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PURIFICATION AND CHARACTERIZATION OF CAMP-FACTOR FROM *STREPTOCOCCUS AGALACTIAE* BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY AND CHROMATOFOCUSING

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SUMMARY

CAMP-factor from *Streptococcus agalactiae* (group B streptococcus) was purified 60-fold from the culture supernatant to electrophoretic homogeneity in 57% yield. The purification procedure involved ammonium sulphate precipitation, ultrafiltration, hydrophobic interaction chromatography on Octyl-Sepharose and chromatofocusing on polybuffer exchanger PBE 94. The purified CAMP-factor consists of a single polypeptide chain with an apparent molecular weight of 25 kD and an isoelectric point of 8.9. The properties of the CAMP-factor demonstrated by charge-shift electrophoresis were consistent with those of an amphiphilic polypeptide.

INTRODUCTION

The CAMP-factor (CAMP-F) is produced by most strains of *S. agalactiae*[1] and was first described by Christie *et al.*[2]. The authors demonstrated that filtrates of *S. agalactiae* contained an agent (CAMP-F) that lysed sphingomyelinase-treated sheep and cow erythrocytes, whereas red blood cells (RBC) from human, horse, rabbit and guinea pig were not lysed[2]. Further studies by Esseveld *et al.*[3,4] and Bernheimer *et al.*[5] revealed that the agent was a polypeptide to which molecular weight values of 33 kD[6], 15 kD[4] and 23.5 kD[5] have been assigned. Part of the mechanism of the CAMP reaction was disclosed in the past by the work of Doery *et al.*[7], Bernheimer *et al.*[5] and Sterzik *et al.*[8]. These authors investigated the role of *S. aureus* sphingomyelinase in the sensitization process of either susceptible RBC or target liposomes.

Attempts to purify CAMP-F resulted in preparations of different grades of purity. This is obvious from the different purification protocols and the considerable differences in the reported amino acid composition[4,5,9]. Moreover, the isoelectric focusing (IEF) data of the purified CAMP-F reported by Bernheimer *et al.*[5] suggested considerable microheterogeneity of the purified CAMP-F. The present study was thus intended not only to clarify the reported discrepancies but also to obtain a homogeneous CAMP-F preparation that would permit further studies of the primary structure and chemical nature of this amphiphilic polypeptide.

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EXPERIMENTAL

Strain and culture conditions for CAMP-F production

S. agalactiae (T.C.C. strain No. 8181) was obtained from Central Public Health Laboratory, Colindale, London, U.K. Organisms were cultured on sheep blood agar plates and inoculated at 37°C for 12–24 h in a 5% carbon dioxide atmosphere.

Fermenter cultivation of *S. agalactiae* was performed as reported earlier[10].

Purification of CAMP-F

Ultrafiltration. The CAMP-F was concentrated in the supernatant by ultrafiltration (Amicon "hollow fiber" dialyser, H5 P2, Fa. Amicon, Witten, F.R.G.) at 4°C to one third of the original volume.

Ammonium sulphate precipitation. The concentrated supernatant was precipitated with solid ammonium sulphate at a saturation of 75%. The precipitation of CAMP-F at pH 8.5 and 8°C was terminated after 96 h. The precipitate was collected by centrifugation (10 000 *g*, 4°C, for 20 min) and dissolved in 0.05 *M* Tris–HCl buffer, 1 *M* potassium chloride (pH 8.0). Ammonium sulphate was removed by hollow fiber ultrafiltration using H1 P2 fibers.

Hydrophobic interaction chromatography (HIC). Binding of CAMP-F to Octyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) was performed in a batchwise procedure. The CAMP-F solution was diluted with 0.05 *M* Tris–HCl buffer (pH 8.0) containing 1 *M* potassium chloride to a protein concentration of 1 mg/ml. Octyl-Sepharose (75 g) equilibrated in the same buffer was used to bind CAMP-F (750 mg) at 4°C after 20 h with constant stirring.

