

# The two *Staphylococcal* bi-component toxins, leukocidin and gamma-hemolysin, share one component in common

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*Staphylococcal* bi-component toxins, leukocidin and  $\gamma$ -hemolysin, consist of two protein components, i.e. F and S for leukocidin and H $\gamma$ I and H $\gamma$ II for  $\gamma$ -hemolysin. In this study we purified H $\gamma$ I and H $\gamma$ II to homogeneity from the culture medium of *Staphylococcus aureus* RIMD 310925 and compared their properties with those of F and S purified from the same source. The N-terminal 59- and C-terminal 2-residue amino acid sequences, apparent molecular mass, and isoelectric point of purified H $\gamma$ I were the same as those of F. In an Ouchterlony double diffusion test a fused line without spur was formed between F and H $\gamma$ I using either anti-F or anti-H $\gamma$ I antibodies. A synergistic action of F and H $\gamma$ II caused hemolysis of human red blood cells, and H $\gamma$ I acted synergistically with S to exhibit leukocidin activity. We conclude that the two toxins share one protein component (F = H $\gamma$ I) in common and leukocidin- and  $\gamma$ -hemolysin-specific activities are determined by S and H $\gamma$ II, respectively. It is also reported that the N-terminal 58-residue sequence of H $\gamma$ II is 72% similar to the corresponding sequence of S.

Leukocidin:  $\gamma$ -Hemolysin: *Staphylococcus aureus*

## 1. INTRODUCTION

Leukocidin and  $\gamma$ -hemolysin are two toxins secreted by *Staphylococcus aureus* and cause cytotoxic changes in polymorphonuclear leukocytes and hemolysis in red blood cells, respectively [1,2]. Both toxin activities are known to result from a synergistic action of the two protein components, i.e. F and S for leukocidin and H $\gamma$ I and H $\gamma$ II for  $\gamma$ -hemolysin [1,2]. The two toxins have so far been studied as distinct entities having no structural or functional similarities. However, bi-component specificity of the two toxins raises the question whether or not there is any relationship between them. To answer this question, it is necessary to obtain homogeneous preparations of the two components of  $\gamma$ -hemolysin. In this study we purified both H $\gamma$ I and H $\gamma$ II components to homogeneity from the culture medium of *S. aureus* and compared their properties with those of F and S components of leukocidin. Here we report evidence that leukocidin F is identical with  $\gamma$ -hemolysin H $\gamma$ I.

## 2. MATERIALS AND METHODS

### 2.1. Purification of H $\gamma$ I and H $\gamma$ II components

Methicillin-resistant *S. aureus* strain No. 4 (RIMD 310925), which was used in previous studies [3,4] was grown in 2.5% heart infusion broth (Difco) at 37°C for 24 h with vigorous aeration, and then the

culture medium was harvested by centrifugation at 4°C. H $\gamma$ I and H $\gamma$ II were partially purified from the culture medium by hydroxylapatite column chromatography as described by Plommet [2], and then further purified by HPLC (TOSOH, Tokyo) equipped with a TSK gel SP-5PW column (TOSOH, Tokyo). H $\gamma$ I and H $\gamma$ II were thereby eluted from the column with a linear gradient of NaCl in 10 mM potassium phosphate buffer (pH 6.8) (Buffer A) at about 0.15 M and about 0.8 M NaCl, respectively. H $\gamma$ I- and H $\gamma$ II-containing fractions were separately pooled, dialyzed against Buffer A and stored at -80°C.

### 2.2. Assay of leukocidin and $\gamma$ -hemolysin activities

Leukocidin and  $\gamma$ -hemolysin activities were determined by the methods of Noda et al. [5] and of Plommet [2], respectively.

### 2.3. SDS-PAGE and Western immunoblot analysis

SDS-PAGE and Western immunoblot analysis were done by the methods described previously [6]. Polyclonal antibodies were raised against leukocidin F and S components purified from *S. aureus* V8 as described earlier [4].

### 2.4. Preparation of antisera against purified H $\gamma$ I and H $\gamma$ II

Rabbit antisera against  $\gamma$ -hemolysin H $\gamma$ I and H $\gamma$ II were prepared by the method described previously [6].

### 2.5. Determination of N- and C-terminal amino acid sequences

The N-terminal sequence was determined by automated Edman degradation using a gas phase protein sequencer (model 473A, ABI Co.) equipped with an on-line PTH amino acid analyzer (model 120A, ABI Co.) and C-terminal sequence was determined by carboxypeptidase Y digestion as described by Hayashi et al. [7]. The amino acids released from the C-terminus were dabsylated by the method of Stocchi et al. [8] and analyzed by an amino acid analyzer (Beckman, System Gold).

