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Production and Purification of *Streptococcus pneumoniae* Hemolysin (Pneumolysin)

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Pneumolysin was found to be produced by 112 of 113 clinical isolates of *Streptococcus pneumoniae* and to be an intracellular hemolysin. A 10-liter-scale fermentor production and purification procedure was developed for this hemolysin. The culture was concentrated by filtration 10 times before centrifugation. The cellular content was purified by ion-exchange chromatography, covalent thiopropyl gel chromatography, and gel filtration. One batch operation resulted in 6 mg of highly purified pneumolysin, with a yield of 66% and a specific activity of 1,400,000 hemolytic units per mg. The pneumolysin had a molecular weight of 53,000 and an isoelectric point of 5.2. The purification method developed will be of value in future studies on this hemolysin.

Streptococcus pneumoniae (pneumococcus) produces a hemolysin which belongs to the family of sulfhydryl (SH)-activated hemolysins and is commonly referred to as pneumolysin (2–4, 7, 18–20). Few data are available in the literature about the frequency with which pneumococcal strains produce this hemolysin, but most strains appear to do so (11, 16, 17). The pneumolysin was shown to be an intracellular protein, but due to the autolysis phenomenon it is always detected extracellularly in broth cultivation as well (8).

The pneumolysin lyses erythrocytes from most species and is inactivated by oxidizing agents and activated by reducing agents; it thus belongs to the family of SH-activated hemolysins, with streptolysin 0 the most studied representative (1, 24). Pneumolysin also damages the cellular membranes of human lymphocytes, polymorphonuclear leukocytes, and platelets (5, 10). It activates the human complement system (22) and has been shown to affect the virulence of pneumococci in experimental infections (9, 21).

The molecular weight of pneumolysin was reported to 52,000 by Paton et al. (21), and Kreger and Bernheimer (15) reported an isoelectric point of 4.9. Specific hemolytic activities have all been reported to be about 500,000 hemolytic units (HU) per mg of protein, and reported purification yields were 10 to 25% (7, 21, 23).

To further investigate the properties of the pneumolysin and to use it as an antigen in a serological assay of pneumococcal disease, it was necessary to improve the methods of production and purification of this protein. Also, a larger-scale production method was needed. The aim of this study was thus to develop a 10-liter-scale cultivation method and to obtain a high yield of highly purified pneumolysin.

MATERIALS AND METHODS

Bacterial strains. From a collection of pneumococci at the National Bacteriological Laboratory (14), 93 strains of the most frequent capsular serotypes were cultivated and tested for pneumolysin production. Furthermore, 20 fresh clinical isolates were obtained from the routine laboratory.

Screening of hemolytic activity. Cultivation was performed without shaking at 37°C overnight (18 h) in 6 ml of brain-heart infusion broth (Difco Laboratories, Detroit, Mich.). After centrifugation, the hemolytic activity was determined in the supernatant and in the bacteria after suspension of the pellet and sonication for 60 s. The specificity of the hemolytic reaction was in all cases verified through inactivation by cholesterol (0.5 mg/ml) and oxidation by 0.25% hydrogen superoxide for 30 min.

Production of pneumolysin in fermentor. One fresh clinical isolate belonging to type 14 was selected because of its high pneumolysin production. The strain was stored frozen at −70°C until used. Brain-heart infusion broth supplemented with 1 g of glucose and 0.15 g of cysteine per liter, pH 7.2, was used in all cases. The stock strain was transferred onto a blood agar plate and incubated overnight at 37°C in 5% CO_2. One hundred milliliters of medium was inoculated with one loop of bacteria, cultivated for approximately 8 h at 37°C, transferred to 900 ml of fresh medium, and maintained for another 8 h in the same conditions. This culture was used to inoculate 9 liters of medium in a 12-liter fermentor (LKB, Solna, Sweden).

The bacteria were grown at 37°C with constant stirring. At the beginning of growth, the pH fell slowly and was then kept constant at 6.8 by gradual addition of 5 M sodium hydroxide. The density and hemolytic activity of the culture were measured at 60-min intervals. The bacteria were harvested about half an hour before maximum growth was reached, as measured by following the optical density (A_{620}) of the culture. The culture was concentrated by filtration (GVPP cassette; Millipore), washed twice on the filter with cold phosphate-buffered saline, pH 6.8, and centrifuged at 4°C (Sorvall RC 2-B, 7,000 × *g*, 15 min). The cells were suspended in 100 ml of Tris buffer (TB; 0.05 M Tris hydrochloride buffer, pH 7.5) and disrupted in an X-press (Biotec, Bromma, Sweden). After centrifugation (20,000 × *g*, 30 min), the pellet was washed twice with the same buffer, and the supernatants were pooled.

