大韓獸醫學會誌(1999) 第39卷 第5號
Korean J Vet Res(1999) 39(5) : 1028~1032

# Production and partial purification of *Staphylococcus aureus* alpha toxin

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(Received Oct 19, 1999)

**Abstract** : Alpha toxin of *S aureus* has cytolytic activity respectively. This antigen has been received the most attention since it is a major virulence factor in pathogenesis of staphylococcal mastitis. Thus, alpha toxin has been focused as potential candidate of vaccine to minimize mastitis in cows.

The purpose of this study was to develop a simple, efficient production and purification methods of sufficient amount of alpha toxin antigen from *S aureus*.

Alpha toxin production measured by hemolytic activity was the highest at 18 hrs postinoculation in yeast extract culture medium supplemented with thiamine, nicotinic acid and casamino acid. Alpha toxin was purified by ammonium sulfate precipitation (65%) and ultrafiltration. Molecular weight of the toxin was 33 kDa in the analysis with SDS-PAGE.

Conclusionally, when alpha toxin was included in the vaccine, the optimal harvest time of alpha toxin was at 18 hrs after inoculation in yeast extract medium supplemented with thiamine and nicotinic acid.

Key words : alpha toxin, *Staphylococcus aureus* , production, purification.

## Introduction

MASTITIS is one of the major economic problems in dairy industry[11,12]. Although the disease is multifactorial, *S aureus* has been considered as the primary agent and it produces several virulence factors such as CPS[4-6] and alpha toxin[8].

Alpha toxin is an exotoxin produced by many strains of *S aureus* from cows. As described by Wood[14], alpha toxin has specific cytotoxic activity on phagocytic cells, neutrophils and macrophages. Since the majority of *S aureus* strains isolated from bovine mastitic milk produce toxins, the role of this toxin in the pathogenesis of bovine mastitis is clear. Re-

본 연구는 한국과학기술부의 1996년도 특정연구개발사업 연구비 지원에 의하여 수행되었음.

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moval of *S aureus* from the mammary gland is accomplished mainly via phagocytosis. Thus, the capability of alpha toxin to kill phagocytes may prolong the infection by reducing the bacterial clearance rate. *In vitro* studies have shown that specific alpha toxin antibodies were able to neutralize the toxin and prevented cytolytic effects on phagocytes[2,3,12]. Therefore, the presence of specific antibodies in milk may be useful in preventing the killing of phagocytes by alpha toxin.

The purpose of this study was to find the optimal conditions for production and purification of alpha toxin with simple, efficient and large scale.

## Materials and Methods

**Bacteria** : *Staphylococcus aureus* Wood 46 strain (ATCC 10832) was used for the production of alpha toxin. This strain was obtained by the courtesy of Dr. Per Jonsson (Section of Bacteriology and Epizootology, Swedish University, Uppsala, Sweden).

**Growth medium for alpha toxin production** : Medium used for alpha toxin production was the yeast extract broth supplemented with casamino acid (20 g) (Difco, USA), D-glucose (2.5 g) (Sigma, USA), thiamine (0.13 mg) (Wako chemicals, Japan), nicotinic acid (1.2 mg) (Wako chemicals). DW was added to this medium to final volume to 1.0 L. The components was dissolved and adjusted pH to 7.5 with 1 to 2 ml of 10 N NaOH.

**Preparation of crude alpha toxin** : Seed culture of *S aureus* was performed aerobically in 100 ml of tryptic soy broth (30 g/liter, pH 7.2) (Difco) in 0.5 liter flasks on a rotary shaker (120 rpm) at 37℃.

After an exponentially growing preculture, 4% (v/v) inoculum was incubated. After 18 hrs incubation, the supernatant of broth culture medium was harvested by centrifugation (10,000×g) (Beckman, USA) for 20 min at 4℃ as the method of Lind *et al*[10] and Harshman *et al*[7]. Briefly, the supernatant was filtered through 0.45-μm filter unit (Nalgene, USA), followed by 0.22-μm filter unit (Nalgene). The total protein concentration of the supernatant was determined by MicroBCA Protein Assay Reagent Kit (Pierce, USA). Supernatants were collected and stored at 4℃ immediately. Solid ammonium sulfate (Wako chemicals) was added (65% saturation) to the supernatant and the precipitate was kept for 2 hrs in a cold room. The precipitate was collected by centrifugation at 4℃ for 15 min at 16,000×g and stored at -20℃ as a stock of crude toxin.