The gel was then washed sequentially on a sintered glass funnel with 1 l of each of the following cold buffer solutions: (a) 0.05 *M* Tris–HCl, pH 8.0; (b) 0.01 *M* Tris–HCl, pH 8.0; (c) 0.001 *M* Tris–HCl, pH 8.0; (d) 0.05 *M* Tris–HCl, pH 9.0; (e) 0.01 *M* Tris–HCl, pH 9.0; (f) 0.001 *M* Tris–HCl, pH 9.0; (g) 0.05 *M* Tris–HCl, pH 8.5.

HIC was carried out by transferring the gel to a column (15 × 2.6 cm I.D.). A sodium desoxycholate gradient (SDOC: 0 → 1%) in 750 ml, 0.05 Tris–HCl (pH 8.5) was applied first to remove extraneous protein. Thereafter, CAMP-F was desorbed by a Triton X-100 gradient (750 ml; 0 → 1% in water). Fractions containing CAMP-F activity were pooled and lyophilized.

Chromatofocusing. The polybuffer exchanger PBE 94 (Pharmacia) was packed into a column (35 × 1 cm I.D.) and equilibrated with 0.025 *M* ethanolamine–hydrochloric acid (pH 9.4). The CAMP-F (3 mg protein) purified by HIC was dissolved in 2 ml of polybuffer PB 96–hydrochloric acid (pH 7.0), and applied to the column for chromatofocusing. CAMP-F was eluted with a 1:10 dilution of polybuffer PB 96–hydrochloric acid (pH 7.0). The elution volume was 250 ml and the flow-rate 12 ml/h. Fractions of 2.5 ml containing CAMP-F were pooled and lyophilized.

Removal of polybuffer PB 96. To remove polybuffer PB 96 from CAMP-F, the freeze-dried protein was dissolved in a minimal volume of 0.05 *M* ammonium bicarbonate (pH 8.2) and applied to a Sephadex G-75 column (50 × 1.6 cm I.D.). Gel filtration was performed in the same solution at a flow-rate of 12 ml/h. Fractions containing CAMP-F were collected, and the absence of polybuffer monitored by reading the absorption at $\lambda = 245$ nm.

Preparation of CAMP-F antibodies. To produce antibodies, 1 mg of purified CAMP-F (specific activity $1.3 \cdot 10^5$ U/mg) was injected in 1 ml of Freund's complete adjuvant (Difco Labs., Detroit, MI, U.S.A.) subcutaneously into a rabbit at multiple sites. A booster injection was given at weeks 5 and 6, and the production of antibodies was followed by the Ouchterlony technique. Blood was drawn from the rabbit's ear, usually 6 days after the last injection.

Determination of protein. Protein was measured according to Peterson[11] with BSA as a standard.

Determination of CAMP-F activity. CAMP-F activity was measured by the kinetic test developed by Sterzik[12].

Characterization of CAMP-F

Analytical polyacrylamide gel electrophoresis (PAGE). Sodium dodecyl sulphate (SDS–PAGE in slab gels (80 × 120 × 1 mm, T = 12%, C = 2.6%) was performed according to Laemmli[13]. Phosphorylase E.C. 2.4.1.1. (M_r 95 000), bovine serum albumin (M_r 68 000), fumarase E.C. 4.1.2.2. (M_r 49 000), carboanhydrase E.C. 4.2.1.1. (M_r 30 000) and lysozyme E.C. 3.2.1.17. (M_r 14 500) were used as standard proteins and purchased from Boehringer (Mannheim, F.R.G.). The proteins were denaturated in the presence of 2.5% (v/v) 2-mercaptoethanol and 1% (w/v) SDS by boiling for 5 min; 5–30 μg of protein were applied and the protein stained with Coomassie brilliant blue.

PAGE of CAMP-F under non-denaturating conditions was carried out in tubes (140 × 5 mm I.D.) using polyacrylamide gels of T = 7.5% and, C = 2.6%. The buffer system of Reisfeld[14] and Laemmli[13] was used and the protein (40 μg/gel) was separated at 3.5 mA per tube.