Purification. In all steps of purification 0.05 M TB pH 7.5, was used. The crude bacterial extract was adsorbed on a column (Pharmacia, C16/20) with DEAE-Sepharose Cl6B (Pharmacia) equilibrated with TB. After a wash with 1 bed volume of TB, the direction of the flow was changed and the column was washed again with 2 to 3 bed volumes of TB. The bound proteins were eluated with a linear gradient of 0 to 0.15 M NaCl. Fractions that exhibited hemolytic activities

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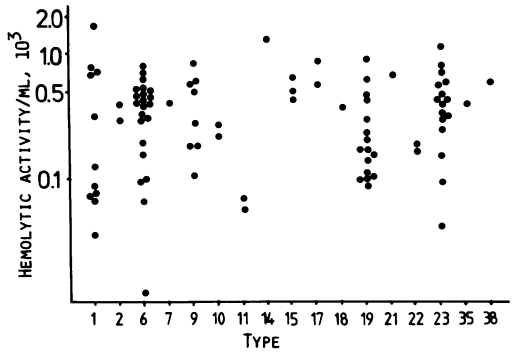

FIG. 1. Production of pneumolysin by 92 strains of the most frequent capsular serotypes. Each dot represents the hemolytic titer of the supernatant obtained for one strain. One strain of type 1 which did not produce pneumolysin is not included.

greater than 1,000 HU/ml were pooled. After this, the toxin was activated by 1 h of incubation with 10 mM dithiothreitol (DTT) at room temperature and concentrated about 15-fold by ultrafiltration (Amicon, PH 10 membrane) to 10 ml. To remove DTT and to increase the concentration of NaCl to 0.5 M, the concentrate was diluted to 50 ml with TB supplemented with 0.5 M NaCl and concentrated again. This procedure was repeated once, which decreased the concentration of DTT to less than 5 μmol. The concentrated sample was applied to a column (Pharmacia C10/10) with 8 ml of thiopropyl-Sepharose 6B (Pharmacia) at a flow rate of 2 ml/h. The column was washed with 10 to 20 ml of TB supplemented with 0.5 M NaCl, and then the pneumolysin was eluted with 20 mM DTT (flow rate, 5 ml/h). Fractions with hemolytic activity were collected, concentrated by ultrafiltration (PM10 membrane, Amicon, followed by Centricon 10) to about 1 ml and applied to a column (Pharmacia C16/100) with Sephacryl S-200, equilibrated with TB. The filtration was performed at a flow rate of 5 ml/h, and fractions that exhibited hemolytic activity were collected and stored frozen at −70°C.

Assay of hemolytic activity. Hemolytic activity was assayed by an interpolation procedure adapted for automatic reading and calculation by a microcomputer (K. Kanclerski and R. Möllby, Acta Pathol. Microbiol. Scand., in press). Serial twofold dilutions of the hemolysin were prepared with isotonic phosphate-buffered saline, pH 7.4, supplemented with 10 mM DTT and 0.1% albumin in microtiter plates with the help of an eight-channel pipette. Before dilution the toxin was activated by 15 min of incubation at room temperature with 10 mM DTT. The micropipette tips were changed after each dilution step.

To each well, 100 μl of a 1% (vol/vol) suspension of three-times-washed rabbit erythrocytes was added, and the plate was incubated for 30 min at 37°C. The hemolytic activity was calculated by reading the density of remaining erythrocytes at 620 nm in a vertical spectrophotometer (Titertec Multiskan MC; Flow Labs, Irvine, Scotland). Before the reading, the erythrocytes were resuspended by stirring with a pipette. A reference value for 50% hemolysis was obtained by reading the A_{620} of a mixture of 0.5% erythrocyte suspension and control buffer. One HU was defined as the dilution of the toxin that resulted in the same density of erythrocytes as the 50% control. This dilution was interpolated as follows: HU/ml = $D \times 2^x$ and $x = d + [A(50) - A(d)]/[A(d + 1) - A(d)]$, where d is the last dilution with an absorbance lower than the 50% control absorbance, $A(d)$ is the absorbance at dilution d, $A(d + 1)$ is the absorbance at dilution $d + 1$, $A(50)$ is the absorbance at 50% hemolysis, and D is the predilution value.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out on Pharmacia gradient gel PAA 4/30 in 0.09 M Tris-borate buffer, pH 8.35, containing 0.2% (wt/vol) sodium dodecyl sulfate (SDS) and 2.5 mM of EDTA. The sample was dissolved in the same buffer supplemented with SDS up to 2.5% (wt/vol) and DTT (15 mM) and heated for 5 min in a boiling-water bath. A total of 20 μg of the tested proteins was applied on the gel. As standards, a Pharmacia low-molecular-weight calibration kit was used.

