

# Vitronectin and its fragments purified as serum inhibitors of *Staphylococcus aureus* $\gamma$ -hemolysin and leukocidin, and their specific binding to the Hlg2 and the LukS components of the toxins

Harue Katsumi, Toshio Tomita, Jun Kaneko, Yoshiyuki Kamio\*

Department of Molecular and Cellular Biology, Graduate School of Agricultural Science, Tohoku University, Aoba-ku, Sendai 981-8555, Japan

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**Abstract** Staphylococcal  $\gamma$ -hemolysin and leukocidin are bi-component cytotoxins, consisting of LukF (or Hlg1)/Hlg2 and LukF/LukS, respectively. Here, we purified serum inhibitors of  $\gamma$ -hemolysin and leukocidin from human plasma. Protein sequencing showed that the purified inhibitors of 62, 57, 50 and 38 kDa were the vitronectin fragments with truncation(s) of the C-terminal or both N- and C-terminal regions. The purified vitronectin fragments specifically bound to the Hlg2 component of  $\gamma$ -hemolysin and the LukS component of leukocidin to form high-molecular-weight complexes with them, leading to inhibition of the toxin-induced lysis of human erythrocytes and human polymorphonuclear leukocytes, respectively. Intact vitronectin also showed inhibitory activity to the toxins. The ability of  $\gamma$ -hemolysin and leukocidin to bind vitronectin and its fragments is a novel function of the pore-forming cytotoxins.

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**Key words:** Staphylococcal  $\gamma$ -hemolysin; Staphylococcal leukocidin; Pore-forming toxin; Serum inhibitor; Vitronectin

## 1. Introduction

*Staphylococcus aureus* is an important pathogen in wound and soft tissue infections, and its nosocomial transmission through asymptomatic carriers and artificial implants is a serious problem. However, the pathophysiology of staphylococcal infections is still enigmatic, partly because of the ability of the bacterium to produce a variety of putative virulence factors including cytotoxins. Staphylococcal  $\gamma$ -hemolysin (Hlg) and leukocidin (Luk) have been isolated as bi-component cytotoxins from the culture fluids of *S. aureus* [1–6], and they are produced by most clinical isolates of the bacterium [7]. Staphylococcal Hlg consists of Hlg1 and Hlg2, which cooperatively lyse human and rabbit erythrocytes [1,5]. Staphylococcal Luk comprises LukS and LukF, and it exhibits leukocytolytic activity towards human and rabbit polymorphonuclear leukocytes [1–4]. Previous studies by us and the other groups showed that Hlg1 is identical with LukF, and that Hlg2 and

LukS are the determinants for the cell specificities of Luk and Hlg although these components have 72% identity in their amino acid sequences [6,8–10].

Washed erythrocytes were used, instead of defibrinized blood, for selection of Hlg-producing colonies of *S. aureus*, because clear zone formation by Hlg-producing *S. aureus* was retarded or inhibited on the plates containing defibrinized blood [5]. In fact, we observed that a Hlg producer, *S. aureus* Smith 5R, showed a marked delay in clear zone formation on blood agar plates with 5% (v/v) defibrinized blood from human or rabbit. In our preliminary experiments, human serum inhibited Hlg activity in a dose-dependent manner, and the inhibitory activity of human serum towards Hlg was significantly reduced when the serum was heated at 100°C for 10 min or treated with proteinase K (5 mg/ml) at 37°C for 1 h. Furthermore, half of the inhibitory activity was recovered in the non- $\gamma$ -globulin fraction, when human serum was fractionated by ammonium sulfate precipitation. These results suggested that proteinaceous Hlg inhibitor(s) other than natural antibodies against Hlg are present in human serum.

In this study, we purified inhibitors of Hlg and Luk from human plasma, and identified them as vitronectin fragments. The purified inhibitors specifically bound to the Hlg2 component of Hlg and the LukS component of Luk, forming high-molecular-weight complexes with these toxin components. The ability of Hlg and Luk to bind vitronectin and its fragments is a novel function of these pore-forming toxins.

## 2. Materials and methods

### 2.1. Staphylococcal Hlg and Luk

The LukF (or Hlg1), the Hlg2, and the LukS components of Hlg and Luk were purified from the culture supernatants of *S. aureus* Smith 5R strain as described previously [6].

### 2.2. Hemolytic assay for Hlg and leukocytolytic assay for Luk

The hemolytic assay was performed using human erythrocytes as described previously [6]. One hundred percent hemolysis was defined as the average value of absorbance at 541 nm obtained from the osmotically lysed erythrocytes. Luk activity towards human polymorphonuclear leukocytes was assayed by the dye exclusion method using trypan blue, as described previously [11].

