in the bottom of the tube **(Figure 3-1)**. This orange precipitate was also observed in the mixing solution of 10 % serum, menaquinone and kaikosin E. Therefore, the addition of serum or ODS column fraction changes color of binding complex of kaikosin E and menaquinone. This result suggested that apolipoprotein A-I in serum may interact with the binding complex of kaikosin E and menaquinone.

3.3 Interaction of apolipoprotein A-I with the complex of kaikosin E and menaquinone

3.3.1 Aggregation of apolipoprotein A-I, kaikosin E and menaquinone

After mixing kaikosin E and menaquinone at a molar ratio of 1:1 and centrifugation, yellow pellet was shown in the bottom of the tube, whereas kaikosin E or menaquinone alone had no pellet. When mixing apolipoprotein A-I, kaikosin E and menaquinone together and centrifugation, orange precipitate was shown in the bottom of the tube **(Figure 3-2)**. Apolipoprotein A-I solution alone was transparent and had no pellet. Therefore, the addition of apolipoprotein A-I changes color of binding complex of kaikosin E and menaquinone. This result suggested that apolipoprotein A-I may interact with the binding complex of kaikosin E and menaquinone.

3.3.2 The existence of apolipoprotein A-I in the precipitate complex

To test whether apolipoprotein A-I interact with the binding complex of kaikosin E with menaquinone, precipitate from mixing apolipoprotein A-I, kaikosin E with menaquinone solution was applied into SDS-PAGE analysis. Apolipoprotein A-I band was detected in the precipitate sample from mixing apolipoprotein A-I, kaikosin E and menaquinone solution **(Figure 3-3)**. Therefore, apolipoprotein A-I interacted with kaikosin E and menaquinone.

3.4 Conclusion

The addition of serum had no effect on the menaquinone production of *S.aureus.* This

finding excludes the possibility that serum enhances antimicrobial activity of kaikosin E by stimulating menaquinone production of *S. aureus*. The addition of apolipoprotein A-I changes color of binding complex of kaikosin E and menaquinone. Apolipoprotein A-I was detected in the precipitate sample from mixing apolipoprotein A-I, kaikosin E with menaquinone solution. These results suggested apolipoprotein A-I interacts with kaikosin E and menaquinone. Apolipoprotein A-I consists of a series of amphipathic helices that are functionally important for protein-lipid interactions as well as protein-protein interactions (Oda *et al.*, 2001). This structural character of apolipoprotein A-I is also reasonable to explain its interaction with kaikosin E and menaquinone. Hence, apolipoprotein A-I may enhance the antimicrobial activity of kaikosin E through interaction with the binding complex of kaikosin E and menaquinone.

Medium	Relative amounts of menaquinone	MIC (μg/ml)
Mueller-Hinton broth	1	8
+10 % serum	1	0.5
+10 % Fr.4 from ODS column	0.8	0.25

Table 3-1. Amounts of menaquinone in *S. aureus* RN4220 growing in the presence of 10 % serum.

Sample	Precipitate (Yes/No)	Precipitate color
Kaikosin E	No	-
Menaquinone	No	-
Serum	No	-
ODS fraction	No	-
Kaikosin E + Menaquinone	Yes	Yellow
Kaikosin E + Serum	No	-
Kaikosin E + ODS fraction	No	-
Menaquinone + Serum	No	-
Menaquinone + ODS fraction	No	-
Kaikosin E + Menaquinone + Serum	Yes	Orange
Kaikosin E + Menaquinone + ODS fraction	Yes	Orange

Table 3-2. Interaction assay between serum, menaquinone and kaikosin E. Each combination group was mixed by vortex and centrifuged at 15 krpm for 5 min. The individual concentration of each sample is as followed: Kaikosin E, 50 μg/ml; Menaquinone, 12.5 μg/ml; ODS fraction, 10 %; Serum, 10 %.