Isoelectric focusing. Isoelectric focusing was performed on an LKB instrument. Ampholine PAG plate with a range of pH 3.5 to 9.5 and Pharmacia isoelectric focusing calibration kit (pH 3 to 10) as the standard as recommended by the manufacturer.

Protein determination. The amount of protein was determined with a Bio-Rad protein assay kit with human albumin grade A (KABI, Stockholm) as the standard.

RESULTS

All but 1 of the 113 pneumococcal strains investigated produced a hemolysin, which was inactivated completely by cholesterol and partly by mild oxidation (Fig. 1). There was no apparent difference between the capsular serotypes or between stored and freshly isolated strains.

After a cultivation time of 6 to 10 h the cells began to lyse and the hemolytic activity increased in the supernatant. Since cultivation for hemolytic screening was performed overnight (18 h), most of the activity was found in the supernatant. However, in all cases there was considerable intracellular activity as well.

During fermentor cultivation the intracellular pneumolysin titer reached its maximum between 5 and 8 h, about 3,000 HU/ml (Fig. 2). After this point, the extracellular pneumolysin titer increased rapidly, presumably due to autolysis of the bacteria. The change in pneumolysin titers is more clearly seen in Fig. 3, where the titer per optical density unit of the culture is plotted.

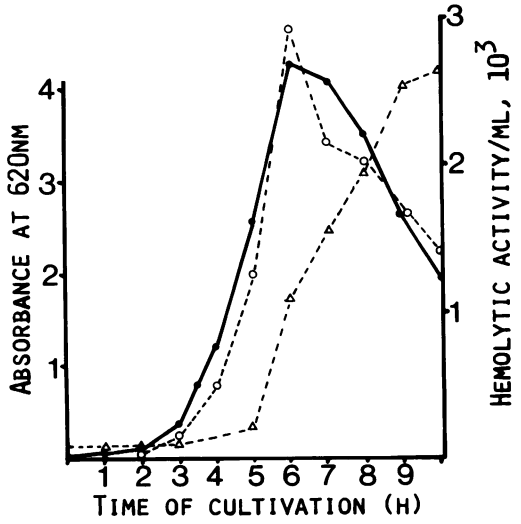

Fig. 2. Growth of *S. pneumoniae* and hemolysin production in fermentor. Symbols: ●, A_{620}; ○, hemolytic activity in cells; △, hemolysin activity (HU) in supernatant.

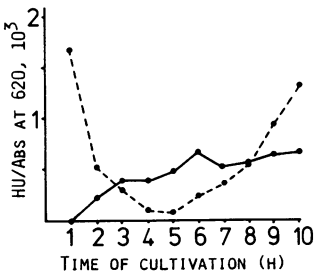

FIG. 3. Hemolytic activity per A_{620} unit of culture during cultivation in fermentor. Symbols: ——, intracellular pneumolysin; - - - -, extracellular pneumolysin.

During concentration and centrifugation of the culture, some of the cells were further disrupted, with concomitant loss of pneumolytic activity. The final crude bacterial extract contained 1.5×10^6 HU/liter of culture.

A simple three-step procedure for purification was used, each step relying on different properties of the pneumolysin. Ion-exchange chromatography on DEAE-Sepharose was used first. A reverse flow increased the yield to 85% in this purification step without decreasing the degree of purification (Fig. 4, Table 1). Affinity chromatography on thiopropyl-Sepharose was used. This method separates proteins through formation of covalent -S-S bridges between the thiol groups on the gel and the proteins. The proteins are then separated through reduction with DTT (Fig. 5). Finally, gel filtration by molecular size was performed, with an excellent yield.

This procedure resulted in a preparation with a specific activity of 1,400,000 HU/mg of protein, with a yield of 66% of the original activity present in the crude bacterial extract (Table 1).

The purified pneumolysin exhibited a single band in analytical SDS-polyacrylamide gradient gel electrophoresis (Fig. 6) at a molecular weight of 53,000. The isoelectric point was determined by isoelectric focusing in a polyacrylamide gel to be 5.2.

The preparation was completely devoid of hemolytic activity after incubation with cholesterol, and oxidation by 0.2% H_2O_2 inactivated 90% of its activity. The latter inactivation was reversed by reduction. These properties are consistent with those reported for the hemolysins of the SH-activated group.