### 2.5. Purification of cloned S and F components of leukocidin

Cloned S and F were prepared from a periplasmic space of *Escherichia coli* DH5 $\alpha$ (pSRK91) [3] and *E. coli* DH5 $\alpha$ (pFRK92) [4], respec-

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tively. The cloned S and F preparations were purified with HPLC equipped with TSK gel SP-5PW column. The F and S were eluted from the column with a linear gradient of NaCl in Buffer A at about 0.15 M and about 0.65 M, respectively. The cloned F and S thus obtained were found to be homogeneous by SDS-PAGE (data not shown).

### 3. RESULTS AND DISCUSSION

#### 3.1. Purification of $\gamma$ -hemolysin components

Purification of the two components H $\gamma$ I and H $\gamma$ II of  $\gamma$ -hemolysin, from 20 liter culture fluid to the degree of electrophoretic homogeneity yielded 5 mg and 60 mg protein, respectively (Fig. 1). The *S. aureus* strain used seemed to secrete a larger amount of H $\gamma$ II than H $\gamma$ I. The molecular mass of purified H $\gamma$ I (34 kDa) was the same as that of leukocidin F component [4]. When co-chromatographed, H $\gamma$ I and F were inseparable on both TSK gel HA-1000 (TOSOH, Tokyo) and SP-5PW columns in HPLC (data not shown). H $\gamma$ II showed a somewhat smaller molecular mass (32 kDa) than leukocidin S (33 kDa) [3] on SDS-PAGE (Fig. 1). The isoelectric points of H $\gamma$ I and H $\gamma$ II were determined by the method described previously [6] to be 9.1 and 9.4, respectively. The value for H $\gamma$ I coincided with that for the leukocidin F component [4].

#### 3.2. Amino acid composition and N- and C-terminal amino acid sequences of H $\gamma$ I and H $\gamma$ II

As shown in Table I, the amino acid composition of H $\gamma$ I was almost the same as that of leukocidin F calculated from its predicted primary structure [4]. The C-terminal amino acid sequences of H $\gamma$ I and H $\gamma$ II were determined to be -Asn-Lys-OH and -Ser-Ile-Thr-Pro-

Lys-OH, respectively. The C-terminal 2-residue and N-terminal 59-residue sequences of H $\gamma$ I were identical with those predicted for F (Table II). On the other hand, the N-terminal 58-residue sequence of H $\gamma$ II determined in this study was 72% identical with the corresponding sequences of leukocidin S (Table II), but no homology was found between the C-terminal 5-residue sequences of H $\gamma$ II and S.

#### 3.3. Immunoblotting analysis and Ouchterlony double diffusion test of $\gamma$ -hemolysin H $\gamma$ I and H $\gamma$ II

In view of the similarity of the N-terminal amino acid sequences of  $\gamma$ -hemolysin H $\gamma$ I and H $\gamma$ II to those of leukocidin F and S, respectively, we performed a comparative immunological study between the components of both toxins. H $\gamma$ I and H $\gamma$ II were positive to anti-F and anti-S antibodies, respectively, whereas they did not cross-react to anti-S and anti-F antibodies, respectively (Fig. 2A and B).

Table I

Amino acid compositions of H $\gamma$ I and H $\gamma$ II components of  $\gamma$ -hemolysin, and S and F components of leukocidin of *S. aureus*

Amino acid	No. of amino acid residues			
	H $\gamma$ I component <sup>a</sup>	F component <sup>b</sup>	H $\gamma$ II component <sup>a</sup>	S component <sup>b</sup>
Lys	28	28	30	27
Arg	9	10	11	8
His	5	5	4	8
Asp	47	17	39	16
Asn		29		26
Glu	26	12	25	13
Gln		13		13
Thr	22	22	18	20
Ser	27	26	23	30
Gly	22	21	17	20
Ala	15	17	14	10
Val	16	15	18	19
Ile	14	14	21	16
Leu	18	18	12	12
Cys	0	0	0	0
Trp	6	6	3	4
Met	3	3	3	3
Tyr	18	18	17	18
Phe	15	16	13	14
Pro	8	8	12	9
Total residue	299	298	280	286
Calculated $M_r$	33,946	33,917	31,921	32,528

<sup>a</sup> Determined by amino acid analysis of purified H $\gamma$ I and H $\gamma$ II components from *S. aureus* RIMD 310925.

<sup>b</sup> Calculated from the predicted amino acid sequence of F and S components [3,4].

Cysteine was determined after performic oxidation and hydrolysis of the preparation for 24 h by the method of Moore [9]. Determination of tryptophan levels was carried out in 6 M guanidine hydrochloride by the method of Bencze and Schmid [10].

