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Characterization of a Protein from Normal Human Polymorphonuclear Leukocytes with Bactericidal Activity against *Pseudomonas aeruginosa*

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Purification of a bactericidal protein (BP) from the cytoplasmic granules of normal human polymorphonuclear leukocytes (PMN) with activity against *Pseudomonas aeruginosa* is described. Bactericidal activity from acid extracts of a mixed granule population was purified 175-fold by a two-step chromatographic procedure. The first step, dye-ligand affinity chromatography with Matrex-Gel Orange A, was followed by cation-exchange chromatography with Bio-Rex 70 resin, and this combination routinely gave a yield near 80%. Only one peak of bactericidal activity against *P. aeruginosa* type I was found after each chromatographic step. Characterization of BP showed a single band with an apparent molecular weight of 55,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Purified BP was most active against the six strains of *P. aeruginosa* tested and against *Escherichia coli* B (a deep rough mutant). Purified BP killed 5×10^6 CFU of *P. aeruginosa* type I per ml at a concentration of 60 to 80 ng/ml. *Proteus mirabilis* and *Staphylococcus aureus* were both resistant to the bactericidal activity of BP. BP was shown to be glycosylated by periodic acid staining after gel electrophoresis and to have an isoelectric point near 7.5 by chromatofocusing. The amino acid composition of BP is presented.

Several laboratories have recently described nonoxidative killing mechanisms of human polymorphonuclear leukocytes (PMNs) active against gram-negative bacteria. The microorganisms sensitive to human PMN cytoplasmic granule extracts have included *Salmonella typhimurium* (4, 23, 25, 28–30), *Escherichia coli* (4, 23, 25, 28–30), *Bacteroides fragilis* (31), *Acinetobacter* sp. strain H01-N (15), and *Neisseria gonorrhoeae* (1, 3, 21). Activity against *S. typhimurium*, *E. coli*, and *N. gonorrhoeae* have been ascribed to proteins which have been purified from chronic myelogenous leukemia (CML) cells. The first was described by Weiss et al. (29) and was referred to as bactericidal/permeability-increasing protein (B/PI), and the second was described by Shafer et al. (23) and was referred to as cationic antimicrobial proteins (CAP). Both of these proteins displayed activity against *S. typhimirium* and *E. coli* (4, 25). It was also determined that granule extract activity against *N. gonorrhoeae* could be attributed to CAP, which has a molecular weight of 57,000 (23).

Here we describe a bactericidal factor from normal human PMNs, referred to as bactericidal protein (BP), which potently kills *Pseudomonas aeruginosa*. *P. aeruginosa* is of particular interest because it causes severe and often fatal infections among immunocompromised patients (17). High mortality rates are largely attributed to inadequate drug therapy because of the broad range of antibiotic resistance of *P. aeruginosa* (17, 19). One would predict that PMNs possess an effective mechanism of killing this bacterium because this ubiquitous, potential pathogen is kept in check, only causing infections as an opportunist when PMN numbers are inadequate or when these leukocytes are unable to reach the site of infection (19, 32). Mandell (14) has observed that, when incubated under anaerobic conditions, normal human PMNs kill *P. aeruginosa* as well as they do when they are incubated in an aerobic environment. We have shown that both normal PMNs incubated in a nitrogen atmosphere and PMNs from patients with chronic granulomatous disease kill *P. aeruginosa* as effectively as normal PMNs (9; N. K. Henry, Ph.D. dissertation, University of Minnesota, Minneapolis, 1980). The results suggest the presence of an important O_2-independent killing mechanism in PMNs against *P. aeruginosa*. This possibility is further supported by the rarity of *P. aeruginosa* infections among patients with chronic granulomatous disease (2, 6).

In this report we describe the purification of a protein with remarkable potency against *P. aeruginosa*. BP was acid extracted from a mixed population of cytoplasmic granules from normal human PMNs and purified approximately 175-fold by a two-step purification scheme by dye-ligand affinity chromatography and cation-exchange chromatography. Characterization of BP by molecular weight, isoelectric point, amino acid composition, and bactericidal potency are presented.

MATERIALS AND METHODS

Bacteria. All *Pseudomonas aeruginosa* strains, *S. typhimurium*, *Pseudomonas cepacia*, and *Proteus mirabilis* were purchased from the American Type Culture Collection, Rockville, Md., with the exception of *Pseudomonas aeruginosa* type I, which was a clinical isolate from the University of Minnesota Hospitals, Minneapolis, Minn. *Staphylococcus aureus* 502A and *E. coli* B, a rough strain, were obtained from Paul Quie, University of Minnesota, Minneapolis, Minn. Bacteria were maintained on blood agar or nutrient agar plates.

