한국임상수의학회지 17(1) : 145-151(2000)
Korean J. Vet Clin Med. 17(1) : 145-151(2000)

Studies on the Production and Purification of Capsular Polysaccharide (CPS) of Staphylococcus aureus

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황색포도상구균의 Capsular Polysaccharide (CPS)의 생산 및 정제에 관한 연구

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요 약 : 황색포도상구균의 Capsular polysaccharide (CPS)는 백혈구의 탐식에 저항하기 때문에 젖소의 유방염 발생의 중요한 병원성 인자로 오랫동안 연구자들의 관심이 집중되어 왔다. 따라서 CPS는 젖소의 유방염을 최소화시키기 위한 유방염 백신개발에 있어 잠재적인 백신 항원물질로 알려져 왔다. 본 연구의 목적은 황색포도상구균의 CPS 항원물질을 좀더 효과적으로 발현시키고 향후 백신항원으로 사용시 좀 더 간편하게 정제하는 방법을 찾아보고자 본 연구를 수행하였다. CPS는 락토스와 덱스트로스를 첨가한 brain heart infusion media에서도 부분적으로 발현되었지만 CPS가 가장 잘 발현되는 배지 조건은 젖소 유래의 유청을 10% 첨가한 조건에서 가장 발현율이 높았다. 또한 CPS발현은 salt agglutination test, india ink법 및 투과전자현미경을 이용하여 확인하였으며 균체표면의 hydrophobicity에서는 락토스, 덱스트로스 및 젖소 유래의 유청을 첨가한 배지조건에서 다른 조건에 비해 높았다. CPS의 가장 손쉬운 검사법은 균체를 절편하지 않고 직접 투과전자현미경상에서 염색없이 관찰하는 것이 가장 간편한 방법이었다. CPS정제는 상층액을 농축시킨 후 이온교환크로마토그라피와 겔 여과법을 이용하여 정제하였으며 CPS의 분자량은 대부분 97 kDa이상이었다. CPS 회수율은 배지와 균체 1리터당 1.5 mg이었다. 결론적으로 CPS를 백신항원으로 포함하고자 할 경우, CPS의 최적 발현조건은 젖소 유래의 유청을 10% 첨가한 Brain heart infusion 배지에서 배양하여 사용하는 것이 가장 좋은 방법이라고 생각된다.

Key words : capsular polysaccharide, Staphylococcus aureus, purification

Introduction

Mastitis is one of the major economic problems in dairy industry[12,13]. Although the disease is multifactorial, *S aureus* has been considered as the primary agent and it produces several virulence factors such as CPS[2-5] and alpha toxin[6].

Of the virulence factors, attention has been paid to CPS since it can inhibit phagocytosis by neutrophils [9,17]. An earlier study[16] reported that encapsulated *S aureus* isolated from milk of cows with mastitis inhibited phagocytosis by neutrophils and capsule production was enhanced by serial passage through bovine mammary glands.

Therefore, the CPS has been considered as a potential candidate for a vaccine to reduce the incidence of mastitis in cows[14,15,18].

The authors wishes to acknowledge the financial support (198071-3) of the Agricultural R&D Promotion Center made in the promotion Center made in the program of 1999."

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

Materials and Methods

Bacterial strain

S aureus Smith strain (ATCC 49519) which highly produces CPS was used. The strain was kindly provided by Dr. Per Jonsson (Section of Bacteriology and Epizootology, Swedish University, Uppsala, Sweden).

Selection of optimal growth medium for CPS production

Several media were prepared as the method of Watson[20]. Media used for CPS production were the nutrient broth, BHI broth and Staphylococcus medium 110 (Difco). And BHI broth supplemented with 10 or 20% bovine milk whey, lactose (100 mg/ml) (Sigma), dextrose (100 mg/ml) (Sigma) and casein (20 mg/ml) (Sigma). These media were adjusted to pH 7.2.

Preparation of bovine milk whey

Milk from lactating quarters of healthy cows ($<10^5$ cells/ml) were defatted by centrifugation (4,000× g, at 4°C for 10 min) and skimmed milk was treated with rennin (0.05 μg/ml) (Sigma) at 37°C for 30 min. Curd was removed by centrifugation (20,000× g, at 4°C for 30 min) and the clear supernatant was adjusted to pH 7.0, and sterilized by passage through a 0.2 μm sterile filter (Millipore) and kept at 4°C.