### 2.3. Assay for Hlg inhibitors

Test samples were serially two-fold diluted with 10 mM phosphate buffer, pH 7.2, with 140 mM NaCl (phosphate-buffered saline, PBS). The serial dilutions were mixed with Hlg (1.2 pmol of LukF and 1.2 pmol of Hlg2), followed by incubation with 0.5% (v/v) human erythrocytes in a total volume of 200  $\mu$ l at 37°C for 20 min. The mixtures were centrifuged at 1000  $\times$  g for 5 min, and the supernatants obtained were assayed for absorbance at 541 nm. Inhibitory activity (U/ml) of a test sample was calculated as the reciprocal of the sample volume which inhibited 50% of the Hlg-induced hemolysis under the conditions described above.

\*Corresponding author. Fax: (81) (22) 717-8780.  
E-mail: ykamio@biochem.tohoku.ac.jp

**Abbreviations:** Hlg,  $\gamma$ -hemolysin from *Staphylococcus aureus*; Hlg1 and Hlg2, the Hlg1 and Hlg2 components of staphylococcal  $\gamma$ -hemolysin; Luk, leukocidin from *Staphylococcus aureus*; LukS and LukF, the LukS and LukF components of staphylococcal leukocidin; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis

#### 2.4. Purification of Hlg inhibitors from human plasma

All purification steps were carried out at 4°C, except for the high performance column chromatography. Inhibitor fractions obtained from each step were treated with 1 mM phenylmethylsulfonyl fluoride (PMSF) unless otherwise stated. Pooled human plasma (600 ml) was mixed with PBS (600 ml) and saturated ammonium sulfate solution (300 ml). After stirring for 10 min, the mixture was centrifuged at  $8000\times g$  for 30 min. The supernatant obtained (1400 ml) was mixed with saturated ammonium sulfate solution (840 ml), and the mixture was stirred for 30 min and centrifuged at  $8000\times g$  for 1 h. The precipitates obtained were dissolved in PBS (200 ml) containing 1 mM PMSF and 10 mM EDTA, and centrifuged with a Hitachi RP42 rotor (Hitachi, Tokyo, Japan) at  $120\,000\times g$  for 3 h. A clean intermediate layer was collected and dialyzed against 30 mM Tris-HCl buffer (pH 8.3) containing 50 mM NaCl, and loaded onto a DEAE-Toyopearl 650M column (diameter 35 mm, height 105 mm) previously equilibrated with the same buffer. Adsorbed fractions were eluted with two linear gradients of NaCl (50–150 mM and 150–500 mM; Fig. 1). Adsorbed inhibitory fractions, or fractions I and II were eluted with approximately 100 and 300 mM NaCl, respectively, and were separately purified. Fraction I or II was mixed with the same volume of 30 mM Tris-HCl buffer (pH 8.3) containing 3 M ammonium sulfate, and the mixture was loaded onto a phenyl-Toyopearl 650M column (diameter 17 mm, height 50 mm). Adsorbed proteins were eluted with a descending linear gradient of ammonium sulfate (1.5–0 M). The inhibitory fractions eluted with 0.2–0 M ammonium sulfate were collected and dialyzed against 20 mM Tris-HCl buffer (pH 8.3), and fractionated on a high performance liquid chromatograph using a TSKgel DEAE-5PW column (diameter 7.5 mm, height 75 mm). Adsorbed proteins were eluted with a linear gradient of 130–300 mM NaCl.

#### 2.5. Polyacrylamide gel electrophoresis (PAGE) and Western immunoblot

Native PAGE was performed as described by Davis [12]. SDS-PAGE under reducing conditions was performed as described by Laemmli [13]. SDS-PAGE under non-reducing conditions was done as described by Laemmli except that samples were pretreated with 2% SDS at room temperature in the absence of 2-mercaptoethanol. Western immunoblot was done using specific antisera and horseradish peroxidase-conjugated protein A (Sigma Chemicals, St. Louis, MO, USA) as described previously [14]. Preparative SDS-PAGE using a 10% acrylamide disc gel (diameter 7 mm, height 40 mm) was performed at 4°C using a Bio-Rad Model 491 Mini Prep Cell (Bio-Rad Laboratory, Hercules, CA, USA) as described by the manufacturer.