Isolation of human PMNs. Venous blood in acid citrate glucose anticoagulant was collected from normal healthy donors by the Red Cross Blood Bank (St. Paul, Minn.). Five volumes of acid citrate glucose venous blood were mixed with one volume of 6% clinical dextran in 0.9% NaCl and were allowed to settle at room temperature for 45 min. The leukocyte-rich plasma was removed and centrifuged at 200 ×

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g for 10 min at 4°C. Erythrocytes remaining in the cell pellet were lysed in two steps by isotonic (0.87%) ammonium chloride and by hypotonic shock with 0.0256 M NaCl (22). Leukocytes were suspended in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution for total cell count and for Wright stained smears. Differential cell populations were 82 to 89% PMNs, 2 to 11% lymphocytes, 0 to 8% monocytes, 0 to 9% eosinophils, and 0 to 1% basophils. Bactericidal activity was not found in the mononuclear cell populations.

Isolation of cytoplasmic granules. Leukocytes suspended in Hanks balanced salt solution were centrifuged at 200 × *g* and suspended at 5×10^8 cells per 2 ml of 0.34 M sucrose. Cell membranes were lysed by homogenization with a motor-driven Teflon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) pestle for two intervals of 3 min at 4°C. The homogenate was centrifuged at 200 × *g* for 10 min at 4°C to remove nuclei and cell debris. The supernatant, rich in cytoplasmic granules, was centrifuged at 8,700 × *g* for 20 min at 4°C. The supernatant was decanted, and the granule pellet was stored at −70°C.

Extraction of granule protein. Granules from 10^{10} PMNs were suspended in 6.0 ml of 0.01 N HCl and sonicated at 45 kHz for three intervals of 30 s each at 4°C to break the granule membranes. The acid extract was centrifuged at 120,000 × *g* for 60 min at 4°C to remove particulates. The supernatant, referred to as the granule extract, contained approximately 10 mg of protein per ml. Protein was measured by the method of Hartree (7), using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as a standard.

Bactericidal assay. Bactericidal activity was determined by a modification of the turbidimetric growth assay described by Muschel and Treffers (16). Bacteria at 5×10^6 CFU/ml in 0.08 M citrate-phosphate (CP) buffer (pH 5.6) were exposed to granule extract or purified BP. Each tube contained a total volume of 1 ml and was incubated in a reciprocal shaking water bath at 37°C. After 30 min, 5 ml of nutrient broth (Difco Laboratories, Detroit, Mich.) was added to provide growth medium for surviving bacteria. Turbidity was measured in a Coleman spectrophotometer at 650 nm when control tubes had an optical density (OD) between 0.3 and 0.4, the equivalent of mid-exponential growth. The percentage of bacteria killed by BP was determined with the following formula: $\%\ \text{death} = [(OD_{control} - OD_{test})/OD_{control}] \times 100$. A bactericidal unit was defined as that amount of protein required to kill 95% of 5×10^6 *P. aeruginosa* CFU/ml. This turbidimetric method was correlated with viable plate counts to ensure accuracy and to rule out bacteriostatic activity.

Affinity chromatography. Sterile solutions were used throughout the steps for purification of BP. Matrex-Gel Orange A dye-ligand agarose beads (Amicon Corp., Lexington, Mass.) were washed 3 times in CP buffer (pH 5.6) and poured into a column (1.7 by 2.5 cm). Granule extract from 2.4×10^{10} PMNs (approximately 130 mg of protein) was diluted 1:1 with distilled water, applied to the Matrex-Gel Orange A column, and allowed to bind at room temperature for 60 min. The column was washed with 150 ml of 0.1 M NaCl in CP buffer (pH 5.6) before elution with a salt gradient of 0.1 M to 1.6 M NaCl in CP buffer (pH 5.6; (total volume, 600 ml). Fractions of 5 ml were collected and stored at 4°C before determinations of protein, bactericidal activity, and enzymatic activity. Conductivities were converted to NaCl molarity with a standard curve.

Cation-exchange chromatography. Bio-Rex 70 (200-400 mesh) cation-exchange resin (Bio-Rad Laboratories, Rockville Centre, N.Y.) was converted with 0.5 N NaOH and washed thoroughly with CP buffer (pH 7.0). This was poured into a column (1.0 by 7.0 cm). The bactericidal fractions from four Matrex-Gel Orange A columns were pooled and diluted 1:2 with distilled water, and approximately 10 mg of partially purified BP was applied to the Bio-Rex 70 column. The column was washed with 100 ml of 0.08 M CP buffer (pH 7.0) before elution with a salt gradient from 0.1 to 0.7 M NaCl in CP buffer (pH 7.0; total volume, 600 ml). Fractions were collected and stored at 4°C before analysis of bactericidal activity, total protein, and conductivity. Fractions containing bactericidal activity were pooled, concentrated, and desalted with an ultrafiltration unit with a YM10 membrane (Amicon).