Transmissible electron microscopy (TEM)

The production of CPS was confirmed by direct examination of the bacteria without section with TEM (Hitachi Ltd., Japan). Briefly, bacterial cultures were centrifuged (3,000× g, 15 min, 4°C), washed once with 0.9% (w/v) sodium chloride containing 0.01% bovine serum albumin (BSAS) and resuspended at a concentration of 10^9 cells/ml in BSAS[7].

From the above preparations approximately 5 μl was deposited on convex, 200 mesh, Formvar coated copper grids. The suspension was left on the grid for 3 min before the excess fluid was drawn off gently with blotting paper. After drying in air the grids were examined without further staining.

Salt aggregation test (SAT)

Cultures of *S aureus* were centrifuged (1,500 rpm, 10 min, 4°C) and the cells were washed once with sterile phosphate buffered saline (PBS, pH 7.2), then resuspended in sterile PBS and adjusted spectrophotometrically to a concentration of 5×10^9 cells ml/ml[7]. A 0.1 ml aliquot of the suspension was added to each well of a U-bottomed microtitre tray. Then 0.1 ml of sodium hydroxide solution prepared with 2-fold dilution was added to each well. These solutions were made by diluting 1.0 M sodium hydroxide with sterile PBS. A PBS control (without sodium hydroxide) was included. The tray covered and allowed to stand for 16 hrs at room temperature. And then the autoagglutination titre was read.

India ink test

This was performed by the method of Guidry et al.[5]. Briefly, *S aureus* were dispersed evenly in a loop of India ink (Difco) on a glass slide. A coverslip was placed over the mixture, and excess ink was blotted out to obtain a nearly transparent brown film. Slides were examined with phase contrast microscope (Olympus, Japan) to determine relative clearing around the each organism.

Analytical methods

The total protein concentration of the purified CPS was determined by MicroBCA Protein Assay Reagent Kit (Pierce). Nucleic acids were determined at 260 nm by GeneQuant II (Pharmacia Biotech, England).

The NaOCl-amylose-potassium iodide method of Sandford et al.[19] was used to detect CPS in column fractions. 0.4 ml of $NaHCO_3$-NaOCl reagent (0.025 M $NaHCO_3$ containing 50 ml of NaOCl solution per liter; pH adjusted to 9) was added to 0.2 ml of fraction samle. After 2 hrs at room temperature, 0.2 ml of 1.5% $NaNO_2$ (Sigma) was added. After 2 min, 0.2 ml of amylose-potassium iodide reagent was added (0.25 g of amylose, Sigma) and boiled for 1 hr in 100 ml of DW and centrifuged at 3,000× g for 20 min. And the supernatant was made 0.03 M potassium iodide. The

absorbance was measured at 615 nm after 10 min.

Determination of CPS concentration was measured by the colorimetric method of Sandford et al.[19]. Two ml of CPS solution was pippeted into a glass tube. 0.05 ml of phenol (Wako chemicals) was added and mixed well. Five ml of concentrated sulfuric acid (Wako chemicals) was added to the tube and stand for 10 min. the tube was shaken for 10-20 min in a water bath (at 25-30°C). The color was measured at 490 nm. Standard curve was drawn by dilution of D-glucuronic acid (100 mg/ml, Sigma).

Extraction of CPS

Bacterial cells grown on whey-supplemented bacterial broth (2 L) were autoclaved at 121°C for 60 min. Cells were pelleted by centrifugation (Beckman) at 25,000×g for 20 min, and the supernatant was pooled. The clear supernatant was filtered with 0.45 μm membrane filter (Millipore), followed by 0.22 μm membrane filter (Millipore). The filtrate was concentrated by polyethylene glycol (MW:8,000, Sigma) and lyophilized.

Purification of CPS

In order to remove nucleic acid and cellular proteins, the filtered solution was extracted with phenol : chloroform : iso-amylalcohol (75:25:1:v/v) (Sigma) after treatment with DNase (80 μg/ml, Sigma), RNase (80 μg/ml, Sigma) and Proteinase K (0.1 mg/ml, Sigma) overnight at 4°C. The solution was applied on anion-exchange chromatography Crude CPS was loaded on a column (2.6×30 cm, Pharmacia Biotech, Sweden) of DEAE-Sephacel (Sigma) equilibrated with 0.05 M sodium acetate buffer (pH 6.0) and eluted with sodium acetate buffer with a linear gradient from 0 to 0.5 M NaCl. The eluted materials were analyzed by a GeneQuant II(Pharmacia Biotech) at 260, 280 nm and 615 nm (Skan Soft I). Elutes containing CPS were pooled and concentrated with PEG 8000 (Sigma).