#### 2.6. Enzyme-linked immunosorbent assay for the binding of toxins to immobilized Hlg inhibitors

Purified inhibitors from fraction II (0.25 µg protein in 50 µl of PBS) were immobilized on the wells of 96-well microplates (Nunc, Roskilde Denmark) at 4°C overnight. After blocking with 1% (w/v) bovine serum albumin, serial two-fold dilutions of Hlg2, LukF or LukS (0–0.15 µg protein in 50 µl) were added to the wells and incubated at room temperature for 2 h. The wells were washed three times with PBS containing 0.05% (w/v) Tween-20. Dilutions (50 µl) of rabbit

antiserum against Hlg2, LukF or LukS were added and incubated at 37°C for 2 h. Alkaline phosphatase-conjugated goat immunoglobulins against rabbit immunoglobulins (Promega Co., Madison, WI, USA) were added to the wells, and incubated at 37°C for 1 h. *p*-Nitrophenylphosphate was used as a substrate for alkaline phosphatase.

#### 2.7. Sucrose gradient centrifugation of toxin-vitronectin complexes

Purified inhibitors from fraction II were concentrated by lyophilization, and rehydrated before the experiments. The inhibitors (100 µg protein in 50 µl) were mixed with Hlg2 or LukS (60 µg protein in 50 µl), and incubated at 37°C for 20 min. The mixture was loaded onto a 5–20% (w/w) sucrose gradient and centrifuged using a Beckman SW55Ti rotor (Beckman Instruments Inc., Palo Alto, CA, USA) at  $120\,000\times g$  at 4°C for 15 h.

#### 2.8. Miscellaneous

Protein was assayed according to Bradford [15], using bovine serum albumin as a standard. Protein sequencing was done for the protein bands blotted onto a polyvinylidene difluoride sheet by using an Applied Biosystems Model 491 Protein Sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) [16]. Human plasma was kindly supplied from Miyagi Red Cross Blood Center (Sendai, Japan). Human plasma vitronectin and human plasmin were from Iwaki Glass Co. (Tokyo, Japan) and Sigma Chemicals, respectively. Anti-porcine vitronectin serum raised in rabbit was from LSL Co. (Tokyo, Japan). Chemicals used were of the highest grade commercially available.

### 3. Results and discussion

#### 3.1. Purification of Hlg inhibitory proteins from human plasma and their identification as vitronectin fragments

Serum inhibitors of Hlg were purified from pooled human plasma by the method including ammonium sulfate precipitation, ultracentrifugation, and successive column chromatography using DEAE-Toyopearl 650M, phenyl-Toyopearl 650M, and TSKgel DEAE-5PW, as described in Section 2. As shown in Fig. 1, Hlg inhibitors were recovered in an unadsorbed fraction and in two adsorbed fractions (fractions I and II) from DEAE-Toyopearl 650M column chromatography. The adsorbed fractions I and II were separately purified by the column chromatography with phenyl-Toyopearl 650M and TSKgel DEAE-5PW (results not shown). SDS-PAGE under reducing conditions showed that the purified fraction II contained one major protein of 57 kDa and two minor proteins of 50 and 38 kDa (Fig. 2A, lane 1), and that the purified fraction I gave one major protein band corresponding to 57 kDa and three minor protein bands corresponding to 62, 50 and 38 kDa (Fig. 2A, lane 2). The protein bands were blotted

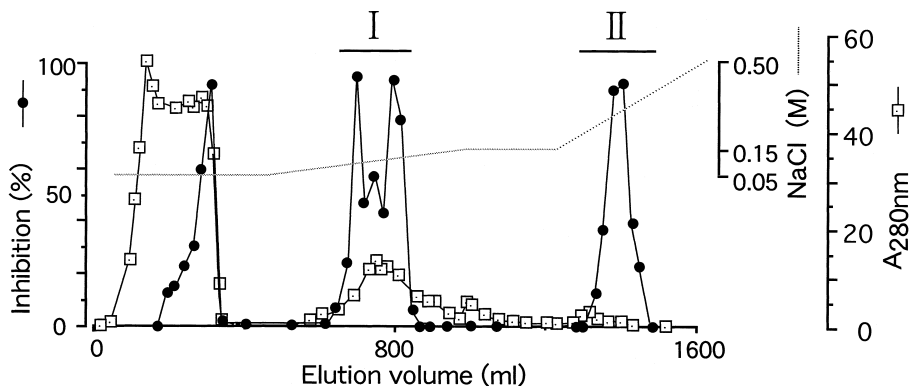


Fig. 1. An elution profile of Hlg inhibitors from DEAE-Toyopearl 650M column chromatography. Percent inhibition of Hlg activity (●) and absorbance at 280 nm (□) were assayed as described in Section 2. Dotted line indicates concentration of NaCl (mM). The bars indicate the inhibitory fractions combined as fractions I and II.