For purification of alpha toxin, the thawed precipitate was dialyzed in dialysis bag (MW: 15,000) (Spectrum, USA) overnight against 10 mM sodium acetate buffer (pH 5.0) containing 20 mM NaCl. Other precipitated proteins were removed by centrifugation at 12,000×g for 20 min at 4℃. The clear supernatant was filtered through a 0.22-μm membrane filter (Nalgene). Finally crude alpha toxin supernatant was collected and concentrated by ultrafiltration apparatus with 10 kDa cutoffs (Amicon, USA)

**Assay of hemolytic activity** : The hemolytic assay was performed as described by Bernheimer and Schwartz[1]. One ml of alpha toxin was diluted to 1 ml with PBS and then one ml was taken for serial two-fold dilution in PBS. One ml of 1% (v/v) PBS-washed rabbit erythrocytes was added to 1 ml of diluted toxin and the dilution series were incubated at 37℃ for 30 min. The tubes were then centrifuged for 5 min at 3000 rpm to remove cells, and the optical densities of the supernatants were determined at 545 nm (Skan soft I, USA). One hundred percent hemolysis was determined from a tube containing 0.1% (w/v) sodium dodecyl sulfate (Sigma). The tube representing 50% hemolysis was also determined spectrophotometrically (Skan soft I) at 412 nm. One hemolytic unit is an amount of alpha toxin producing 50% lysis. The concentration of hemolytic units in a sample assayed was calculated from the known dilution factors. Usually, interpolation of values between adjacent assay tubes was done. Rabbit erythrocytes were obtained by cardiac puncture and were stored in Alsever solution (pH 6.9).

**Electrophoresis and western blot** : The crude alpha toxin was analyzed by 12% SDS-PAGE described by Laemmli[9]. Electrophoresis was carried out as follows. After centrifugation of culture supernatant, 50 μl (1 μg of total protein) of sample was loaded on each well. And after concentration of 2 L of culture supernatant, 100 μl (100 μg of total protein) of alpha toxin concentrate was loaded.

Amperage was at 100 mA for 10 min until the dye was moved into the resolving gel and decreased amperage to 50 mA and run the gel until the bromophenol blue reaches the bottom of the resolving gel. After electrophoresis, gels were stained with Coomassie brilliant blue (Bio-Rad, USA).

After electrophoresis without staining, the gels were electroblotted on nitrocellulose membranes (Millipore, USA) and blocked with gelatin (Sigma). And then reacted with a monoclonal antibody specific to alpha toxin (Sigma). Peroxidase conjugated anti-rabbit IgG (Sigma) was added and then reacted with 4-chloro-1-naphthol as substrate peroxidase (Bio-Rad).

## Results

Growth medium for alpha toxin production was the yeast extract medium supplemented with casamino acid, glucose, thiamine and nicotinic acid.

Comparisons of number of bacterial cells, protein concentration and hemolytic units (HU) of alpha toxin were shown in Table 1. *S aureus* strain Wood 46 exhibited exponential growth in the first 11 hrs when grown aerobically on yeast extract medium. Hemolytic activity was peaked at 18 hrs and protein concentration was the highest at 8 hrs after inoculation.

Table 1. Comparisons of bacterial growth rate, protein concentrations and alpha toxin hemolytic activity after inoculation in yeast extract medium

| Postinoculation | A(cells/ml) | B(mg/ml) | C(HU/ml) |
|---|---|---|---|
| 0 hr | $2.3\times10^{2}$ | 9.5 | 0 |
| 8 hrs | $1.7\times10^{7}$ | 13.0 | 24 |
| 11 hrs | $2.2\times10^{8}$ | 12.0 | 18 |
| 16 hrs | $3.5\times10^{8}$ | 11.5 | 48 |
| 18 hrs | $16\times10^{10}$ | 9.0 | 96 |
| 33 hrs | $3.2\times10^{10}$ | 7.0 | 36 |
| 48 hrs | $2.7\times10^{10}$ | 8.0 | 12 |

A : number of bacterial cells.
B : total protein concentration of 1 ml culture medium.
C : hemolytic activity using 1% rabbit erythrocyte suspension was measured at 412nm periodically (end-point method).