Protein titration curve. The titration curves in slab gels (125 × 125 mm, T = 5%, C = 2.6%) were obtained according to Righetti *et al.*[15].

RESULTS

Purification of CAMP-F

Concentration of CAMP-F and ammonium sulphate precipitation. Fermenter cultivation of *S.* agalactiae[10] in Trypticase Peptone broth (Becton Dickinson, Heidelberg, F.R.G., No. 11 921) in the presence of glucose (2%) and carbon dioxide resulted in the production of CAMP-F in the supernatant with a specific activity (SA) of $3.9 \cdot 10^3$ (U/mg). After ultrafiltration, concentration, ammonium sulphate precipitation, the SA increased to $9 \cdot 10^3$ (U/mg). A further increase was achieved by a second ultrafiltration step, resulting in the CAMP-F preparation of SA = $2.2 \cdot 10^4$ (U/mg). The yield of CAMP-F at this stage of purification was still 94% of the starting material.

Hydrophobic interaction chromatography. The successful purification of *S. aureus* lipase by HIC[16,17] suggested that HIC could be useful in the purification of the amphiphilic CAMP-protein. Preliminary batch experiments with Octyl-Sepharose CL-4B confirmed the binding of CAMP-F to this matrix. The experiments also revealed that CAMP-F was separated from extraneous protein on Octyl-Sepharose by a combination of a batchwise and a column procedure. Quantitative binding of CAMP-F to Octyl-Sepharose was achieved in batch experiments after 20 h at 8°C in

0.05 *M* Tris–HCl buffer (pH 8.0), containing 1.0 *M* potassium chloride, at a gel/protein ratio of 1 (g):10 (mg). The washing procedure (steps a–g, *cf.* Experimental) with buffers of different pH and molarities removed contaminating material but not CAMP-F.

CAMP-F was then further purified by gradient elution chromatography after packing the gel into a column. When the SDOC gradient (0 → 1%) was applied in the first step, 20% of contaminating protein and a small amount of CAMP-F (5%) of total activity) were eluted (Fig. 1). CAMP-F activity was then removed from the gel with a Triton X-100 gradient (0 → 1%). After HIC, 89% of the CAMP-F activity (SA = $113.3 \cdot 10^3$, U/mg) was recovered.

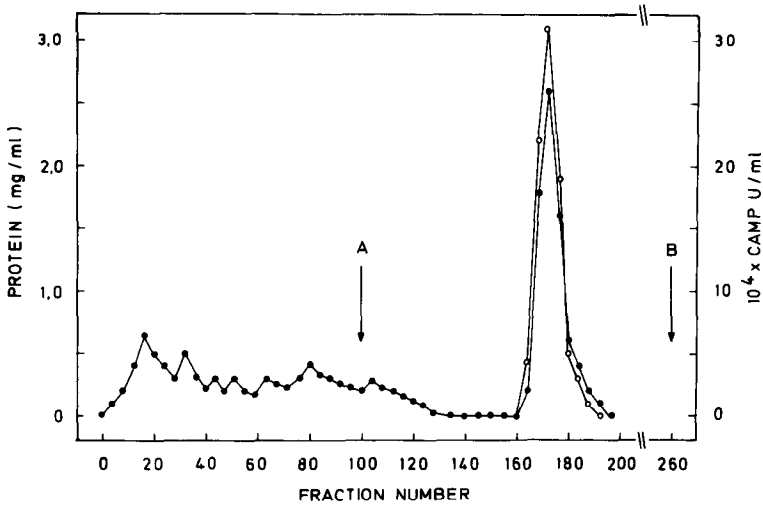

Fig. 1. Purification of CAMP-factor by hydrophobic interaction chromatography on Octyl-Sepharose CL-4B (column, 40 × 2.6 cm I.D.). Arrow indicates change of eluent. (A) Gradient elution of extraneous protein with sodium desoxycholate, 0 → 1% (w/v) in 0.05 *M* Tris–HCl buffer (pH 8.5) (fraction Nos. 1–100 ≙ 750 ml); flow-rate, 20 ml/h. (B) Gradient elution of CAMP-factor with Triton X-100, 0 → 1% (w/v) in water (fraction Nos. 101–260 ≙ 750 ml); flow-rate, 20 ml/h. Protein mg/ml (–●–●–); activity of CAMP-factor U/ml (–O–O–).