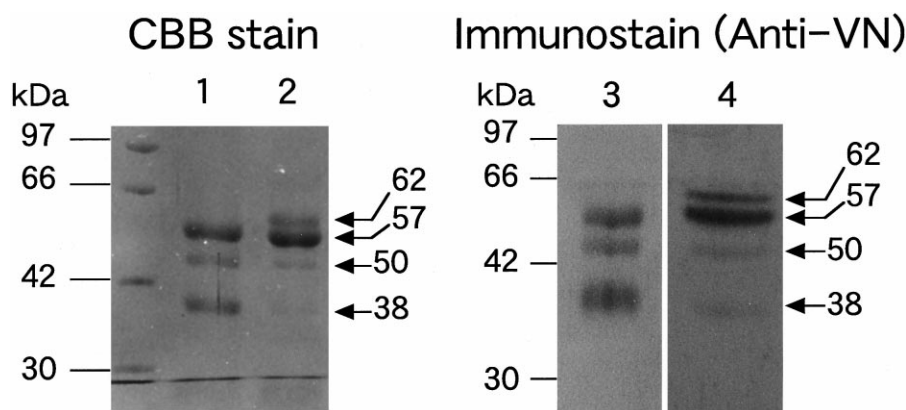


Fig. 2. SDS-PAGE and Western immunoblot for the purified Hlg inhibitors. Lanes 1 and 2: purified Hlg inhibitors (5  $\mu$ g protein) from fractions II and I, respectively, were electrophoresed on a SDS-polyacrylamide gel under reducing conditions, followed by stain with Coomassie brilliant blue R-250. Lanes 3 and 4: purified Hlg inhibitors (0.3  $\mu$ g protein) from fractions II and I, respectively, were subjected to SDS-PAGE under reducing conditions, followed by Western immunoblot using anti-vitronectin serum. VN: vitronectin.

onto a polyvinylidene difluoride sheet, and their N-terminal amino acid sequences were determined: Asp-Gln-Glu-Ser-X-Lys-Gly-Arg-X-Thr-Glu-Gly-Phe-Asn-Val-Asp-Lys-Lys- for the proteins of 62 and 57 kDa, Gly-Asp-Val-Phe-Thr-Met-Pro-Glu-Asp-Glu-Tyr-X-Val-Tyr-Asp-Asp-Gly-Glu- for the 50-kDa protein, and Gly-Asn-Pro-Glu-Gln-Thr-Pro-Val-Leu-Lys-Pro-Glu-Glu-Glu- for the 38 kDa protein (where X indicates unspecified amino acid residue). A similarity search on the DDBJ/GenBank/EMBL nucleic acid sequence database indicated that all these proteins were vitronectin fragments: the proteins of 57 and 62 kDa had an N-terminal amino acid sequence identical to that of intact human vitronectin, and the N-terminal amino acid sequences of the 50 and 38 kDa proteins were identical with the internal amino acid sequences of vitronectin which start from Gly<sup>46</sup> and Gly<sup>89</sup> of the molecule, respectively. All these protein bands of fractions I and II were immunostained with a specific antiserum raised against porcine vitronectin (Fig. 2A, lanes 3 and 4). Thus, the Hlg inhibitors from human plasma were identified as vitronectin fragments. Approximately 2 and 8 mg of the purified vitronectin fragments were obtained from fractions I and II, respectively, when 600 ml of pooled human plasma was used as the starting material. Recovery of total inhibitory activity was approximately 5%. Specific activity of the Hlg inhibitors from fraction II was increased up to 630-fold, compared with that of the human plasma used. Fifty percent of the Hlg-induced hemolysis was inhibited by 0.37  $\mu$ g protein of the purified inhibitors from fraction II under standard conditions.

To study whether or not each of the vitronectin fragments has the inhibitory activity to Hlg, the purified inhibitors from fraction II were concentrated by lyophilization, treated with 2% SDS at room temperature for 5 min, and fractionated on a preparative SDS-PAGE. As shown in Fig. 3, the 38 kDa fragment was purified to homogeneity, and it showed a dose-dependent inhibitory activity to Hlg (Fig. 3). The 57 kDa fragment was purified nearly to homogeneity, and it showed a dose-dependent inhibitory activity (Fig. 3). The results also suggested that the 50 kDa fragment had inhibitory activity to Hlg, because fraction 58 showed a higher inhibitory activity than fraction 52, although both fractions contained similar amounts of the 38 kDa fragment (Fig. 3). Taken together with the N-terminal amino acid sequence and the molecular size of the 38 kDa fragment, an essential domain for the

inhibitory activity of vitronectin to Hlg may be located in its central part consisting of the C-terminal half of the connecting region, the hemopexin 1 domain, and the N-terminal quarter of the hemopexin 2 domain [17].