TABLE 1. Purification steps

Step	Total activity (HU)	Total protein (mg)	Sp act (HU/mg)	Recovery (%)	Purification (fold)
Crude extract	1.3×10^7	1,560	8.1×10^3	100	
DEAE-Sepharose	1.1×10^7	334	3.2×10^4	85	4
Thiopropyl-Sepharose	8.5×10^6	47	1.8×10^5	67	22
Sephacryl S-200	8.4×10^6	6	1.4×10^6	66	173

DISCUSSION

The characteristic phenomenon for *S. pneumoniae* on the blood agar plate is the presence of a zone of green (alpha) hemolysis around the colony. However, the substance responsible for this effect is not the pneumolysin (11, 16). This can be shown by adding a drop of purified pneumolysin to a blood agar plate, which results in a zone of clear (beta) hemolysis.

All but one of the 113 strains tested in the present work were found to produce intracellular pneumolysin. Furthermore, Lorian et al. showed that 100 of 100 isolates of *S. pneumoniae* produce a beta-hemolytic zone when exposed to certain antibiotics (16). This hemolysis does not appear when the strains are cultivated so as to prevent autolysis. These facts suggest that practically all isolates produce the pneumolysin, without correlation to capsular serotypes.

During the fermentor cultivation the intracellular hemolytic titer per cell was almost constant. After maximum growth was reached the extracellular titer increased rapidly while the intracellular activity per optical density unit remained constant. These facts are in accordance with an intracellular localization of pneumolysin (8). This is in contrast to all other SH-activated hemolysins, which are extracellular (24).

Earlier purification methods for this hemolysin have used preparative electrophoresis as a last step (7, 21, 23). We did not use this method since it resulted in a poor yield.

A simple three-step method of purification used in the present work resulted in a high yield (66%) with a specific activity as high as 1,400,000 HU/mg of protein. The result could be compared only to those of Johnson et al. (12). The molecular weight of 53,000 determined in polyacrylamide gel electrophoresis was similar to that of Paton et al. (21). Other methods such as gel filtration and sucrose density gredient centrifugation have resulted in findings of different molecular

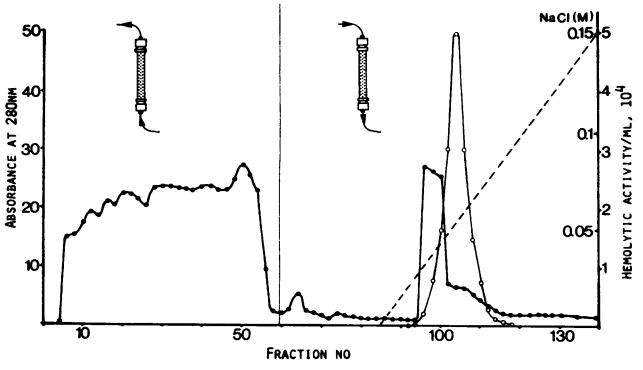

FIG. 4. Ion-exchange chromatography of pneumolysin on DEAE-Sepharose. Symbols: ●, A_{280}; ○, hemolytic activity (10^4 HU/ml); - - - -, NaCl gradient.

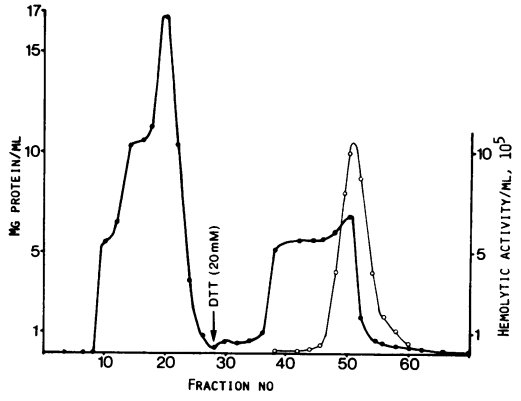

FIG. 5. Covalent chromatography on thiopropyl-Sepharose 6B. Symbols: ●, protein concentration; ○, hemolytic activity (HU/ml).

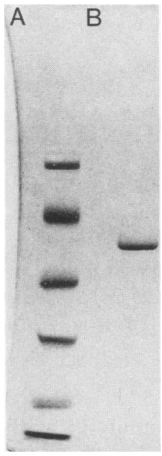

FIG. 6. SDS-polyacrylamide gradient gel electrophoresis of purified pneumolysin. Lane A, Molecular weight markers (phosphorylose b, 94,000; albumin, 67,000; ovalbumin 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100; α-lactalbumin, 14,400). Lane B, Purified pneumolysin (20 μg).

weights (15). The isoelectric point of 4.9 obtained by isoelectric focusing in a column (15) was similar to ours, 5.2.

In conclusion, we have developed a 10-liter-scale production and purification method for pneumolysin. The procedure results in 6 mg of highly purified pneumolysin per culture, with a specific activity of 1,400,000 HU/mg. Since the hemolysin seems to be produced by most, if not all, clinical isolates, the potential of the purified pneumolysin as an antigen in serological diagnosis of pneumococcal infections was investigated in further studies (13).

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