Subsequent analysis of CAMP-F by native electrophoresis at different pH values[13,14] exhibited two protein bands only as shown in Fig. 2A and B. In contrast, protein titration[15] revealed five polypeptides of very similar hydrophobicity (Fig. 2C), electrophoretic mobility and isoelectric points.

Chromatofocusing of CAMP-F. It was shown by Sluyterman *et al.*[18–20] and Kopetzki and Entian[21] that chromatofocusing is an excellent technique for the separation of polypeptides with similar isoelectric points. Thus, CAMP-F was separated from its "satellite proteins" by chromatofocusing using the polybuffer exchanger PBE 94 (pH 7–9.4). A typical elution profile is depicted in Fig. 3. CAMP-F was eluted with polybuffer 96–hydrochloric acid at pH 9 and thus successfully separated from its contaminants.

The separation steps are summarized in Table I.

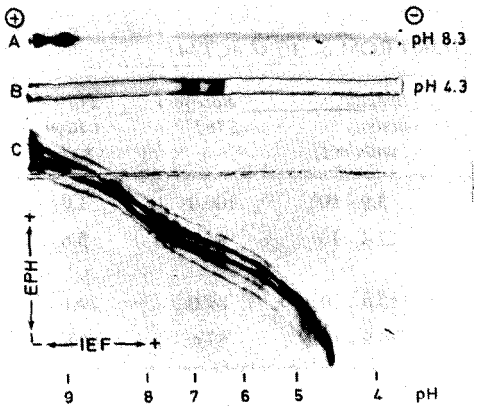

Fig. 2. Native PAGE of partially purified CAMP-factor was performed in tubes (T = 7.5%; C = 2.6%; 140 × 5 mm I.D.) at 3.5 mA per tube using the following buffer systems: (A) system of Laemmli[13]; (B) system of Reisfeld[14]. (C) Protein titration curves were obtained with the PAGE technique (slab gels; T = 5%; C = 3%; 125 × 125 mm) in the presence of 2% ampholine (pH 3.5–10). First dimension: isoelectric focusing (IEF), 50 min at 8°C; 10 W/gel; second dimension: electrophoresis (EPH) of 100 μg of CAMP-factor preparation, 10 min at 8°C; 700 V/gel.

Characterization of CAMP-F

Homogeneity and isoelectric point of CAMP-F. The technique to characterize proteins by "protein titration"[15] provides an excellent means to determine both the isoelectric point and the degree of homo- or heterogeneity of an individual polypeptide. "Protein titration" of the purified CAMP-F revealed an isoelectric point of pI = 8.9 ± 0.2. The titration curve demonstrated in addition the presence of a single