Quantitation of enzymes. Myeloperoxidase activity was determined as described by Klebanoff (11, 12) except that *o*-dianisidine dihydrochloride was used as substrate. Lysozyme was measured as described by Shugar (24).

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method described by Laemmli (13). A 5 to 15% polyacrylamide gradient-separating gel was used in combination with a 3% stacking gel. Approximately 1.0 μg of protein of purified BP was applied to the gel and electrophoresed at 15 mA of constant current for 4 to 5 h or until the bromphenol blue dye was at the bottom of the separating gel. Molecular weight standards for SDS-PAGE were purchased from Sigma.

Two gel techniques to determine the isoelectric point of BP were used. Preformed Ampholine PAGplates (pH 3.5 to 9.5) supplied by LKB Instruments, Inc. (Houston, Tex.), were electrophoresed for approximately 2 h with a constant current of 50 mA. A Sephadex G-50 gel bed mixed with a natural buffer system was prepared and electrophoresed as described by Prestidge and Hearn (20). Isoelectric focusing calibration kit standards (pI range, 5 to 10.5) purchased from Pharmacia Fine Chemicals (Piscataway, N.J.) were included in each electrophoresis gel.

Gel stain. SDS-PAGE gels were stained overnight in Coomassie blue R-250 protein stain (1.25 g of R-250 in 45% methanol–10% acetic acid) and destained in 30% methanol–8% acetic acid. Gels were washed free of destain with gentle agitation in distilled water for 2 h. Faint bands were then visualized by silver staining as described by Oakley et al. (18), except that 10 μl of 20% glutaraldehyde was added directly to the silver nitrate solution. The gels were stained for carbohydrate by the procedure of Fairbanks et al. (5). Before carbohydrate staining, BP was applied to nondenaturing discontinuous polyacrylamide gels, as described by Thomas and Hodes (26), and was electrophoresed with 15 mA of constant current for 3 h. Human PMN elastase and cathepsin G (a gift from N. Wehner, University of Minnesota) were included as positive controls, and cytochrome *c* (Sigma) was included as a negative control.

Chromatofocusing. Chromatofocusing was run from pH 9 to 6 as described in the instructions for use which accompanies the Pharmacia chromatofocusing kit. A column of Polybuffer exchanger (PBE 94; Pharmacia) was prepared and equilibrated at pH 9.4. A total of 1 mg of partially purified BP, 1 mg of cytochrome *c*, and 1 mg of human carbonic anhydrase B were pooled and applied to the column and eluted with Polybuffer 96 (pH 6.0; Pharmacia). Fractions of 5 ml were collected and assayed for total protein, pH, and bactericidal activity.

Amino acid analysis. Amino acid composition analysis of BP was done by standard procedures described by Hausinger and Howard (8). Total protein was analyzed, after