Detection of CPS

Fractions which contained CPS were detected by the method of capillary precipitation[9]. Briefly, the polyclonal antibody was mixed with melted agar solution in buffer at 45-50°C. The final agar concentration was 0.3-0.5% (w/v). This sufficient mixture was placed in a small pre-warmed glass test tube (3~5 mm internal diameter) to give a liquid column 30-50 mm high. Tubes was cooled so that gelation occurs and then equilibrated at the desired incubation temperature. The antigen sample either in free solution or incorporated in 0.3-0.5% agar was then added and the tubes sealed and incubated. And fractions which contained teichoic acid were detected by method of capillary precipitation with anti-teichoic acid serum provided by Dr. Per Johnson (Sweden Universtiy, Uppsala, Sweden)

Electrophoresis

The CPS was analyzed by 12% SDS-PAGE of Laemmli[10]. The CPS positive fractions confirmed by capillary precipitation were loaded on each well. Standard molecular marker (sigma) ranging 10 kDa to 96 kDa was used. Electrophoresis was run as follows. After anion chromatography, 100 μl (4 μg of CPS) of fraction sample was loaded. And gel filtration, 100 μl (0.2 μg of CPS) of fraction sample was loaded on each well. 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 silver stain kit (Strategene, USA).

Results

Optimal condition for production of CPS

S aureus was grown in several BHI media supplemented with whey, lactose, dextrose and casein, respectively for 24 hrs at 37°C with agitation at 300 rpm. CPS was partially produced in BHI supplemented with lactose and dextrose, respectively. But the highest production of CPS was observed in BHI supplemented with 10% bovine milk whey (Table 1).

Purification of CPS

CPS was purified from bacterial extracts and culture supernatant. The bacteria grown in the media was filtered twice after autoclaving. The filtrates were ex-

Table 1. Comparisons of capsular polysaccharide expression rate with three tests in various culture media.

Culture media	TEM[a]	India ink	Autoagglutination
Nutrient broth	<10[b]	-[c]	N[d]
BHI	<10	-	N
Staphylococcus medium 110	20~30	-	N
BHI + whey (10%)	>90	-	P[e]
BHI + whey (20%)	>80	-	P
BHI + lactose (100 mg/ml)	<20	-	P
BHI + dextrose (100 mg/ml)	<20	-	P
BHI + casein (20 mg/ml)	<10	-	N

[a]transmissible electron microscopy.
[b]percents which showed expression of capsular polysaccharide when counting 100 bacterial cells.
[c]*S aureus* showed no clear halo around the bacterial cells was considered negative.
[d]negative for autoagglutination.
[e]positive for autoagglutination

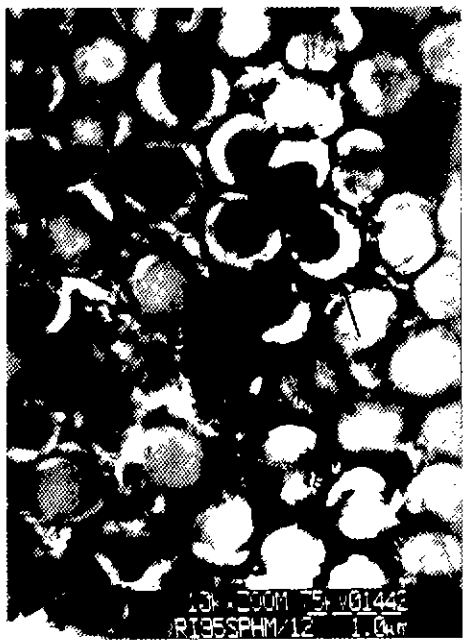

Fig 1. Transmissible electron microcopy of capsular polysaccharide of Staphylococcus aureus grown on brain heart infusion supplemented with 10% bovine milk whey. Large arrow : capsular polysaccharide-expressing cells. Small arrow : non-capsular polysaccharide-expressing cells.

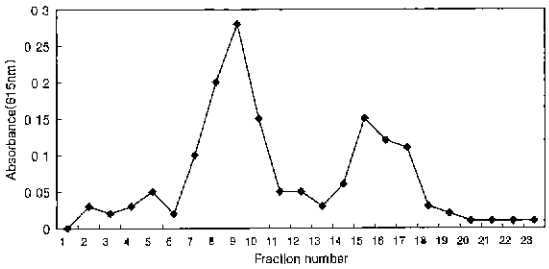

Fig 2. Anion exchange chromatoography after elution of a DEAE-Sephacel column in acetate buffer (pH 5.0) with increasing NaCl gradient (0.1 M to 0.5 M). Note fraction number (6-11) containing CPS and fraction number (13-18).