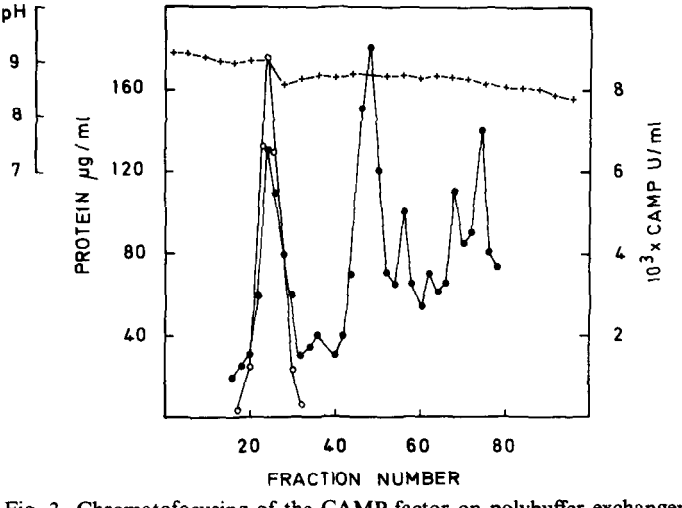

Fig. 3. Chromatofocusing of the CAMP-factor on polybuffer exchanger PBE 94 (column, 40 × 1 cm I.D.). Separation of CAMP-factor (fraction Nos. 18–32) from contaminating material (fraction Nos. 40–53) was done by elution with polybuffer 96–hydrochloric acid at pH 7.0. Starting buffer, 0.025 *M* ethanolamine–hydrochloric acid (pH 9.4); flow-rate, 12 ml/h. Protein μg/ml (–●–●–); activity of CAMP-factor U/ml (–O–O–).

TABLE I

SUMMARY OF PURIFICATION OF CAMP-FACTOR FROM *S. AGALACTIAE*

Purification step	*Total activity (units)*	*Specific activity (units/mg)*	*Recovery (%)*	*Purification x-fold*
(1) Culture supernatant	$35.8 \cdot 10^6$	$3.9 \cdot 10^3$	100.0	1.0
(2) Ultrafiltration after ammonium sulphate precipitation	$33.6 \cdot 10^6$	$22.4 \cdot 10^3$	94.0	5.6
(3) Hydrophobic chromatography	$31.9 \cdot 10^6$	$113.0 \cdot 10^3$	89.0	29.1
(4) Chromatofocusing	$20.6 \cdot 10^6$	$236.9 \cdot 10^3$	57.6	60.8

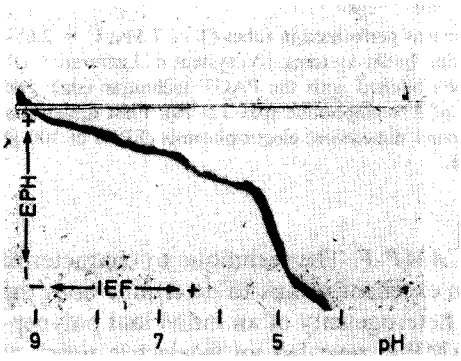

Fig. 4. Protein titration of the purified CAMP-factor (see legend of Fig. 2).

polypeptide band within a wide range of pH (Fig. 4). In addition, analytical SDS-PAGE confirmed that CAMP-F isolated by chromatofocusing appeared as a single protein band (Fig. 5A).

Molecular weight of purified CAMP-F. Estimation of the molecular weight of

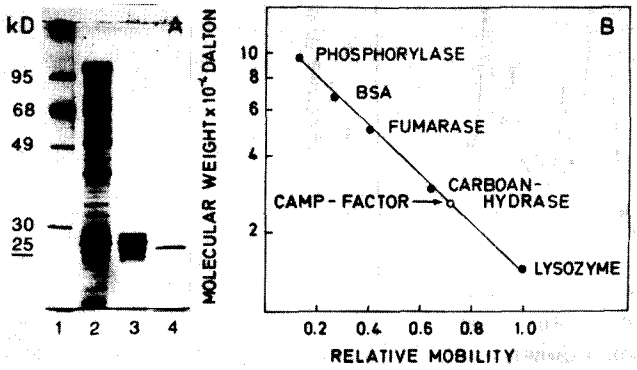

Fig. 5. (A) Analytical SDS–PAGE of CAMP-factor after different purification steps (T = 12%; C = 2.6%; 40 × 120 × 1 mm). Channels: 1 = marker proteins, phosphorylase (M_r 95 000), BSA (M_r 68 000), fumarase (M_r 49 000), carboanhydrase (M_r 30 000); 2 = ammonium sulphate precipitate of culture supernatant; 3 = CAMP-factor after hydrophobic interaction chromatography; 4 = CAMP-factor after chromatofocusing. (B) Determination of molecular weight of purified CAMP-factor by SDS–PAGE.