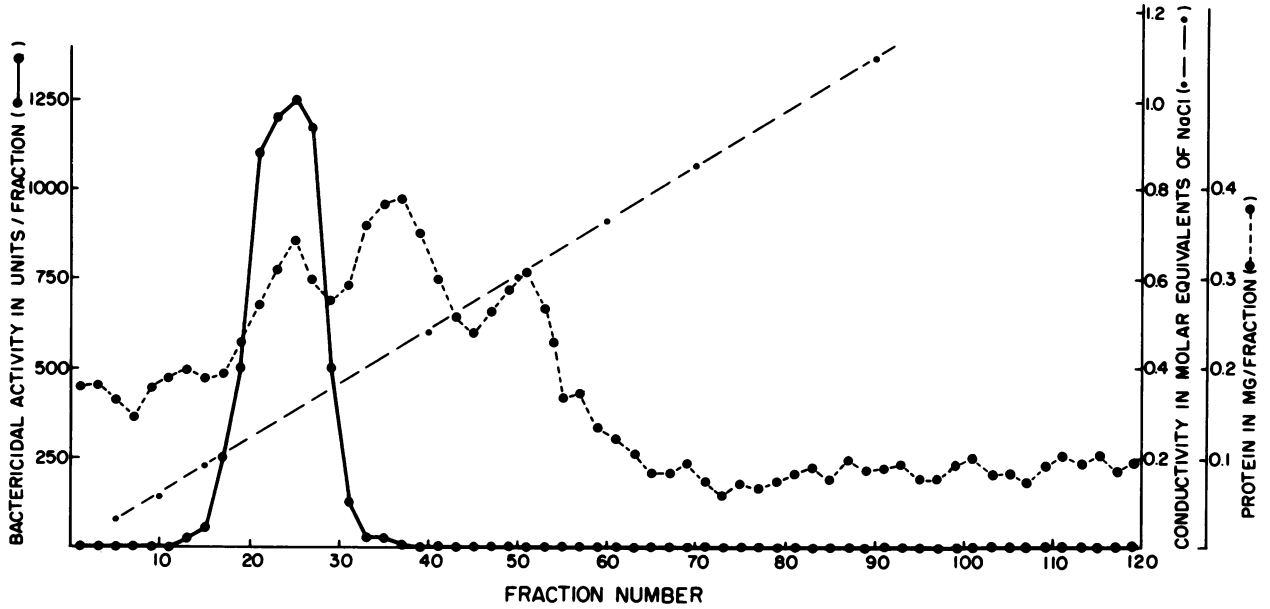

FIG. 1. Dye-ligand affinity chromatography. A column of Matrex-Gel Orange A was prepared and eluted as described in the text. The results of a representative column are shown here. A total of 122 mg of granule protein was applied to the column; and 5.0-ml fractions were collected and tested for bactericidal activity against *P. aeruginosa* type I (——), total protein (– – –), and conductivity (— —). One bactericidal unit was that amount of protein required to kill 95% of 5 × 10^6 *P. aeruginosa* type I CFU/ml.

hydrolysis at 110°C for 24, 48, and 72 h, in an amino acid analyzer (119 CL; Beckman Instruments, Inc., Fullerton, Calif.).

RESULTS

Fractionation of granule proteins by dye-ligand affinity chromatography. The bactericidal activity in acid extracts from PMN granules was routinely purified 40- to 50-fold with Matrex-Gel Orange A. All BP bound to the Matrex-Gel Orange A and was eluted in a single peak between 0.25 and 0.35 M NaCl in a salt gradient of 0.1 to 2.0 M NaCl. In Fig. 1 are shown the elution profiles of protein and bactericidal activity determined for a representative Orange A column. A summary of the purification is shown in Table 1. The bactericidal activity from four Matrex-Gel Orange A columns was pooled to give about 11 mg of protein and applied to one Bio-Rex 70 cation-exchange column. Recent lots of Matrex-Gel Orange A have shown a significant improvement in the flow rates that can be achieved. For this reason it would be possible to change to one Matrex-Gel Orange A column of 4 times the size of the columns described here. When the column was washed with 0.1 M NaCl in CP buffer (pH 5.6) prior to the salt gradient, both myeloperoxidase and lysozyme were eluted. These enzymes were well separated from BP and most other granule proteins. The serine proteases, elastase, and cathepsin G, which were present in the PMN granule extracts, were bound to the column but eluted in the salt gradient after collection of BP.

Purification of BP by cation-exchange chromatography. The bactericidal activity, partially purified by Orange A dye-ligand affinity chromatography, was purified a further 3.7-fold by cation-exchange chromatography. All BP bound to the resin, and the bactericidal activity eluted between 0.37 and 0.39 M NaCl. In Fig. 2 are shown the total protein, bactericidal activity, and conductivity of the 120 fractions collected from a representative column. A summary of the two-step purification is shown in Table 1. The first step of purification, Orange A dye-ligand chromatography, routinely gave a 40- to 50-fold purification; and the second step, Bio-Rex 70 cation-exchange chromatography, gave a further 3- to 4-fold purification for a total purification near 170-fold (Table 1). After the combination of these two purification techniques, 80% of the bactericidal activity was recovered routinely.

Bactericidal activity of purified BP. All bactericidal activity against *P. aeruginosa* in PMN granule extract could be

TABLE 1. Summary of the two-step purification scheme

Fraction	Total BU[a]	Total protein (mg)	Sp act (BU/mg)	Yield of BU (%)[b]	Fold purification[c]	One BU equivalent (μg of protein)
PMN granule extract	11,800	122.7	96.2			10.0
Matrex-Gel Orange A	11,040	3.12	3,538.5	93.6	37.1	0.28
Matrex-Gel Orange A[d]	50,000	11.0	4,545.4	90.0	47.3	0.22
Bio-Rex 70	46,500	2.77	16,787	91.4	3.7	0.060

[a] BU, Bactericidal unit. BU is that amount of protein required to kill 95% of 5 × 10^6 CFU of *P. aeruginosa* type I per ml, as determined by the turbidimetric assay.
[b] Total yield, 82.3%.
[c] Total purification, 175.0-fold.
[d] Pooled BP from four Matrex-Gel Orange A purifications applied to one Bio-Rex 70 column.