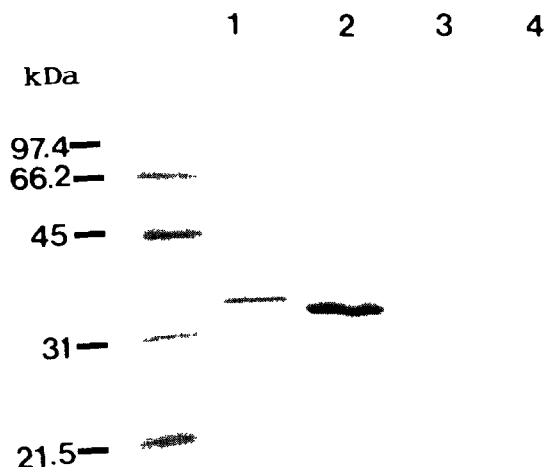


Fig. 1. SDS-PAGE of F, S, H $\gamma$ I and H $\gamma$ II. The gel was stained with Coomassie brilliant blue R-250. Molecular weight standards used were phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (21.5 kDa). Lanes: MW, molecular weight standards; 1, S; 2, H $\gamma$ II; 3, F; 4, H $\gamma$ I.

Table II  
Comparison of N-terminal amino acids sequence of F, S, H $\gamma$ I and H $\gamma$ II components from *S. aureus*

Protein	Residue	Sequence <sup>a</sup>
F	1-29	EGKITPVSVKKVDDKVTLYKTTATADSDK
H $\gamma$ I	1-29	EGKITPVSVKKVDDKVTLYKTTATADSDK
F	30-59	FKISQILTFNFIKDKSYDKDTLVLKATGNI
H $\gamma$ I	30-59	FKISQILTFNFIKDKSYDKDTLVLKATGNI
S	1-29	ANDTEDIGKKGSDIEI IKRTEDKTSNK-WGV
H $\gamma$ II	1-27	ENKIEDIGQG--AEI IKHTQDITS-KHLAI
S	30-60	TQNIQFDFVKDTKYNKDALILKMQGFISSRT
H $\gamma$ II	28-58	TQNIQFDFVKDKKYNKDALVVKMQGFISSRT

<sup>a</sup> Identical residues at corresponding positions in the compared sequence are boxed.

In an Ouchterlony double diffusion test a fused line without spur was formed between F and H $\gamma$ I using either anti-F or anti-H $\gamma$ I antibodies (Fig. 2C and D),

indicating that there is no apparent difference in antigenic structure between them. S as well as H $\gamma$ II cross-reacted with anti-H $\gamma$ II serum (data not shown).