CAMP-F was performed by SDS–PAGE with the aid of appropriate calibration proteins. As can be seen from Fig. 5B, an apparent molecular weight of 25 kD ± 5% was found. The value was in good agreement with that of Bernheimer *et al.*[5] and corresponded well to the figure calculated from the amino acid analysis by Jürgens *et al.*[9]. It differed considerably, however, from the molecular weights reported earlier[4,6].

Antigenicity of CAMP-F and detergent binding. CAMP-F produced antibodies in the rabbit when used for immunization with complete Freund's adjuvant. When the purified CAMP-F was analysed in immunodiffusion, one precipitation line was observed. The antiserum was used in addition to detect CAMP-F in charge-shift electrophoresis according to Helenius and Simons[22]. Charge-shift electrophoresis revealed binding of cationic and anionic detergents resulting in a shift of the direction of migration and electrophoretic mobility of CAMP-F (Fig. 6). This type of immunoelectrophoresis indicated also that the purified CAMP-F, after detergent binding, migrated as a homogeneous protein fraction.

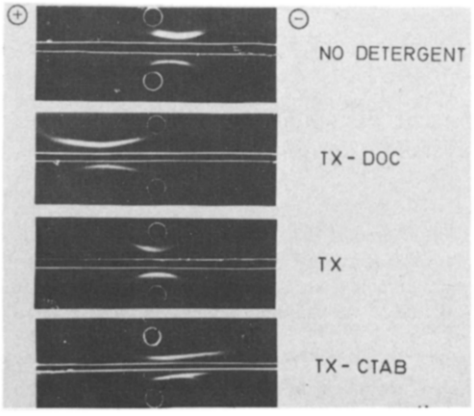

Fig. 6. Characterzation of CAMP-factor by charge-shift immunoelectrophoresis at pH 8.7[20]. Electrophoretic mobility of purified CAMP-factor in the presence of Triton X-100 and sodium desoxycholate (TX-SDOC), Triton X-100 (TX), Triton X-100 and cetyltrimethylammonium bromide (TX-CTAB), respectively.

DISCUSSION

Purification and characterization of CAMP-factor of group B streptococci by different groups in the past gave rise to discussions of the chemical nature of this extracellular polypeptide. Besides the molecular weights determined in different laboratories[4–6], the isoelectric point and particularly the amino acid composition[4,5,9] were at variance. These results are difficult to explain because the CAMP-F derived from different strains of group B streptococci may well represent a different gene product with differences in the amino acid composition. Thus, Bernheimer *et al.*[5] found 8 lysine, 18 alanine, 22 valine, and 4 methionine residues per mol, whereas 22, 31, 33, and 1 methionine residue(s) per mol, respectively, were found in our laboratory[9]. The existence of one methionine residue, however, is of considerable interest since BrCN cleavage should result in two peptide fragments. Recent experiments in

our laboratory[23] confirmed this view and demonstrated that, instead of the five fragments predicted from an earlier amino acid analysis[5], only two fragments are found.

Purification protocols suggested also that the CAMP-F preparation obtained by different groups[3–6] varied considerably in their degree of purification. Proofs of homogeneity by stringent methods were either lacking or indicated that the purified material still exhibited considerable microheterogeneity, as revealed by IEF analysis[5].

It is consistent with our findings that polypeptides with flat titration curves[15] are not readily separated and characterized by IEF. When the CAMP-F purified by gel filtration was subjected to isoelectric focusing, the active material was detected within a wide pH range (7–9.5) with maximum activity at pH 8.6 SDS–PAGE revealed, however, in contrast to earlier work[5] that the peak fraction still contained five polypeptides, visualized by silver staining[24], which have not been separated from CAMP-F. These contaminants could be separated successfully from CAMP-F by chromatofocusing[18–20] in polybuffer exchanger PBE 94. Thus the CAMP-F fraction eluted from the column at pH 9.0 was homogeneous as judged by SDS–PAGE, charge-shift electrophoresis[22] and protein titration[15]. The apparent molecular weight of 25 kD was in good agreement with that of 23.5 kD determined earlier by Bernheimer[5].

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