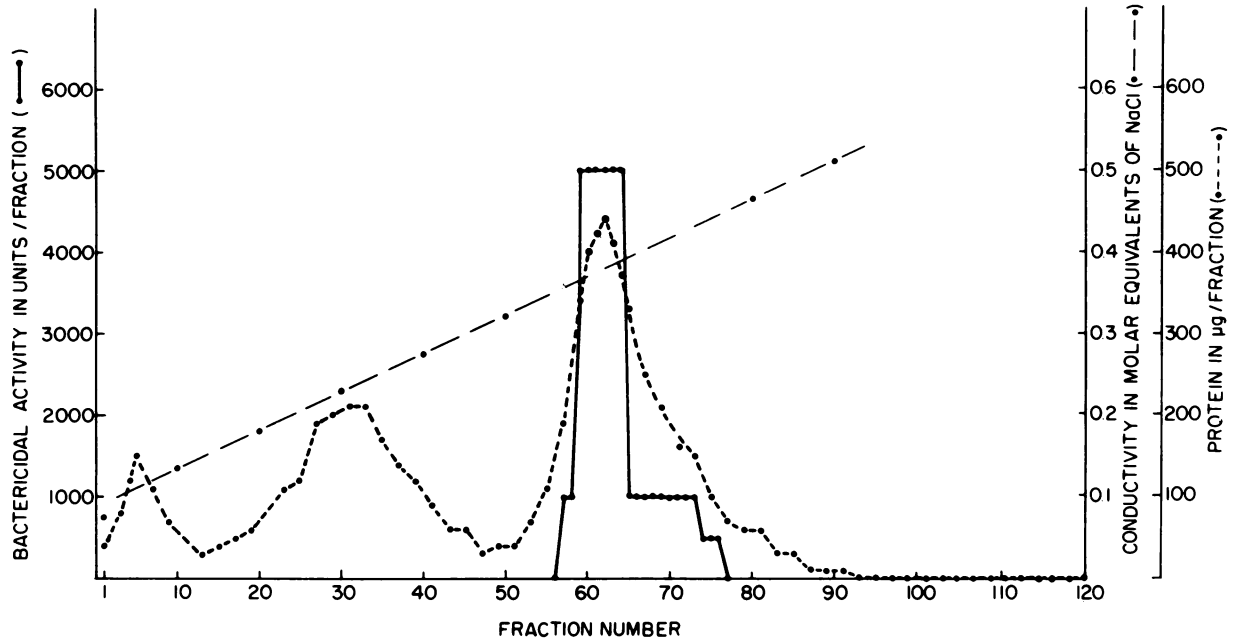

FIG. 2. Cation-exchange chromatography. A Bio-Rex 70 cation-exchange resin column was prepared and eluted as described in the text. A total of 11 mg of protein, pooled from four Matrex-Gel Orange A partial purifications, was applied to the column; and 5.0-ml fractions were collected and analyzed for bactericidal activity against *P. aeruginosa* type I (——), total protein (– – –), and conductivity (— —). One bactericidal unit was that amount of protein required to kill 5×10^6 *P. aeruginosa* type I CFU/ml.

attributed to a single protein, BP. Bactericidal activity against all *P. aeruginosa* strains tested was present in purified BP when measured by the turbidimetric assay (Table 2). Each strain differed in sensitivity, but between 78 and 290 ng of BP per ml killed 95% of 5×10^6 CFU/ml. *Pseudomonas cepacia*, *Proteus mirabilis*, *Staphylococcus aureus*, and *Salmonella typhimurium* were also tested. All of these organisms were resistant to granule extract in the range of 300 ng/ml. Approximately 24 μg of BP was required to kill 95% of 5×10^6 CFU of *S. typhimurium* per ml. Of the remaining strains, only *Pseudomonas cepacia* was killed, but 12.1 μg of BP was required, a dramatic 42-fold increase compared with the least sensitive strain of *Pseudomonas aeruginosa*. The bactericidal effect of granule extract and purified BP were not bacteriolytic since Petroff-Hausser cytometer counts did not change after cells were killed by BP (data not shown).

TABLE 2. Bactericidal activity of purified BP

Bacteria[a]	LD_{95} of BP (μg/ml)[b]
Escherichia coli B	0.070 ± 0.01
Pseudomonas aeruginosa	
type I	0.078 ± 0.01
27853	0.142 ± 0.01
15692	0.172 ± 0.06
25619	0.234 ± 0.10
15152	0.247 ± 0.06
27312	0.290 ± 0.02
Pseudomonas cepacia	12.1 ± 0.10
Salmonella typhimurium 14028	24.0 ± 3.10
Proteus mirabilis	≥30.0
Staphylococcus aureus	≥30.0

[a] Bacteria were used in the turbidimetric bactericidal assay, as described in the text.