The purified Hlg inhibitors from fraction II gave immunostained bands corresponding to 57, 50 and 38 kDa irrespective of the treatment with 2-mercaptoethanol at 100°C (results not shown). However, the purified Hlg inhibitors gave immunostained bands corresponding to 180, 150–130, and 115 kDa on SDS-PAGE under non-reducing conditions, when they had been lyophilized or frozen and thawed (results not shown). The lyophilized and the frozen-and-thawed inhibitors gave protein bands corresponding to 57, 50, and 38 kDa on SDS-PAGE under reducing conditions (results not shown). These results suggested that the vitronectin fragments were not disulfide-linked with one another in the fresh preparations, and significant portions of the vitronectin fragments became disulfide-linked and/or non-covalently linked with one another through lyophilization and freezing and thawing. However, no significant difference was found between the inhibitory activities of the non-treated, the lyophilized, and the frozen-and-thawed vitronectin fragments to Hlg, suggesting that the vitronectin fragments were functionally active as Hlg inhibitors irrespective of the molecular forms.

The other Hlg inhibitors, which were eluted in the unadsorbed fraction from DEAE-Toyopearl 650M column chromatography (Fig. 1), were also analyzed by Western immunoblot analyses using antisera against human immunoglobulins and porcine vitronectin. The results showed that the unadsorbed fraction contained immunoglobulins but no significant amount of vitronectin (results not shown), indicating that the unadsorbed Hlg inhibitors were natural antibodies to Hlg.

### 3.2. Inhibitory activity of intact vitronectin towards Hlg

Inhibitory activity of intact vitronectin to Hlg was studied using an authentic vitronectin preparation, which was purified from human plasma essentially as described by Hayman et al. [18]. The vitronectin preparation inhibited the Hlg-induced hemolysis in a dose-dependent manner, and 5.5  $\mu$ g of the intact vitronectin caused 50% inhibition of the Hlg activity under standard assay conditions. Thus, intact vitronectin had an inhibitory activity to Hlg, but it was 15-fold less active than the purified Hlg inhibitors on protein basis. Further-