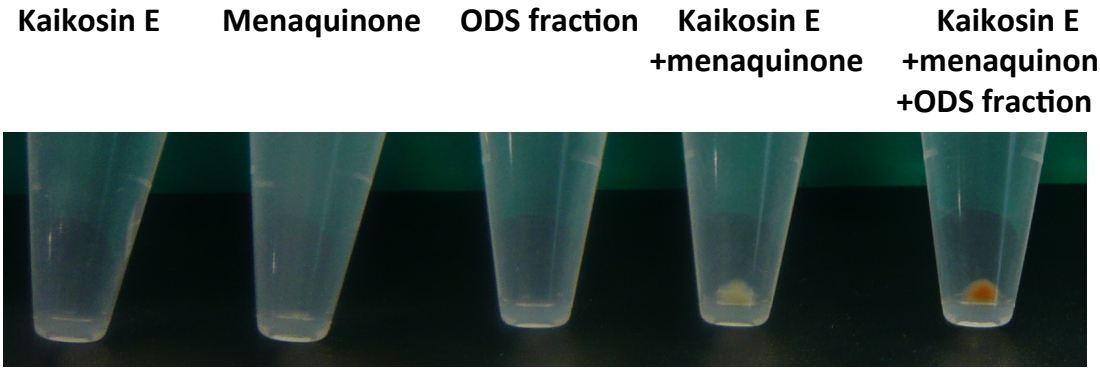

Figure 3-1. Aggregation assay between ODS fraction, menaquinone and kaikosin E. Each combination group was mixed by vortex and centrifuged at 15 krpm for 5 min. The individual concentration of each sample is as followed: kaikosin E, 50 μg/ml; menaquinone, 12.5 μg/ml; ODS fraction, 10 %.

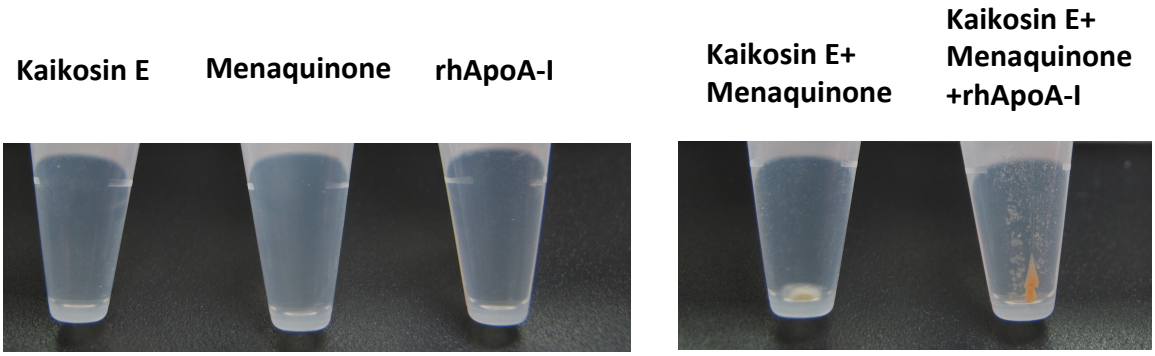

Figure 3-2. Aggregation of rhApo A-1, kaikosin E and menaquinone. rhApo A-I: human recombinant apolipoprotein A-I.