[b] The mean ± standard error ($n = 3$) of the LD_{95} for bacterial strains was derived from linear regression analysis, using at least four data points between 1 and 95% bacteria killed. One LD_{95} is one bactericidal unit.

Chromatofocusing. Another method of protein separation that was attempted after initial purification with the Orange A column was chromatofocusing on a column in the range of pH 9 to 6. A single peak of bactericidal activity was eluted between pH 7.3 and 7.5 (Fig. 3). Other fractions were not bactericidal when up to 0.5 ml was tested. Although an estimated 95% of sample protein was recovered, less than 1% of the bactericidal activity applied to the column was recovered in this peak. The poor recovery was not due to inhibition of bactericidal activity by the polybuffer present in column fractions. When bactericidal fraction from the Orange A column was added to chromatofocusing fractions collected over the pH range, no direct inhibition of bactericidal activity by polybuffer-ethanolamine eluates was observed. The basis for the poor recovery is not understood but was considered to be a serious drawback to the use of chromatofocusing for purification of BP. It did, however, indicate that BP is weakly cationic, with an isoeletric point near 7.5. Purified BP from Bio-Rex 70 columns was applied to LKB isoelectric focusing gels (pH 3.5 to 9.3) and to Sephadex electrofocusing gels with a natural buffer system to confirm this finding. Although the standards banded well, neither technique was successful for determining the pI of purified BP. No protein or bactericidal activity was recovered from the Sephadex isoelectric focusing gels, and purified BP applied to LKB ampholine PAGplates smeared near any point of application and appeared to have precipitated even with the additions of 8 M urea and 25% Triton X-100 to the sample (data not shown).

Molecular weight determination. SDS-PAGE was performed to analyze purity and derive a molecular weight for the purified BP. A single band with an apparent molecular

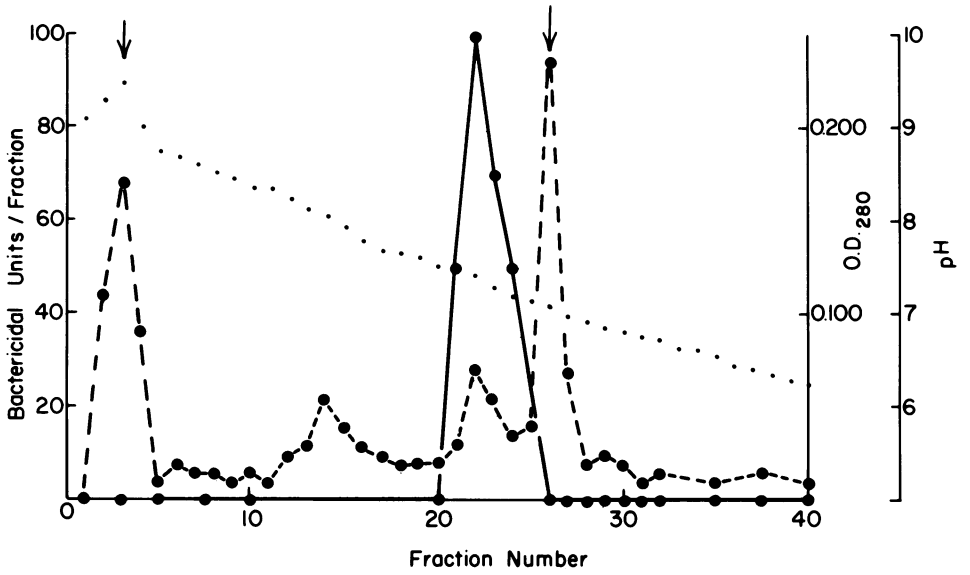

FIG. 3. Chromatofocusing of the bactericidal fraction. A column of polybuffer exchanger (PBE 94; Pharmacia) was prepared and equilibrated at pH 9.4 as described in the text. A sample containing the Matrex-Gel Orange A bactericidal fraction (1 mg of protein), 1 mg of cytochrome *c*, and 1 mg of human carbonic anhydrase B was applied; and elution was carried out with Polybuffer 96 (pH 6.0) while 5-ml samples were collected. The profiles of sample pH (· · · ·), bactericidal activity against *P. aeruginosa* type I (——), and A_{280} (– – –) are shown and are representative of results of three closely similar experiments. Peaks corresponding to marker proteins are indicated by arrows. pIs are 10.25 for cytochrome *c* (left arrow) and 6.55 for carbonic anhydrase (right arrow). The apparent pI for BP was 7.5.

weight of 55,000 resulted (Fig. 4). Protein (1 μg) was applied to the gel stained by a silver stain technique in which 1 to 10 ng of protein was detectable. By nondenaturing PAGE 50 μg of protein of purified BP, elastase, cathepsin G, or cytochrome *c* was stained for carbohydrate by the method of Fairbanks et al. (5). BP stained positive for carbohydrate but with less intensity than did elastase (data not shown). Cytochrome *c*, the negative control, did not stain and cathepsin G stained faintly. The reported quantities of carbohydrates for elastase and cathepsin G are 25 and 1%, respectively (27).