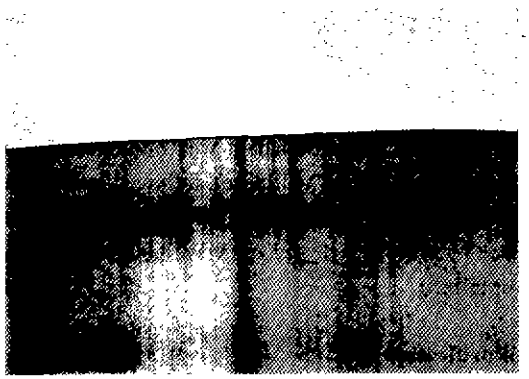

Fig 3. Analysis of elutes (6-11 of fraction number in Fig 2) from DEAE-Sephacel anion chromatography. The bands of capsular polysaccharide (closed arrow) were observed at approximately 97 kDa.

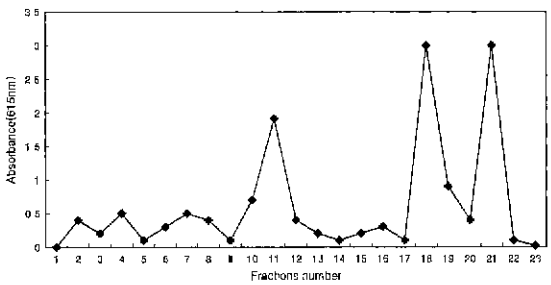

Fig 4. Analysis of Sephacryl S-300 HR column (1.5 × 90 cm) with 0.05 M sodium acetate buffer (pH 6.0). Note the fraction number (17-20) containing capsular polysaccharide.

tracted with phenol : chlorform : isoamyl alcohol after enzyme digestion. The solution was applied on anion-exchange chromatography, followed by concentration

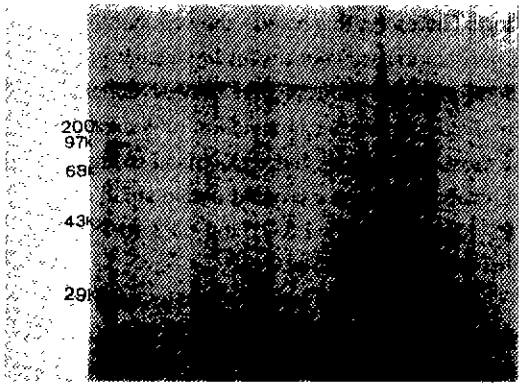

Fig 5. Analysis of elutes (17-20 of fraction number in Fig 4) from Sephacryl S-300 HR chromatography. The bands of capsular polysaccharide (closed arrow) were observed at approximately 97 kDa.

with PEG 8000 (crude CPS).

The elutes after anion exchagne chromatography showed two peaks. First peak containing CPS was at 6 to 11 of fraction number (Fig 2). On SDS-PAGE, CPS was present at 97 kDa (Fig 3). The elutes after gel filtration showed two peaks. First peak containing CPS was at 17 to 20 of fraction number (Fig 4). A major band of CPS was observed at 97 kDa by SDS-PAGE following by silver staining after removing teichoic acid from the solution by oxidation with sodium metaperiodate and purifying CPS by gel filtration (Fig 5). Concentration of crude CPS after autoclaving and enzyme digestion was 10 mg/liter of culture. The yield of purified CPS was obtained 1.5-2.5 mg/liter of culture. The CPS contained less than 0.3% of protein and 0.1% of nucleic acid.

Bacterial cell-surface hydrophobicity

Bacteria grown in BHI supplemented with several nutrients were determined by the salt aggregation test (Table 2). *S aureus* grown in the presence of milk whey showed its hydrophilic surface properties. BHI-grown bacteria exhibited hydrophobic surface properties. The surface of the bacteria was altered from hydro-phobic to hydrophilic in lactose or whey-supplemented media. Weak surface hydrophilicity was also observed in groups supplemented with thiamine and glucose.

Confirmation of CPS

The CPS expression of *S aureus* cultured in various media are shown in Table 2. Direct examination of the bacteria in TEM proved to be a reliable method for visualising CPS around the bacteria.

