Protein Characterizations and Immunological Properties of the Low-Molecular-Mass Protein A Isolated from *Staphylococcus aureus* KS 1034

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ABSTRACT. The extracellular low-molecular-mass protein A isolated from *Staphylococcus aureus* KS 1034 was analyzed for its amino acid contents and terminal amino acid sequences. The C-terminal and the N-terminal amino acid sequences of the low-molecular-mass protein A were -Asn-Ala-Phe and Ala-Gln-His-Asp-Glu-Ala-Gln-, respectively. For immunochemical properties, the low-molecular-mass protein A was similar to protein A isolated from *S. aureus* Cowan I. Extracellular protease activity of KS 1034 strain was considerably lower than *S. aureus* C-30 isolated from chicken that produced the extracellular low-molecular-mass protein A.—**KEY WORDS:** extracellular protein A, *Staphylococcus aureus*.

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Most strains of *Staphylococcus aureus* contain protein A [3, 24] which is covalently linked to the cell wall [2, 14, 18, 19] or released extracellularly [3, 8, 11, 15]. In our previous report, we described the purification of the extracellular low-molecular-mass protein A isolated from *S. aureus* KS 1034 strain, a mutant of *S. aureus* Cowan I [8]. The mutant is useful for producing high levels of the low-molecular-mass protein A (102–205 µg/ml). On the other hand, we isolated the extracellular low-molecular-mass protein A from *S. aureus* isolated from chickens that digested cell-bound protein A with extracellular protease [9]. In the present report, some chemical and immunological properties of the low-molecular-mass protein A are described and evaluated for digesting cell-bound protein A with extracellular protease of KS 1034 strain.

MATERIALS AND METHODS

Bacterial strains: The strains of *S. aureus* used were KS 1034 isolated by mutagenesis from Cowan I [8], C-30 isolated from chicken (provided by Dr. S. Takeuchi, Department of Agriculture, Fukui Prefectural College) [9] and Cowan I.

Protein characterizations: The low-molecular-mass protein A was prepared as described in our previous report [8], and lyophilized. In the preparation of the lyophilized protein A, a minor protein with molecular weight of 24 kDa was observed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Therefore, we purified the

major band of 27 kDa as follows. The lyophilized protein A (2 mg) was dissolved in 1 ml of 10 mM Tris-HCl buffer (pH 6.8) containing 1% (wt/vol) of SDS and 20% (vol/vol) of glycerol, and analyzed by SDS-PAGE according to the method of Weber and Osborn [23]. After the electrophoresis, the low-molecular-mass protein A in a band (27 kDa) of gel was extracted by the method of Tsugita *et al.* [1, 21]. Amino acid composition of the low-molecular-mass protein A was determined according to the method of Spackman *et al.* [20] by an ATTO MLC-203 amino acid analyzer. C-terminal amino acids of the low-molecular-mass protein A were determined by digestion with carboxypeptidase Y (Takara Shuzo Co., Ltd.) in 0.1 M-pyridine acetate buffer, pH 5.3 [5, 6]. A 64 nmol of the protein was digested with carboxypeptidase Y [enzyme substrate ratio, 1/33 (w/w)] at 30°C for 10, 30 and 50 min. The reaction was terminated by heating the reaction mixture in a boiling water bath for a few minutes. The pyridine acetate buffer was removed by sparging N₂ gas. The samples were dissolved in 0.1 N-HCl and applied to the amino acid analyzer. N-terminal amino acid sequence of the low-molecular-mass protein A was determined by a PQS-1 protein sequencer equipped with an on-line PHT identification system (Shimadzu Co., Ltd.).