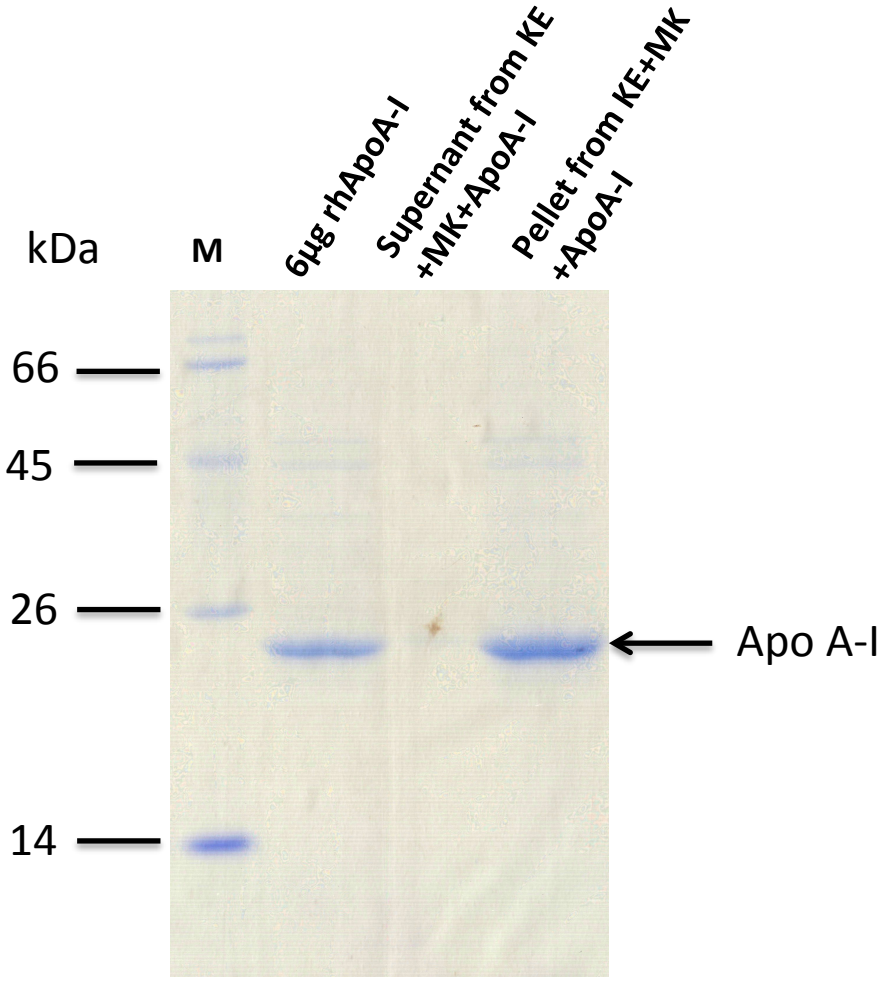

Figure 3-3. SDS-PAGE analysis on the existence of Apo A-1 in the precipitate from mixing Apo A-I, kaikosin E and menaquinone solution. Apo A-I: apolipoprotein A-I.

Materials and methods

1. Bacterial culture condition

Cation-adjusted Mueller-Hinton broth (Ca^{2+}: 25 mg/L, Mg^{2+}: 12.5 mg/L) is used for minimum inhibitory concentration (MIC) assay of antibiotics except for testing daptomycin whose broth needs to be supplemented with 50 mg/L of Ca^{2+}. Bacteria were incubated at 37 ˚C except kaikosin E-resistant mutants were incubated at 30 ˚C.

2. Chemicals and reagents

Daptomycin was purchased from Sequoia Research Products (UK). DiS-C_3 (5) was purchased from AnaSpec Inc. Bovine calf serum was obtained from SAFC Biosciences (Lot No.11A216). Sep-Pak C18 open column was purchased from Waters (Ireland). Trypsin was supplied by Invitrogen Life Science Technologies. Coomassie Plus-The Better Bradford Assay Reagent was obtained from Thermo Scientific. Sil-best stain was purchased from Nacalai Tesque (Japan). Human recombinant apolipoprotein A-I (rhApoA-I) was supplied by Wako. Kaleidoscope Polypeptide Standards marker was purchased from BIO-RAD.

3. Potassium leakage assay

S. aureus FDA 209P was used for cell viability measurement and potassium leakage assay. Bacterial culture was prepared as previously (Katsu *et al.*, 1989). Washed cells were resuspended in buffer (100 mM choline chloride and 50 mM 4-morpholinepropanesulfonic acid (Mops)/2-amino-2-hydroxy-methypropane-1,3-diol (Tris), pH 7.2) at 2×10^9 CFU/ml and incubated with the antibiotic at 37 ˚C for 30 min. Cell viability was determined by enumeration of CFU/ml. The amounts of K^+ leakage

were measured with a K^+-sensitive electrode as previously described (Katsu *et al.*, 1989 and Komagoe *et al.*, 2011). The total amounts of K^+ were determined by 10 μM melittin-mediated disruption of the cytoplasmic membrane of cells.

4. Hemolytic activity assay

Antibiotics were incubated with sheep red blood cell for 1 hour at 37 ˚C. Treated with 1 % Triton X-100 as 100 % hemolytic.

5. Determination of antimicrobial activity

MIC determination was performed in MHB supplemented with calcium following the broth microdilution assay guideline set by CLSI. Mueller Hinton broth was supplemented with 50 mg/L Ca^{2+} for daptomycin test.

6. Measurement of menaquinone amounts in *S. aureus*

S. aureus RN4220, *fni* mutant or *menA* mutant was incubated at 30 ˚C with shaking overnight. Then menaquinone from *S. aureus* was isolated following the standard quinone extraction method. Bacterial culture was centrifuged at 8 krpm for 1 min and pellet was collected. The collected pellet was resuspended in 1 ml PBS, 250 μl 5×buffer (25 mM EDTA, 5 μM α-Tochopherol in PBS) and 6 ml Hexane/EtOH (5:2) solution. The suspended solution was mixed by vortex and centrifuged at 4000×g for 3 min at room temperature. The hexane layer was transferred into a new tube, rinsed with 1 ml hexane and dry up with evaporation. The dry sample was suspended with ethanol and filtered with 0.22 μm PVDP filter. The extracted menaquinone was analyzed by reverse-phase high-performance liquid chromatography (HPLC) senshu-

pak C18 (column 4.6×250 mm, Ex: 320 nm, Em: 430 nm) after reduction of sample by a platinum column (Reduction column, 10×4.6 mm, Toa, Tokyo) and eluted by Diisopropyl ether: Methanol (1:4) at a flow rate of 1.0 ml/min. MK8 from *E.coli* was used as the standard sample.