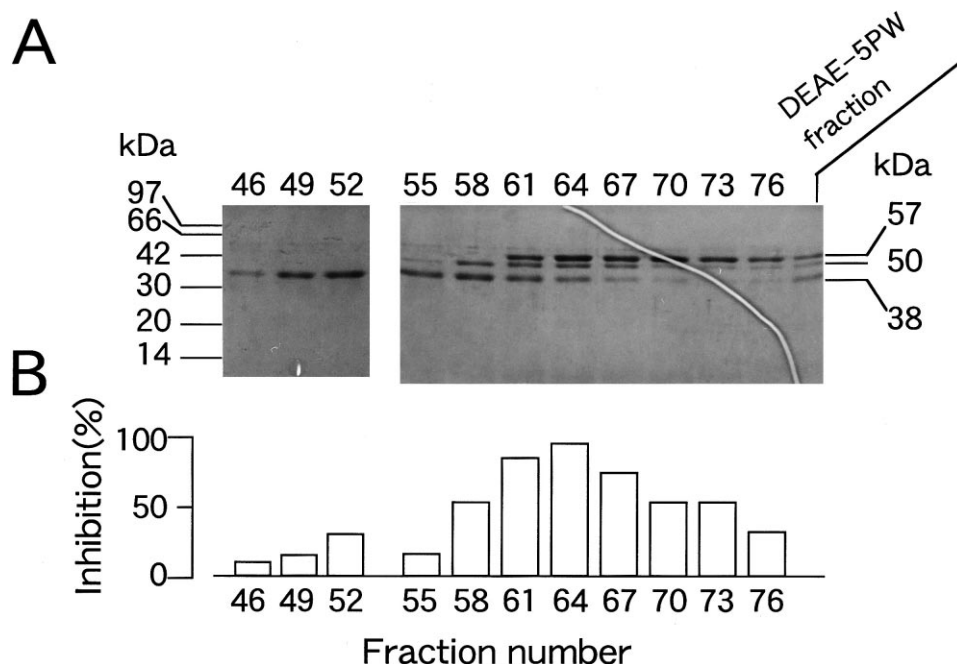


Fig. 3. Separation of the vitronectin fragments by preparative SDS-PAGE (A) and inhibitory activity of the separated vitronectin fragments towards Hlg (B). A preparation of Hlg inhibitors was lyophilized and fractionated on preparative SDS-PAGE under non-reducing conditions as described in Section 2. A: A portion of the fractions obtained were analyzed by SDS-PAGE under reducing conditions. The protein bands were stained with Coomassie brilliant blue R-250. B: The rest of the fractions were used for assay of their inhibitory activity to Hlg after removal of SDS by electro dialysis against PBS.

more, inhibitory activity of intact vitronectin was raised to a comparable level to that of the purified vitronectin fragments when partially digested with human plasmin, i.e. 0.8  $\mu$ g of the plasmin-treated vitronectin inhibited 50% of the Hlg-induced hemolysis under the standard conditions, and SDS-PAGE for the plasmin-treated vitronectin showed the presence of protein bands corresponding to 62 and 38 kDa.

### 3.3. Specific binding of the vitronectin fragments to the Hlg2 component of $\gamma$ -hemolysin and the LukS component of leukocidin

Binding of LukF and Hlg2 to human erythrocytes was assayed in the presence of Hlg inhibitors. As shown in Fig. 4, the Hlg inhibitors blocked the binding of Hlg2 to erythrocytes as well as the Hlg-induced hemolysis in a dose-dependent manner (Fig. 4, lanes 2, 5–7). In contrast, the Hlg inhibitors did not affect the binding of LukF to the cells irrespective of the presence of Hlg2 (Fig. 4, lanes 2–4). These results suggested that the Hlg inhibitors specifically bound to Hlg2 and inactivated the component. An enzyme-linked immunosorbent assay using antisera against LukF and Hlg2 showed that Hlg2 bound to immobilized Hlg inhibitors in a dose-dependent manner and showed half-maximal binding at a concentration of approximately 0.3  $\mu$ g/ml, while LukF showed no significant binding to the Hlg inhibitors at 30  $\mu$ g/ml under the same conditions.

Since Hlg2 has 72% identity with the LukS component of Luk in amino acid sequence [6,8], we studied effects of the purified vitronectin fragments on leukocidin activity. LukS (1.6 pmol) and LukF (1.6 pmol) were incubated with or without the vitronectin fragments at 37°C for 10 min, followed by the incubation with human polymorphonuclear leukocytes ( $1 \times 10^4$  cells) in a total volume of 20  $\mu$ l at 37°C for 10 min. In the absence of the vitronectin fragments, LukS and LukF

induced 100% lysis of the leukocytes under the conditions. In contrast, addition of the vitronectin fragments to the reaction mixtures inhibited the leukocytolytic activity in a dose-dependent manner: 10, 25 and 50  $\mu$ g of the vitronectin fragments inhibited 55, 58, and 100% of the leukocidin activity, respectively (average values from three independent experiments). An enzyme-linked immunosorbent assay also showed that LukS bound to the immobilized vitronectin fragments in

LukF	+	+	+	+	+	+	+
Hlg2	+	+	–	–	+	+	+
Inhibitor ( $\mu$ g)	0	15	0	15	10	5	2.5

Immunostain								
LukF	—	—	—	—	—	—	—	—
Hlg2	—	—	—	—	—	—	—	—

Hemolysis (%)	100	3.4	0	0	4.8	37	100
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Fig. 4. Effect of the Hlg inhibitors on the binding of Hlg to human erythrocytes. Hlg (0.15  $\mu$ g of LukF and 0.15  $\mu$ g of Hlg2) was mixed with the Hlg inhibitors (2.5, 5, 10 or 15  $\mu$ g protein), and then incubated with human erythrocytes ( $5 \times 10^8$  cells) in 0.4 ml of PBS at 37°C for 20 min. A small portion of the mixtures were withdrawn and assay for percent hemolysis. The rest of the mixtures were centrifuged to collect erythrocyte membranes, and the erythrocyte membranes obtained were subjected to SDS-PAGE under reducing conditions, followed by Western immunoblot using a mixture of diluted antisera against LukF and Hlg2. +: Added. –: Not added.