Amino acid composition. The amino acid composition of purified BP was done on total protein hydrolysate, as described above. The results in Table 3 are reported in both the number of residues per mole of purified BP and in the percentage of total amino acids for easy comparison with the amino acid compositions of BP/I and CAP (see discussion).

DISCUSSION

We have described a two-step scheme for purification of BP from normal human PMNs which has potent activity against all strains of *P. aeruginosa* tested. The dye-ligand affinity purification step with Matrex-Gel Orange A is a new and reliable first step for purifying BP and has broad applications for fractionating other human PMN granule proteins. For example, this procedure would be an excellent first step in the purification of both myeloperoxidase and lysozyme. Both of these enzymes bound to Matrex-Gel Orange A, eluted in 0.1 M NaCl, and were well separated from most other proteins. BP was purified by following this initial step with Bio-Rex 70 cation-exchange chromatography. It was determined that BP is glycosylated and has a molecular weight of 55,000 and an isoelectric point of approximately 7.5.

Two proteins described by other laboratories are similar to BP. Weiss et al. (29) purified a 58,000- to 60,000-molecular-weight protein from human chronic myelogenous leukemia cells with activity against *S. typhimirium* and *E. coli* and referred to this protein as B/PI. It was reported that 90% of 10^7 CFU of rough-strain mutants of these bacteria per ml were killed with 0.5 to 2.0 μg of B/PI (26). Wild-type smooth parent strains were more resistant in that 2.0 to 20 μg of B/PI was required to kill 90% of 10^7 CFU/ml (29). After a 40-fold purification, B/PI was estimated to be 1.5% of the total granule extract protein. Characterization of B/PI showed its isoelectric point to be 9.8 (29).

Also with chronic myelogenous leukemia cells, Shafer et al. purified a 57,000-molecular-weight protein and a 37,000-

TABLE 3. Amino acid composition of BP

Amino acid	Content of:			
	BP		B/PI (% of total)	CAP (% of total)
	Residues/mol	% of total		
ASX[a]	43	8.6	8.5	9.3
Threonine	26	5.2	4.2	4.5
Serine	41	8.2	7.8	8.5
GLX[b]	48	9.6	9.1	9.4
Proline	37	7.4	7.9	6.1
Glycine	39	7.8	5.9	6.3
Alanine	32	6.4	5.5	6.4
Half-cystine	ND[c]	ND	ND	ND
Valine	42	8.4	7.9	6.9
Methionine	12	2.4	3.3	2.6
Isoleucine	24	4.8	5.5	5.5
Leucine	49	9.8	10.6	10.4
Tyrosine	15	3.0	3.3	2.7
Phenylalanine	27	5.4	5.8	5.7
Histidine	13	2.6	3.6	4.4
Lysine	28	5.6	7.9	7.8
Arginine	23	4.6	3.3	3.0

[a] Aspartic acid and asparagine.
[b] Glutamic acid and glutamine.
[c] ND, Not detected.

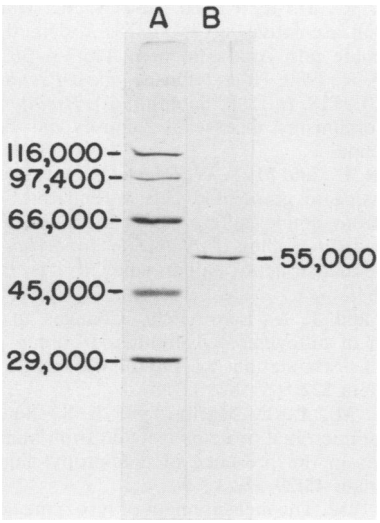

FIG. 4. Determination of purified BP molecular weight by SDS-PAGE. Lane A, molecular weight marker proteins from the top: β-galactosidase, 116,000; phosphorylase *b*, 97,400; bovine serum albumin, 66,000; ovalbumin, 45,000; carbonic anhydrase, 29,000. Lane B, BP with a calculated molecular weight of 55,000. Approximately 1 μg of protein was applied per band. The gel was visualized for protein with a modified silver stain described originally by Oakley et al. (18).