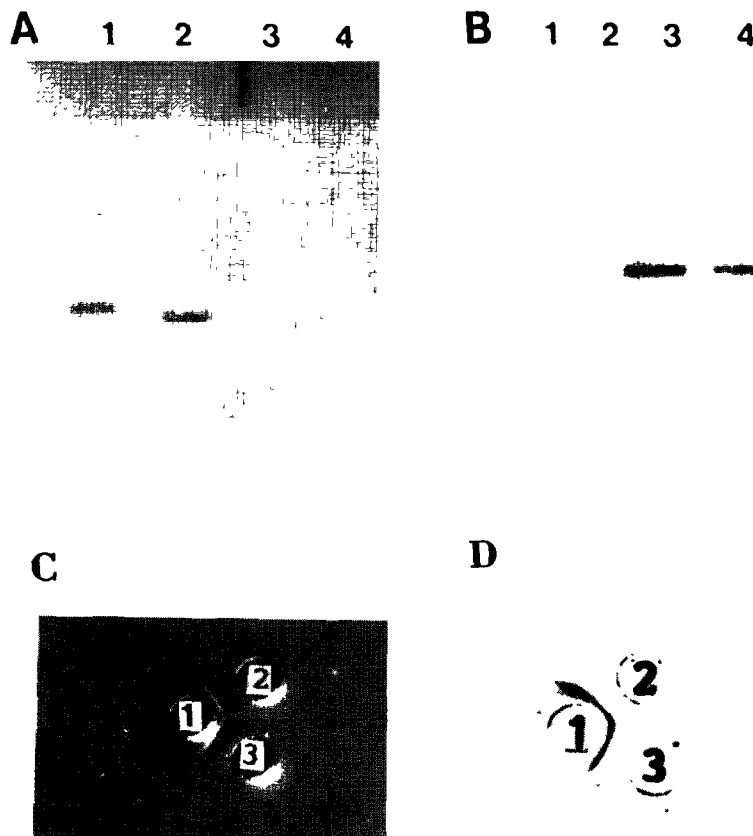


Fig. 2. Western blot (A and B) and Ouchterlony double diffusion test (C and D) of F, S, H $\gamma$ I and H $\gamma$ II preparations, using specific anti-serum against S (A) and F (B and C) or H $\gamma$ I (D). Lanes of both (A) and (B): 1, S; 2, H $\gamma$ II; 3, F; 4, H $\gamma$ I. For double diffusion test, the well, No. 1 of (C) and (D) contain anti-F and anti-H $\gamma$ I antibodies, respectively. The wells, No. 2 and No. 3 of both (C) and (D) contain 12  $\mu$ g (10  $\mu$ l) F and H $\gamma$ I, respectively. In the case of (D), the specimen was stained with Coomassie brilliant blue R-250.

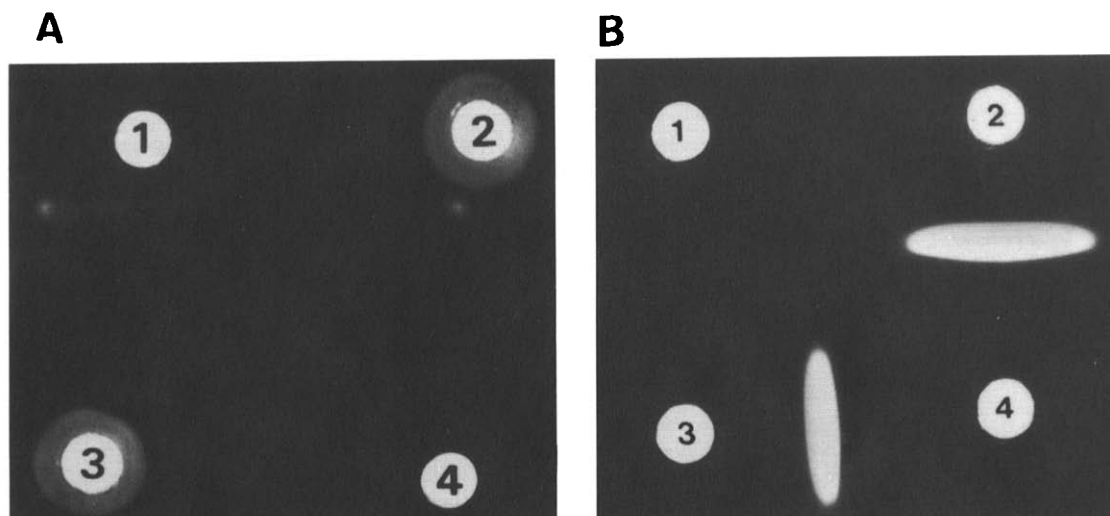


Fig. 3.  $\gamma$ -Hemolysin synergism on human blood agarose plates, (A) and (B). The human blood agarose plate was prepared by the method of Plommet [2]. Plate A: 1, F + S; 2, H $\gamma$ I + H $\gamma$ II; 3, F + H $\gamma$ II; 4, H $\gamma$ I + S. Plate B: 1, cloned S; 2, cloned F; 3, H $\gamma$ I; 4, H $\gamma$ II. The concentration of each component was adjusted to 100 ng/ $\mu$ l in Buffer A. The amount of each component applied to the well was 3  $\mu$ g. The plates (A) and (B) were incubated at 37°C for 2 h and 18 h, respectively.

#### 3.4. Change from leukocidin activity to hemolysin activity by substituting H $\gamma$ II for leukocidin S component

The findings described above suggest that leukocidin F and  $\gamma$ -hemolysin H $\gamma$ I components are identical. If F is a common factor for both leukocidin and  $\gamma$ -hemolysin, and if the leukocidin- and  $\gamma$ -hemolysin-specific activities are determined by S and H $\gamma$ II, leukocidin activity should change to hemolytic activity by substituting H $\gamma$ II for S in combination with F. Accordingly, we examined the effect of S and H $\gamma$ II on the specificity of toxin activity in combination with leukocidin F component. A synergistic action of F and H $\gamma$ II caused hemolysis of human red blood cells (Fig. 3A). Cloned F also had hemolytic activity in combination with H $\gamma$ II (Fig. 3B). H $\gamma$ I acted synergistically with S to exhibit leukocidin activity without hemolytic activity (data not shown). From these findings, we conclude that the two toxins share one protein component (F = H $\gamma$ I) in common and the leukocidin- and  $\gamma$ -hemolysin-specific activities are determined by S and H $\gamma$ II, respectively. We have reconfirmed the above conclusion by cloning and nucleotide sequencing of the  $\gamma$ -hemolysin gene(s) (submitted elsewhere).

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