Immunochemical properties: The low-molecular-mass protein A and protein A isolated from Cowan I were tested for the reactivity to human immunoglobulins [IgG (Organon Teknika Corporation), IgM (Organon Teknika Corporation), IgE (Chemicon International, Inc.) and IgA (Organon Teknika

Corporation)], normal human serum and several normal animal sera (dog, pig, rat, rabbit and chicken) by the Ouchterlony's diffusion test [17]. For the human immunoglobulins, dot immunobinding assay was also performed according to the modified method of Jahn *et al.* [7]. Nitrocellulose membrane filters (Bio Rad Lab.) were rinsed with distilled water for 5 min and air-dried. Each of the human immunoglobulins was applied to the respective filter sheets in a small amount. After drying, the sheets were immersed in 10% (vol/vol) acetic acid / 25% (vol/vol) isopropyl alcohol for 10 min for fixation. Subsequently, the sheets were rinsed several times with distilled water, and preincubated in Tris-buffered saline (200 mM NaCl / 50 mM Tris-HCl, pH 7.4) for 5 min. The sheets were then incubated for 1 hr in Tris-buffered saline containing 0.5% (wt/vol) of gelatin. The sheets were then incubated for 2 hr in Tris-buffered saline containing the low-molecular-mass protein A or protein A (5 μ g/ml) and 0.5% of gelatin, and rinsed three times with Tris-buffered saline containing 0.05% (vol/vol) of Tween 20 for 10 min, respectively. Next, the sheets were incubated for 2 hr in Tris-buffered saline containing peroxidase-conjugated IgG (Organon Teknika Corporation, 10 μ g/ml) and 1.5% of gelatin. Binding activity with the human immunoglobulins was tested with 4-chloro-1-naphthol by the method of Hawkes [4].

Extracellular protease: We previously reported a strain (C-30) isolated from chicken to produce the low-molecular-mass protein A which digested cell-bound type protein A with extracellular protease [9]. The strain of KS 1034 was also tested whether the extracellular protease digest cell-bound type protein A as described in the previous report [9]. The condensed culture solution of the KS 1034 strain, from which bacterial cell had been removed, was incubated with fluorescein isothiocyanate (FITC) protein A, and the sample was then analyzed by SDS-PAGE. The protein bands in SDS-PAGE gel were detected by irradiating with an ultraviolet lamp (254 nm).

RESULTS

Protein characterizations: The C-terminal sequence was identified as -Asn-Ala-Phe (Table 1). The N-terminal sequence of the low-molecular-mass protein A was identified as Ala-Gln-His-Asp-Glu-Ala-Gln-. Further, amino acid composition of the

Table 1. Release of carboxyl terminal amino acid residues from the low-molecular-mass protein A by carboxypeptidase Y

Incubation time (min)	Amino acid released (nmol per nmol substrate)		
	Phe	Ala	Asn
10	0.24	0.20	0.05
30	0.70	0.52	0.15
50	0.96	0.73	0.35

Table 2. Amino acid composition of the low-molecular-mass protein A purified from KS 1034 strain and protein A deduced from DNA sequence of 8325-4 strain

Amino acids	Protein A purified from KS 1034 strain	Protein A deduced from 8325-4 ^{a)}
Lysine	20	22
Histidine	1	2
Arginine	5	4
Aspartic acid	49	54
Threonine ^{b)}	2	1
Serine ^{b)}	16	17
Glutamic acid	49	49
Proline	12	12
Glycine	9	6
Alanine	26	27
Valine ^{c)}	7	3
Methionine	3	3
Isoleucine ^{c)}	8	7
Leucine	25	23
Tyrosine	3	4
Phenylalanine	12	12

a) Deduced amino acid composition is based on the DNA sequence from the gene of strain 8325-4 [22].

b) Linear extrapolation to zero time.

c) The highest value taken from the two hydrolysates.

low-molecular-mass protein A was shown in Table 2. The protein was not analyzed for tryptophan and half-cysteine. The most probable numbers of amino acid residues of the low-molecular-mass protein A was 247.