For measurement of menaquinone amounts in *S. aureus* growing in the presence of serum, RN4220 was incubated at 37 ˚C overnight with 10 % serum or 10 % ODS column fraction. Other steps are same as measurement of menaquinone amounts in *S.aureus*.

7. Dissipation of membrane potential

Dissipation of membrane potential was determined by using a fluorescent assay with a membrane potential sensitive dye DiS-C_3 (5) as described previously (Wu *et al.*, 1999). Full growth of *S. aureus* (RN4220 and #5 *menA* mutant) was diluted 1 in 100 with MHBc and subcultured with shaking at 37 °C to an exponential phase (OD_{600}, 0.5 to 0.6). The cells were centrifuged and washed with buffer (5 mM HEPES, pH 7.2, 5 mM Glucose) once. Then the pellet was resuspened in the same buffer to an OD_{600} of 0.05. Bacterial suspensions were transferred to a cuvette containing a stir bar and placed in a heated chamber of a FP-6200 spectrofluorometer (JASCO) for 30 seconds to measure background. Then 0.4 μM DiS-C_3 (5), nisin and kaikosin E were added in order and monitored at indicated time point. The excitation and emission wavelengths were 622 nm and 670 nm, respectively. The pore-forming antimicrobial nisin (1×, 2×MIC) was used as positive control.

8. Liposome leakage assay

Calcein-encapsulated unilamber liposome was prepared as previously described (Zhang *et al.*, 2001). Equimolar mixure of phosphatidylcholine (PC) and phosphatidylglycerol (PG) was dissolved in chloroform with or without menaquinone (5 mM) and was dried under evaporation. The lipid film was rehydrated with 5 mM sodium HEPES, pH 7.5, containing 25 mM calcein and freeze-thawed by liquid N_2 for 5 cycles. The liposome suspension was passed through a Sephadex G-50 column and eluted with buffer (20 mM sodium HEPES, 150 mM NaCl, 1 mM sodium EDTA, pH 7.5) to remove the free calcein. The calcein-encapsulated PC/PG (1:1) liposomes were applied onto spectrofluorometer for calcein release assay. Ten-fold dilution of liposome (2 ml) was transferred into cuvette with a stir bar and measured for 2 min to establish a baseline. Fluorescence triggered by test compounds was detected for additional 5 min before addition of 0.1 % Triton X-100. The value collected by Triton X-100 was as 100 % calcein release.

9. Binding assay between kaikosin E and menaquinone

Kaikosin E-menaquinone interaction was measured with isothermal titration calorimetry (ITC) instruments. Calorimetric titrations were carried out at 25 °C, by repeatedly injecting 2 µl aliquots of 3.1 mM menaquinone dissolved in 5 % DMSO into 0.28 mM kaikosin E dissolved in 5 % DMSO solution every 300s intervals, with stirring. Ubiquinone was used as control. ITC measures heat released or absorbed upon binding and provides the value of reaction molar ratio (n).

10. The effect of serum on antimicrobial activity of kaikosin E

Serum was treated with heat inactivation (at 56 ˚C for 30 min) and used for MIC assay. MIC determination was performed in MHB with 10 % prepared serum sample following the broth microdilution assay.

11. Enhancing activity assay

Enhancing activity measurement was modified from MIC assay. Full growth of *S.aureus* was adjusted to 10^4-10^5 CFU/ml with MHB and added into each well for 100 μl aliquots with 1 μg/ml kaikosin E in a 96-well microtiter plate. Then serum sample was added into the first well and serially diluted at 2-fold. The plate was incubated at 37 ˚C for 18-20 hr. Enhancing activity was evaluated by visible inhibition of bacterial growth. One unit of activity was defined as the minimum amount of enhancing factor which can inhibit bacterial growth in the presence of 1 μg/ml kaikosin E. Total activity was calculated as followed:

$$\text{Total activity}\ (\text{U}) = 1\text{unit} \times \frac{\text{Sample volume}\ (\text{mL})}{\text{Minimum amount}\ (\text{mL})}$$

12. EtOH extraction

After heat inactivation (at 56 ˚C for 30 min), bovine calf serum was used for each assay. Serum was mixed with different concentrations of ethanol and centrifuged at 15 krmp for 14 min at 4 ˚C. The supernatant and pellet were dried with a centrifugal evaporator and resuspended in water respectively for the enhancing activity assay.