molecular-weight protein referred to as CAP. Our discussion of CAP will be restricted to the 57,000-molecular-weight CAP because of its similarity to B/PI and BP. The purification scheme used by Shafer et al. (23) required the addition of a serine protease inhibitor, diisopropyl fluorophosphate, to the granule extracts before chromatography. It should be noted that throughout our BP-purification procedures, with normal PMN, we saw only one peak of bactericidal activity against *P. aeruginosa* strains and *E. coli* B. The fact that we did not find a 37,000-molecular-weight bactericidal protein may be due to the lack of serine protease inhibitors in our starting extract or to the use of normal PMNs for granule isolation. Shafer et al. (23) reported that the 50% lethal dose for 1×10^3 to 5×10^3 CFU of *S. typhimurium* SL-1004 (Rd_1 LPS chemotype) was 0.25 μg of CAP. Calculated from the 40-fold purification scheme, CAP represented 0.1% of the total granule protein extracted (23), which is well below the 1.5% calculated for B/PI (29) but not that different from BP, which we found to be about 0.5% of the total granule protein.

To compare the relative potencies of B/PI, CAP, and BP we extrapolated the reported values for bactericidal activity to conform with 5×10^6 CFU/ml. The 95% lethal dose (LD_{95}) of BP for *E. coli* B, a rough strain, was 70 ng of protein, and the LD_{95} for the six *P. aeruginosa* strains tested ranged from 78 to 290 ng of BP. These values are strikingly below calculated values for B/PI and CAP. Estimated LD_{95}s for rough strains of *S. typhimurium* were 0.5 to 1.0 μg of protein for B/PI (28) and 500 μg of protein for CAP (23). We tested wild-type *S. typhimurium* 14028 and found an LD_{95} of 24 μg of protein. This is close to the value we have calculated for the B/PI LD_{95} against smooth *S. typhimurium* MS395 which was greater than 15 μg of protein (28).

An evident variation between BP and B/PI is in isoelectric point. B/PI was reported as being strongly cationic, with a PI of 9.8 (29). Spitznagel (25) and Shafer et al. (23) have often referred to the strong cationic nature of CAP but have not reported the isoelectric point of the protein. The basis for the initial interaction of these proteins with bacteria has been suggested to be dependent on their strong cationic charge (4, 25). By the use of chromatofocusing we determined that the isoelectric point of BP is very near 7.5, and based on cellulose acetate paper electrophoresis (data not shown), we determined that this protein is not strongly cationic. It should be noted that although less than 1% of the bactericidal activity was recovered from the chromatofocusing column, it was coincident with a substantial protein peak which, when applied to nondenaturing PAGE, migrated as expected for BP (data not shown). Thus, it seems unlikely that BP merely comigrated with other proteins at a pI of 7.5. Attempts to verify the pI of BP by other techniques were unsuccessful. On both isoelectric focusing gels with ampholines and Sephadex isoelectric focusing gels with a natural buffer system, BP did not form a focused band, even with the addition of urea or Triton X-100 detergent. It is clear that BP displayed unusual problems associated with the use of these two techniques to determine the isoelectric point. The amino acid compositions of B/PI, CAP, and BP (Table 3) are within 1% of each other for all amino acids, except for nonpolar glycine and the positively charged amino acids histidine, lysine, and arginine. The total for these three basic amino acids was 12.8%, the total for B/PI was 14.8%, and the total for CAP was 15.2%. The discrepancy between the isoelectric points of B/PI and BP may not be explained by the lower content of basic amino acids in BP since this difference seems to be too slight to account for the very different isoelectric points.

The most striking difference between BP and B/PI were their respective physiological effects on *P. aeruginosa* and *S. typhimurium*. B/PI created discrete damage of *S. typhimurium*, which increased outer membrane permeability without affecting protein synthesis for 2 to 3 h after irreversible loss of the ability to divide (29, 30). By contrast, BP caused dramatic decreases in amino acid transport, respiration, and cytoplasmic membrane potential within minutes of exposure of *P. aeruginosa* to the protein (10). Even when this difference and the lower isoelectric point of BP are considered, it may be reasonable to expect that BP, B/PI, and CAP are identical because of their similar molecular weights and amino acid compositions. An exchange of bactericidal proteins and antisera to BP, CAP, and B/PI by our laboratories should resolve this question. In spite of the possible identity of BP and bacterial proteins described previously (23, 29), our description of a glycoprotein from normal PMNs which has bactericidal activity against *P. aeruginosa* expands our understanding of the oxygen-independent bactericidal mechanisms of human PMNs and of the nonspecific immune system. Our observations may at some time contribute to the design of effective synthetic antibiotics to combat *P. aeruginosa* infections.

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