Immunochemical properties: We studied binding activity of the low-molecular-mass protein A to the human immunoglobulins and the normal animal sera, referring to the results published previously [9]. It was found that the binding patterns were similar to those of the protein A isolated from Cowan I by the Ouchterlony's diffusion test and the dot immunobinding assay (Table 3). In contrast, the protein A isolated from Cowan I formed a spur with the low-molecular-mass protein A against human, dog, and pig sera, although it formed no spur against IgG, IgM, IgE and rat serum (Table 3). Furthermore, the low-molecular-mass protein A

Table 3. Binding patterns of the low-molecular-mass protein A and Cowan I protein A with human immunoglobulins and animal sera

		Low-molecular-mass protein A		Cowan I protein A	
		Ouchterlony precipitations	Dot blotting	Ouchterlony precipitations	Dot blotting
Human IgG		+ ^{a)}	+ ^{c)}	+	+
Human IgM		+	+	+	+
Human IgE		+	+	+	+
Human IgA		— ^{b)}	+	—	+
Human serum		+		(+) ^{d)}	
Dog serum		+		(+)	
Pig serum		+		(+)	
Rat serum		+		+	
Rabbit serum		—		—	
Chicken serum		—		—	
Chicken antiserum of Cowan I protein A		+ ^{e)}		+	

a) Precipitation line was formed.

b) Precipitation line was not formed.

c) Spot was stained with 4-chloro-1-naphthol.

d) Precipitation line was formed and spur was formed with the low-molecular-mass protein A.

e) Precipitation line fused with that of Cowan I protein A.

Fig. 1. Protein A-FITC digested by extracellular enzymes from KS 1034 and C-30 strains. Lanes: 1, KS 1034 strain; 2, C-30 strain; 3, Relative molecular mass standards (94, 67, 43, 30, 20.1, and 14.4 kDal. from top to bottom) stained in Coomassie Brilliant Blue R-250.

showed a fused precipitation line with the protein A isolated from Cowan I against chicken antiserum of Cowan I protein A (Table 3).

Extracellular protease: Digested fluorescent bands of protein A-FITC were observed in the lane of C-30 strain (Fig. 1, Lane 2). But no clear fluorescent band of protein A-FITC with lower molecular mass was observed in the lane of KS 1034 strain (Fig. 1, Lane 1).

DISCUSSION

The N-terminal amino acid sequence of the low-molecular-mass protein A was identical to the amino acid sequence deduced from the DNA sequence of *S. aureus* 8325-4 [22], but differs from that of *S. aureus* A676, a methicillin-resistant strain, which shows Ala-Gln-Asp-Glx-Lys- [11]. The C-terminal sequence of the low-molecular-mass protein A, -Asn-Ala-Phe, appears in the amino acid sequence of the protein A isolated from *S. aureus* 8325-4 [22]. Though a direct comparison of amino acid composition between purified and deduced proteins is difficult due to strain difference, amino acid composition of the low-molecular-mass protein A (Table 2) were nearly identical to that expected from the DNA sequence corresponding to 246 amino acids of mature type of protein A isolated from strain 8325-4 [22]. Hence, it is indicated that this low-molecular-mass protein A lacks in a C-terminal region which is responsible for binding to the cell wall [11, 18] and thus is secreted extracellularly.

No antigenic difference was found between the low-molecular-mass protein A and protein A isolated from Cowan I. These results indicate that both of the protein A have the same antigenic determinants. The binding patterns of the low-molecular-

mass protein A to the human immunoglobulins and the normal animal sera were similar to those of the protein A isolated from Cowan I. The relative activity of IgG from different species with the protein A is rabbit ~ human > pig > dog > rat and chicken IgG has no significant binding activity in the fluid phase reaction [10]. It is not considered that, because a rabbit IgG forms only a soluble complex with protein A [10], the protein A in this experiment formed the precipitation line in the Ouchterlony's diffusion test. It was elucidated that the precipitation line formed by protein A isolated from Cowan I against normal human, dog and pig sera formed a spur with the low-molecular-mass protein A by the Ouchterlony immunodiffusion test. Masuda *et al.* [12, 13, 16] also reported the spur formation among two forms of extracellular protein A produced by mutants from Cowan I against normal canine serum. Although the productivity of extracellular protein A by KS 1034 strain [8] was higher than that by C-30 strain [9], no clear fluorescent band of protein A-FITC with low molecular mass was observed in the lane of SDS-PAGE for the strain KS 1304. These data suggest that the low-molecular-mass protein A isolated from KS 1034 was not produced by digesting cell-bound protein A with extracellular protease.

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