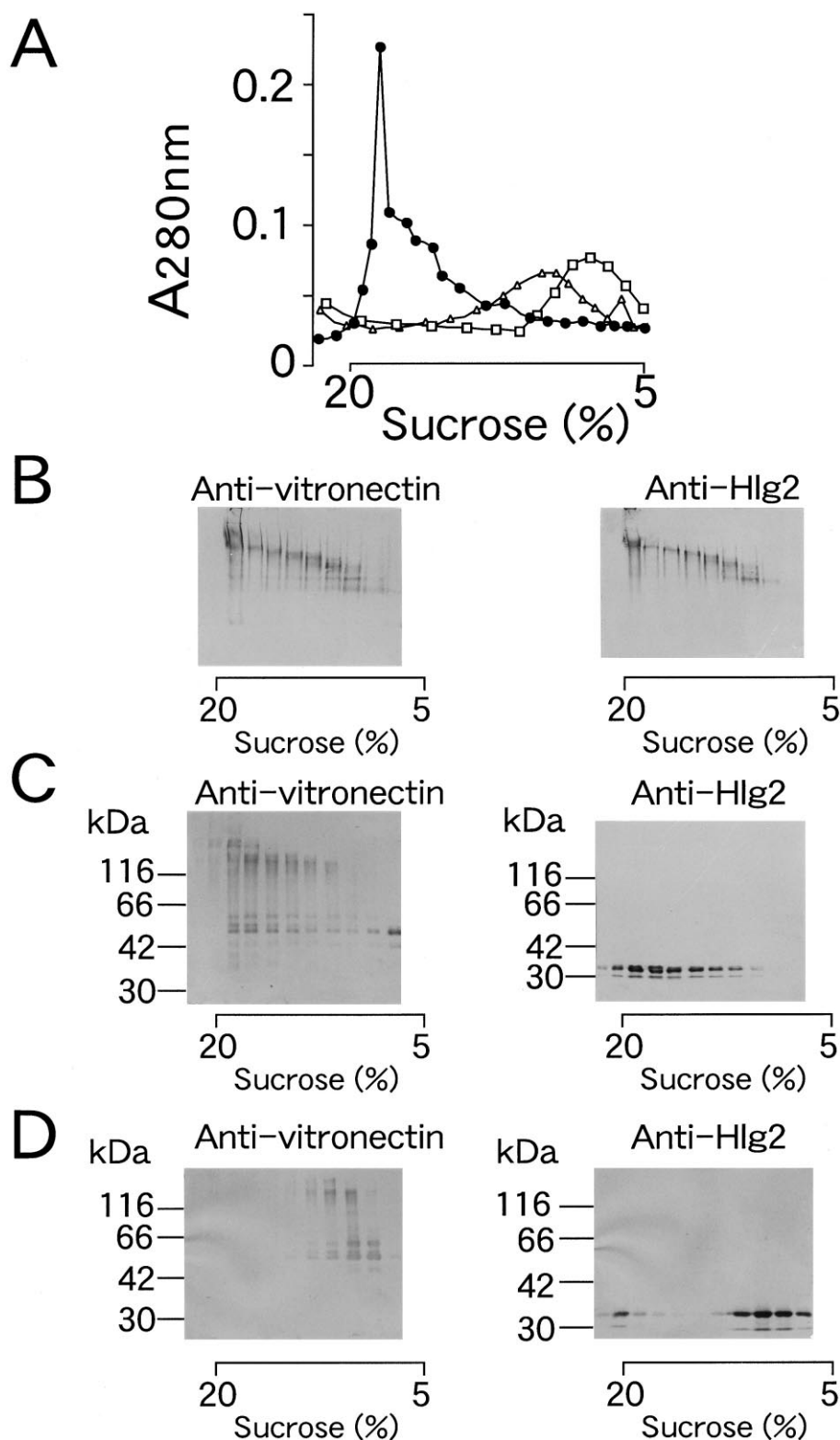


Fig. 5. Sedimentation profiles of vitronectin-Hlg2 complexes, the vitronectin fragments, and Hlg2 in a sucrose gradient centrifugation (A), native PAGE/Western immunoblot (B), and SDS-PAGE/Western immunoblot (C and D) for the fractions from the centrifugation. A: A mixture of the Hlg inhibitors (100 µg) and Hlg2 (60 µg) was incubated in TBS (100 µl) at 37°C for 20 min, and fractionated using a 5–20% (w/w) sucrose gradient centrifugation, as described in Section 2. Absorbance at 280 nm was assayed for the fractions obtained from the centrifugation. A representative sedimentation profile of the vitronectin-Hlg2 complexes (●) is illustrated. Sedimentation profiles of the vitronectin fragments alone (△) and Hlg2 alone (□) are also illustrated. B and C: A portion of the fractions from the centrifugation of the mixture of vitronectin and Hlg2 was withdrawn and subjected to native PAGE (B) or SDS-PAGE under non-reducing conditions (C), followed by Western immunoblot using anti-vitronectin (left panels) or anti-Hlg2 serum (right panels). D: The Western immunoblot was done for the fractions from sucrose gradient centrifugation of the vitronectin fragments alone (left panel) and Hlg2 alone (right panel).

a dose-dependent manner, and the binding of LukS to the inhibitors took place at a comparable level to that of Hlg2 (i.e. the concentration of LukS for half-maximal binding was approximately 0.75 µg/ml).