When *S aureus* was cultured in media supplemented with bovine milk whey or lactose, most bacteria showed a thick, well-defined CPS around the cell wall (Fig 1). In contrast, when this bacteria was cultured in other media, the CPS was absent or present only as a thin, marginal structure on some bacteria.

Discussion

The SAT has been used to measure the cell surface hydrophobicity of *S aureus* grown on various medium. This confirmation method provided a simple measure of CPS expression in bacterial suspension after incubation. There was correlation between expression of CPS by the organism and autoagglutination in the

Table 2. Autoagglutination test for detecting the degree of hydrophobicity in Staphylococcus aureus in various media .

Culture media	Molarity of sodium hydroxide (2-fold dilutions with 0.1 N NaOH)										
	1	2	3	4	5	6	7	8	9	10	11
BHI	-	-	-	-	-	-	-	-	-	-	-
BHI + thiamine (100 mg/ml)	+	+	+	+	+	+	+	+	+	-	-
BHI + dextrose (100 mg/ml)	+	+	+	+	+	+	+	+	+	+	-
BHI + whey (10%)	+	+	+	+	+	+	+	+	+	+	+
BHI + nicotinic acid (100 mg/ml)	+	+	+	-	-	-	-	-	-	-	-
BHI + lactose (100 mg/ml)	+	+	+	+	+	+	+	+	+	-	-

BHI : brain heart infusion broth.
+ : positive for agglutination.
- : negative for agglutination.

presence of sodium hydroxide. Thus, the autoagglutination assay was a useful preliminary test for expression of CPS by *S aureus* in culture.

In addition, it is suggested that, for testing CPS expression in vaccine cultures of *S aureus*, the autoagglutination test should be used in conjuction with examination of cells in the TEM.

For transmissible electron microscopy, an earlier report[20] washed one time with the below same buffer. However, in our study, bacterial suspension was washed twice with 0.9% saline containing 0.01% bovine serum albumin (BSAS). Since expressed CPS was fragile, it was required to stabilize the CPS with antiserum before washing the suspension. And also, when BSAS was made, albumin should be solubulized to reduce proteinous materials. And it was reliable method to use 200 mesh Fomvar coated grids when visualizing the CPS. Therefore, it was more helpful to visualize the CPS, after drying in air the grids without further section and staining. *S aureus*-expressed CPS had a clear halo around the cell wall and the CPS was very fragile during the TEM processing.

The bacteria grown in BHI supplemented with bovine milk whey showed more predominant CPS expression ability than the bacteria grown on casein-, dextrose- and thiamine-supplemented BHI. This results of the study aggrees with that in the experiment of Mamo et al.[11]. Presumably, CPS expression was disappeared in *in vitro* culture medium or subculturing on agar or broth but could be restored by growth in bovine milk whey. Thus this result suggested that growth in milk whey was an appropriate condition to induce the expression of CPS. In contrast, Dassy and Fournier[1] reported that CPS was produced by *S aureus* during the exponential phase of growth under all culture conditions and post-exponential-phase CPS production. This suggestion revealed that CPS expression was produced by carbon substrate.

As reports of Poutrel et al.[17] and International Dairy Federation[8], most CPS from bacteria was released by autoclaving. Thus CPS was obtained by centrifugation of autoclaved bacterial culture. In CPS purification, the method of enzyme digestion and column chromatography was simple. But this purification method with culture broth was time consuming and laborious effort. Therefore, this results showed that this method of purification was not useful in vaccine preparation. Therefore, it is thought that harvest collected from blood agar plate after inoculation of *S aureus* will be the most time saving and economical method in CPS preparation because we could obtain small amount of CPS (approximately 1.5-2.5 mg CPS of liter of culture) from broth cultrue.

The CPS and alpha toxin preparate could be important as antigen of mastitis vaccine. Because vaccine antigen with higher antigenicity in antigen preparation is crucial to increase the higher immune response in vaccinated cows, it is thought that alpha toxin with high hemolytic unit and highly expressed-CPS should be used in mastitis vaccine preparation.

Conclusion

Capsular polysaccharide was partially expressed in brain heart infusion media supplemented with lactose and dextrose. But production of CPS was the highestest in brain heart infusion medium supplemented with 10% bovine milk whey. Cell surface hydrophobicity was higher in brain heart infusion broth supplemented with lactose, dextrose and bovine milk whey than any other group.

Conclusionally, when CPS was included in mastitis vaccine, optimal growth medium of CPS expression was BHI broth supplemented with 10% bovine milk whey.

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