Other proteins other than 33 kDa of alpha toxin were present (Fig 1). The crude toxin was harvested after 18 hrs. After dialysis of precipitate with ammonium sulfate (65% saturation), insoluble proteins were removed by ultrafiltration. Alpha toxin band after concentration of ultrafiltration was present at 33 kDa (Fig 2).

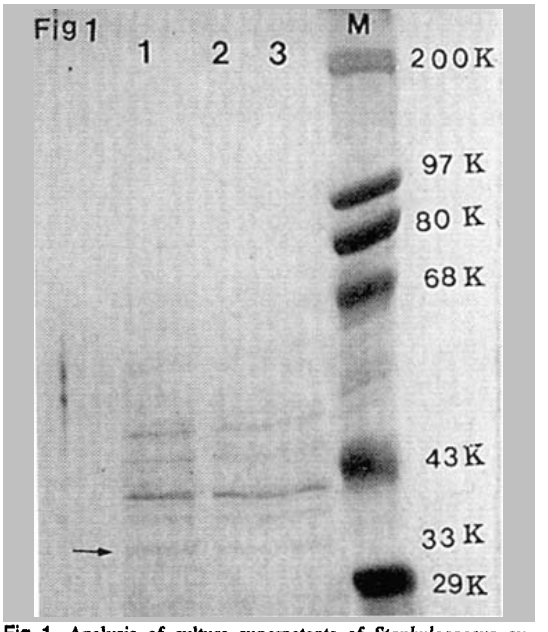


Fig 1. Analysis of culture supernatants of *Staphylococcus aureus* Wood 46 with sodium dedecyl sulfate-polyarcrylamide gel electrophoresis after baterial cells were removed by centrifugation. Alpha toxin bands (closed arrow) were shown at approximately 33 kDa. Lane 1 : culture supernatant after centrifugation (3,000×g), Lane 2 : culture supernatant after centrifugation (10,000×g), Lane 3 : culture supernatant after centrifugation (16,000×g), M : standard molecular marker.

Alpha toxin was also observed at 33 kDa in the analysis of western blot. The yield of alpha toxin was 2.0-4.5 mg/liter of culture medium.

## Discussion

The highest hemolytic activity of alpha toxin was observed at 18 hrs postincubation. This result of the study agrees with a report of Lind *et al*[10]. *S aureus* showed exponential growth in the first 11 hrs when grown aerobically on yeast extract broth. Toxin production also started during

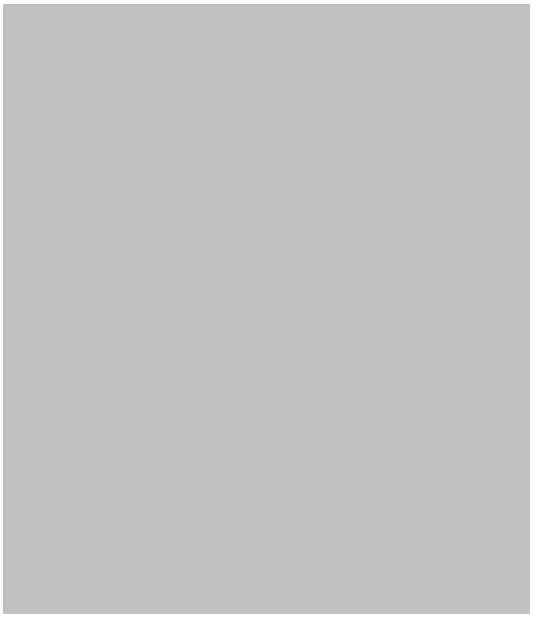

Fig 2. Analysis of purified alpha toxin of *Staphylococcus aureus* after ammonium sulfate concentration and ultrafiltration. Thick alpha toxin band (closed arrow) was shown at approximately 33 kDa. M : standard molecular marker.

this period. But protein concentration was not continually increased for 48 hrs. Our study results showed that protein concentration was slightly decreased, compared with that at 8 hrs after inoculation. This may be due to destruction of protein by a variety of proteinase secreted by bacterial cells. This postulation was also suggested by Lind *et al*[10]. Thus, when this toxin from culture supernatant was used in vaccine preparation or when alpha toxin was added to the vaccine by hemolytic units, because harvest after 48 hrs may cause the destruction of alpha toxin by several proteinases. it is thought that the optimal harvest time of alpha toxin preparation will be at 18 hrs after inoculation. While, Opdebeeck and Norcross[13] had used alpha toxin in vaccine as protein concentration at 48 hrs after inoculation. This earlier experiment disaggrees with results of my study.

