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

Yoshiyuki Kamio, Arifur Rahman, Hirofumi Nariya, Toshiko Ozawa and Kazuo Izaki

Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, Amamiya-machi 1-1, Aoba-ku, Sendai 981, Japan

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Staphylococcal bi-component toxins, leukocidin and γ-hemolysin, consist of two protein components, i.e. F and S for leukocidin and HγI and HγII for γ-hemolysin. In this study we purified HγI and HγII 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 HγI were the same as those of F. In an Ouchterlony double diffusion test a fused line without spur was formed between F and HγI using either anti-F or anti-HγI antibodies. A synergistic action of F and HγII caused hemolysis of human red blood cells, and HγI acted synergistically with S to exhibit leukocidin activity. We conclude that the two toxins share one protein component (F = HγI) in common and leukocidin- and γ-hemolysin-specific activities are determined by S and HγII, respectively. It is also reported that the N-terminal 58-residue sequence of HγII is 72% similar to the corresponding sequence of S.

Leukocidin; γ-Hemolysin; *Staphylococcus aureus*

1. INTRODUCTION

Leukocidin and γ-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 HγI and HγII for γ-hemolysin [1,2]. The two toxins have sofar 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 γ-hemolysin. In this study we purified both HγI and HγII 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 γ-hemolysin HγI.

2. MATERIALS AND METHODS

2.1. *Purification of HγI and HγII 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. HγI and HγII 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). HγI and HγII 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. HγI- and HγII-containing fractions were separately pooled, dialyzed against Buffer A and stored at −80°C.

2.2. *Assay of leukocidin and γ-hemolysin activities*

Leukocidin and γ-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 HγI and HγII*

Rabbit antisera against γ-hemolysin HγI and HγII 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* DH5α(pSRK91) [3] and *E. coli* DH5α(pFRK92) [4], respec-

Correspondence address. Y. Kanio, Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, Amamiya-machi 1-1, Aoba-ku, Sendai 981, Japan. Fax: (81) (22) 272 1870.

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 γ-hemolysin components*

Purification of the two components HγI and HγII of γ-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 HγII than HγI. The molecular mass of purified HγI (34 kDa) was the same as that of leukocidin F component [4]. When co-chromatographed, HγI and F were inseparable on both TSK gel HA-1000 (TOSOH, Tokyo) and SP-5PW columns in HPLC (data not shown). HγII showed a somewhat smaller molecular mass (32 kDa) than leukocidin S (33 kDa) [3] on SDS-PAGE (Fig. 1). The isoelectric points of HγI and HγII were determined by the method described previously [6] to be 9.1 and 9.4, respectively. The value for HγI coincided with that for the leukocidin F component [4].

3.2. *Amino acid composition and N- and C-terminal amino acid sequences of HγI and HγII*

As shown in Table I, the amino acid composition of HγI was almost the same as that of leukocidin F calculated from its predicted primary structure [4]. The C-terminal amino acid sequences of HγI and HγII 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 HγI were identical with those predicted for F (Table II). On the other hand, the N-terminal 58-residue sequence of HγII 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 HγII and S.

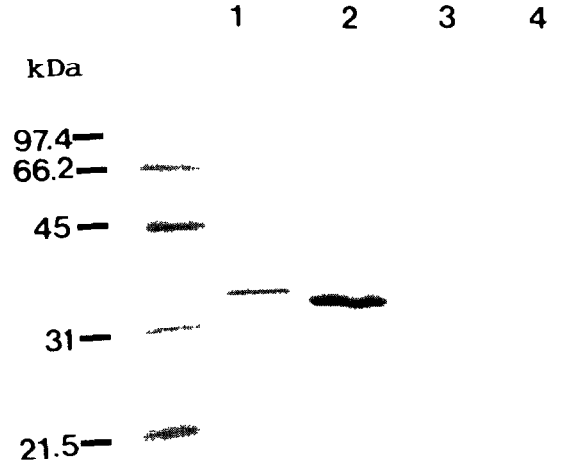

Fig. 1. SDS-PAGE of F, S, HγI and HγII. The gel was stained with Coomassie brilliant blue R-250. Molecular weight standards used were phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (21.5 kDa). Lanes: MW,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