13. Purification of the enhancing factor using 60 % EtOH extraction

Bovine calf serum was mixed with ethanol at a ratio of 2:3 by vortex and centrifuged at 15 krmp for 14 min at 4 ˚C. The supernatant was collected and dried up with evaporation. The dry sample was resuspended in water, equal with same volume of serum, and centrifuged at 15 krmp for 14 min at 4 ˚C. The supernatant was collected, evaporated and resuspended in water followed by filtering with 0.22 μm filter. The 60 % EtOH extract fraction was stored at 4 ˚C for usage.

14. ODS open column chromatography

The 60 % ethanol extract was applied into octadecyl silica (ODS) open column at a ratio of 1:2 (loading volume: bed volume) and eluted by 0 %, 25 %, 50 %, 75 % and 100 % ethanol with 0.1 % formic acid in step-wise manner. Each fraction was dried for weight measurement and then resuspended in water for enhancing activity assay.

15. Size exclusion column

Fraction from ODS open column was further applied into size exclusion column (Inertsil HPLC column WP300 Diol, 4.6 × 250 mm) and elute by methanol with 0.1 % TFA at a flow rate of 0.5 ml/min. Each fraction was collected per 1min and used for enhancing activity assay. Column was kept at 40 ˚C.

16. Trypsin treatment

Trypsin was used for digestion of protein. Effect of trypsin on antimicrobial activity of kaikosin E against *S. aureus* was determined by MIC assay following broth

microdilution method. Kaikosin E was treated with 2.5 % trypsin and incubated at 37 °C for 30 min. MIC assay of kaikosin E with or without trypsin was performed. Semi-purified sample (fraction from ODS open column) was treated with 2.5 % trypsin and incubated at 37 °C for 30 min. Then serum sample was applied into enhancing activity assay.

17. Measurement of protein amounts

Protein concentration of serum samples was determined with Bradford assay. Fifty microliter dilute protein sample was mixed with 500 µl Bradford reagent and incubated at 37 °C for 10 min. Absorbance of the mixture was read within 30 min at 595 nm using spectrophotometer. Protein concentration was calculated using absorbance reading data from standard curve of known BSA (Bovine serum albumin) solution with 0-100 µg/ml protein concentration range.

18. SDS-PAGE analysis

Sodium dodecyl sulfate (SDS) - polyacylamide gel electrophoresis was used to various protein from serum sample. The molecular weight of standard marker ranges from 14 to 66 kDa. Serum samples are run on 15 % SDS-polyacylamide gel. Electrophoresis condition: 100 v, 120 min. CBB dye was employed for gel staining.

19. SDS-Tricine gel electrophoresis

Fractions from size exclusion column using HPLC were applied onto 16.5 % sodium dodecyl sulfate (SDS)-Tricine gel electrophoresis. The molecular weight of standard marker (Kaleidoscope Polypeptide Standards, BIO-RAD) ranges from 3.5 to 32 kDa.

Electrophoresis condition: 100 v, 180 min. Silver dye was employed for gel staining.

20. Peptide mass fingerprinting analysis of 24 kDa protein

The 75 % EtOH elute fraction from ODS open column was applied into SDS-polyacrylamide gel electrophoresis. The 24 kDa protein band was cut from the above gel and used for peptide mass fingerprinting analysis.

21. Aggregation assay among serum, kaikosin E and menaquinone

Kaikosin E (50 μg/ml), menaquinone (12.5 μg/ml), 10 % serum and 10 % ODS column fraction were mixed alone or in different combination method and centrifuged at 15 krpm for 5 min at room temperature.

22. Interaction assay of apoA-I, kaikosin E and menaquinone

Kaikosin E (50 μg/ml) and menaquinone (12.5 μg/ml) were mixed with or without human recombinant apolipoprotein A-I (15 μg/ml) and centrifuged at 15 krpm for 5 min at room temperature. Kaikosin E (50 μg/ml), menaquinone (12.5 μg/ml) or human recombinant apolipoprotein A-I (15 μg/ml) alone was used as control. Supernatant and pellet were separately collected and the pellet was rinsed with 1 ml water. Then supernatant and pellet were evaporated and suspended with 1×SDS electrode buffer. The prepared sample was applied into SDS-PAGE analysis.

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