Complex formation between the vitronectin fragments and Hlg2 was analyzed by sucrose gradient centrifugation (Fig. 5A), followed by native PAGE/Western immunoblot (Fig. 5B) and SDS-PAGE/Western immunoblot (Fig. 5C) using antisera against vitronectin and Hlg2. When a mixture of the vitronectin fragments and Hlg2 was incubated and centrifuged in a 5–20% (w/w) sucrose gradient, a broad peak comprising the vitronectin fragments and Hlg2 sedimented faster than those of the vitronectin fragments alone and Hlg2 alone (Fig. 5A,D). Native PAGE/Western immunoblot showed that the vitronectin fragments and Hlg2 co-sedimented on the sucrose gradient and moved together in the native gel (Fig. 5B), indicating complex formation between the vitronectin fragments and Hlg2. SDS-PAGE/Western immunoblot confirmed the co-sedimentation of the vitronectin fragments and Hlg2 in the sucrose gradient centrifugation (Fig. 5C). However, Hlg2 did not migrate together with the vitronectin fragments in the SDS-polyacrylamide gel, suggesting that the complexes of the vitronectin fragments with Hlg2 were dissociated in the presence of 2% SDS (Fig. 5C). Experiments using sucrose density gradient centrifugation and Western immunoblot under the same conditions showed that the vitronectin fragments also formed complexes with the LukS component of Luk (results not shown).

In the present study, we purified serum inhibitors of Hlg and Luk from human plasma and identified them as vitronectin fragments. Since vitronectin exists primarily as an intact 75 kDa molecule and a nicked molecule consisting of an N-terminal 65 kDa fragment and a C-terminal 10 kDa fragment in human plasma [18–21], the purified inhibitors of Hlg and Luk may have derived proteolytically from the intact and the nicked molecules during purification, probably by the action of serum protease(s) such as plasmin. In fact, intact and nicked vitronectin molecules were detected by Western immunoblot for fraction I of the DEAE-Toyopearl 650M column chromatography (results not shown), and the limited digestion of intact vitronectin by human plasmin produced inhibitory fragments with molecular masses of 62, 42, and 38 kDa (results not shown). Furthermore, vitronectin is one of the major serum proteins, and its concentration in human serum has been estimated to be 300–500 µg/ml [19,20] (i.e. >10-fold higher concentrations than that causing 50% inhibition of Hlg under standard conditions). Taken together, vitronectin and its fragments are considered to be the major serum inhibitors of Hlg and Luk.

Vitronectin is present in the extracellular matrix of many tissues as well as in plasma. So, it should be noted that limited proteolysis of vitronectin may occur in the extracellular matrix of normal and lesional human tissues, because vitronectin fragments of 61–63, 58, 50, 42, and 35 kDa were detected by Western immunoblot analyses for freshly excised samples of human normal skin [22] and for retina extracts from patients with proliferative diabetic retinopathy [23]. The vitronectin fragments might play some physiological and/or pathological roles in normal and lesional tissues. Since vitronectin is considered to regulate proteolytic enzyme cascades including the

complement, coagulation and fibrinolysis systems [17], it would act as an ambivalent factor for hosts in the loci of staphylococcal infections. (i) Vitronectin and its fragments capture Hlg2 and LukS in the extracellular matrix of fibroblasts and tissue macrophages, leading to integrin-mediated endocytosis and degradation of the toxins by the cells [24]. (ii) However, the resultant lower level of vitronectin would cause tissue injuries by an excess level of terminal complex of complement and hyperproduction of plasmin. (iii) Vitronectin has been shown to be a binding molecule for *S. aureus* [25]. Binding of Hlg and Luk to vitronectin in the extracellular matrix would cause spreading of tissue-bound staphylococci. Thus, not only cytolytic activity but also vitronectin binding activity of Hlg and Luk are the putative pathophysiological functions of the staphylococcal bi-component toxins.

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