3.3. *Immunoblotting analysis and Ouchterlony double diffusion test of γ-hemolysin HγI and HγII*

In view of the similarity of the N-terminal amino acid sequences of γ-hemolysin HγI and HγII to those of leukocidin F and S, respectively, we performed a comparative immunological study between the components of both toxins. HγI and HγII 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 HγI and HγII components of γ-hemolysin, and S and F components of leukocidin of *S. aureus*

Amino acid	No. of amino acid residues			
	HγI component[a]	F component[b]	HγII component[a]	S component[b]
Lys	28	28	30	27
Arg	9	10	11	8
His	5	5	4	8
Asp	47	17	39	16
Asn		29 (46)		26 (42)
Glu	26	12	25	13
Gln		13 (25)		13 (26)
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

[a] Determined by amino acid analysis of purified HγI and HγII components from *S aureus* RIMD 310925.

[b] 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].

Table II

Comparison of N-terminal amino acids sequence of F, S, HγI and HγII components from *S. aureus*

Protein	Residue	Sequence[a]
F	1-29	EGKITPVSVKKVDDKVTLYKTTATADSDK
HγI	1-29	EGKITPVSVKKVDDKVTLYKTTATADSDK
F	30-59	FKISQILTFNFIKDKSYDKDTLVLKATGNI
HγI	30-59	FKISQILTFNFIKDKSYDKDTLVLKATGNI
S	1-29	ANDTEDIGKGSDIEIIKRTEDKTSNK-WGV
HγII	1-27	ENKIEDIGQG--AEIIKHTQDITS-KHLAI
S	30-60	TQNIQFDFVKDTKYNKDALILKMQGFISSRT
HγII	28-58	TQNIQFDFVKDKKYNKDALVVKMQGFISSRT

[a] 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 HγI using either anti-F or anti-HγI antibodies (Fig. 2C and D), indicating that there is no apparent difference in antigenic structure between them. S as well as HγII cross-reacted with anti-HγII serum (data not shown).