One advantage of the procedure described here is that partially purified alpha toxin can be obtained within 24 hrs by starting with dialyzed ammonium sulfate precipitate of the culture supernatant. Furthermore, the partially purified toxin was stable for several weeks at 4℃ after the addition of ammonium sulfate (65% saturation). Reportedly[10], although use of liquid nitrogen was not performed in this experiment, alpha toxin was stable for several months when alpha toxin was rapidly frozen in liquid nitrogen and kept at -20℃. A partially purification method of alpha toxin of this study was simpler than any other method. This purification method was not time consuming and laborious efforts. However, the confirmation step was necessarily needed when purified by ammonium precipitation method.

## References


1. Bernheimer AW, Schwartz LL. Isolation and composition of staphylococcal alpha toxin. *J Gen Microbiol*, 30:455-457, 1963.
2. Cifrian E, Guidry AJ, O'Brien CN, Marquardt WW. Effect of alpha-toxin and capsular exopolysaccharide on the adherence of *Staphylococcus aureus* to cultured teat, ductal and secretory mammary epithelial cells. *Res Vet Sci*, 58(1):20-5, 1995.
3. David AL, Neil LN. Enzyme-linked immunosorbent assay for detection of milk immunoglobulins to leukocidin toxin of *Staphylococcus aureus*. *Am J Vet Res*, 46:1728-1732, 1985.
4. Fattom A, Schneerson R, Karakawa WW, Fitzgerald D, Pastum I, Li X. Shiloach J, Bryla DA, Robbins JB. Laboratory and clinical evaluation of conjugate vaccines composed of *Staphylococcus aureus* type 5 and 8 capsular polysaccharides bound to *Pseudomonas aeruginosa* recombinant exoprotein A. *Infect Immun*, 61:1023-1032, 1993.
5. Fattom AI, Sarwar J, Ortiz A, Naso R. A *Staphylococcus aureus* capsular polysaccharide (CP) Vaccine and CP-specific antibodies protect mice against bacterial challenge. *Infect Immun*, 64(5):1659-1665, 1996.
6. Guidry AJ, Oliver SP, Squiggins KE, Erbe EF, Dowlen HH, Hambleton CN, Berning LM. Effect of anticapsular antibodies on neutrophil phagocytosis of *Staphylococcus aureus*. *J Dairy Sci*, 74:3360-3369, 1991.
7. Harshman S, Sugg N, Cassidy P. Preparation and Purification of Staphylococcal Alpha Toxin. *J Enzymol*, 165:3-7, 1988.

8. Herbelin C, Poutrel B, Gilbert FB, Rainard P. Immune recruitment and bactericidal activity of neutrophils in milk of cows vaccinated with staphylococcal alpha toxin. *J Dairy Sci*, 80:2025-2034, 1997.
9. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage $T_4$. *Nature*, 227:680-685, 1970.
10. Lind I, Ahnert-Hilger G, Fuchs G, Gratzl M. Purification of Alpha-Toxin from *Staphylococcus aureus* and Application to Cell Permeabilization. *J Enzymol*, 164:84-89, 1987.
11. Norcross NL, Opdebeeck JP. Encapsulation of *Staphylococcus aureus* isolated from bovine milk. *Vet Microbiol*, 8:397-404, 1983.
12. Opdebeeck JP, Norcross NL. Frequency and immunologic cross-reactivity of encapsulated *Staphylococcus aureus* in bovine milk in New York. *Am J Vet Res*, 44:936-988, 1983.
13. Opdebeeck J, Norcross NL. Enzyme immunoassay for detection of antibodies specific for staphylococcal alpha-haemolysin in bovine milk. *Res Vet Sci*, 30:83-86, 1981.
14. Wood AM. Assay of the two components of Staphylococcal leucocidin and their antibodies. *J Pathol Bacteriol*, 81: 63-68, 1961.