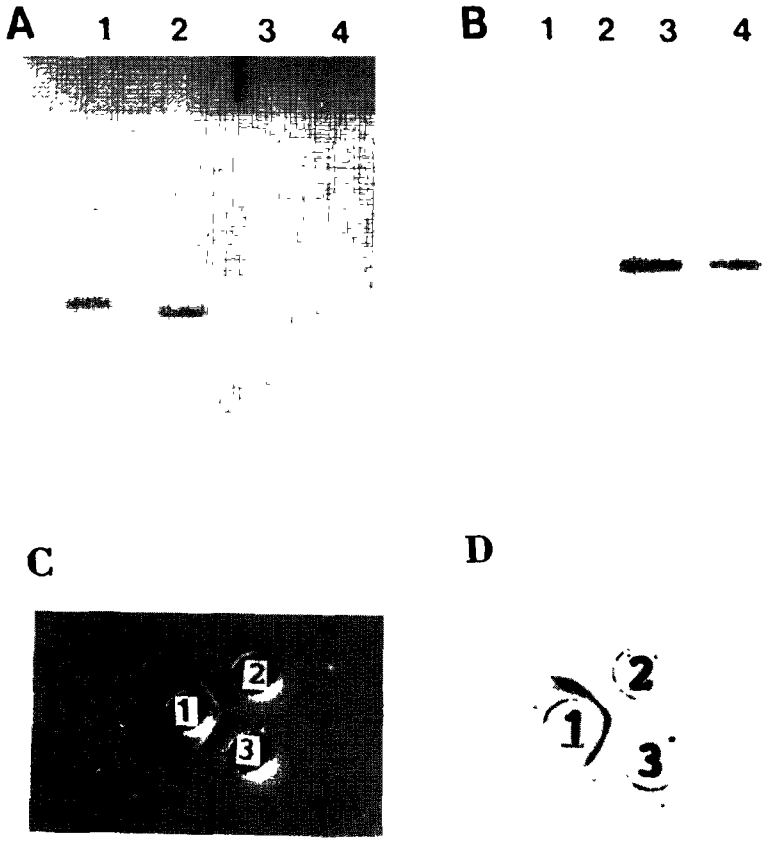

Fig. 2. Western blot (A and B) and Ouchterlony double diffusion test (C and D) of F, S, HγI and HγII preparations, using specific anti-serum against S (A) and F (B and C) or HγI (D). Lanes of both (A) and (B): 1, S; 2, HγII; 3, F; 4, HγI. For double diffusion test, the well, No. 1 of (C) and (D) contain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

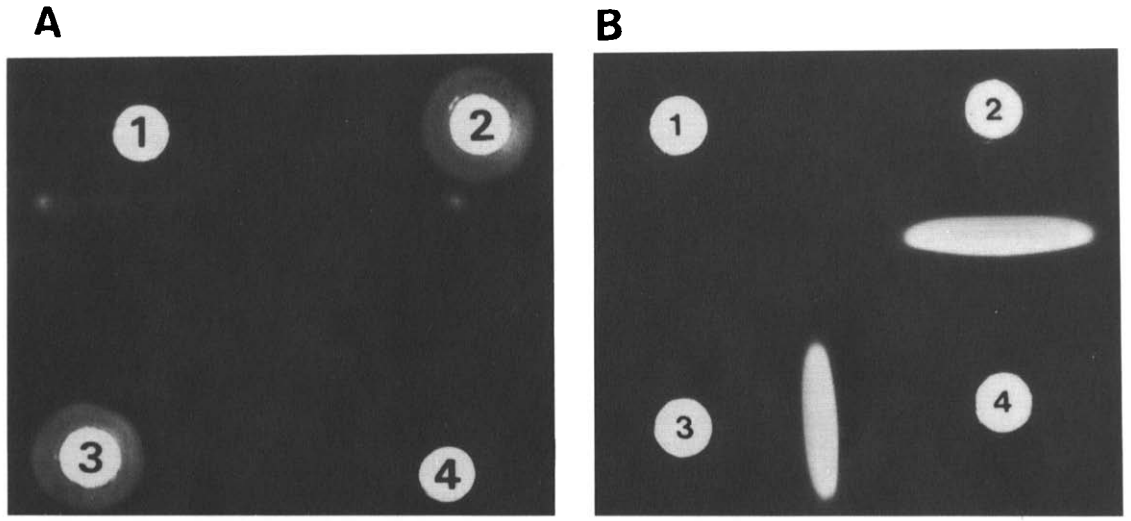

Fig. 3. γ-Hemolysin synergism on human blood agarose plates, (A) and (B). The human blood agarose plate was prepared by the method of Plommet [2]. Plate A: 1, F + S; 2, HγI + HγII; 3, F + HγII; 4, HγI + S. Plate B: 1, cloned S; 2, cloned F; 3, HγI; 4, HγII. The concentration of each component was adjusted to 100 ng/μl in Buffer A. The amount of each component applied to the well was 3 μg. 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 HγII for leukocidin S component*

The findings described above suggest that leukocidin F and γ-hemolysin HγI components are identical. If F is a common factor for both leukocidin and γ-hemolysin, and if the leukocidin- and γ-hemolysin-specific activities are determined by S and HγII, leukocidin activity should change to hemolytic activity by substituting HγII for S in combination with F. Accordingly, we examined the effect of S and HγII on the specificity of toxin activity in combination with leukocidin F component. A synergistic action of F and HγII caused hemolysis of human red blood cells (Fig. 3A). Cloned F also had hemolytic activity in combination with HγII (Fig. 3B). HγI 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 = HγI) in common and the leukocidin- and γ-hemolysin-specific activities are determined by S and HγII, respectively. We have reconfirmed the above conclusion by cloning and nucleotide sequencing of the γ-hemolysin gene(s) (submitted elsewhere).

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