Journal of Chromatography A, 657 (1993) 357–363
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 593

Ion-exchange–immunoaffinity purification of a recombinant baculovirus *Plasmodium falciparum* apical membrane antigen, PF83/AMA-1

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(First received June 28th, 1993; revised manuscript received September 27th, 1993)

ABSTRACT

A two-step purification regime has been developed for a quantitatively minor, putatively transmembrane, M_r 83 000, apical membrane blood stage vaccine candidate antigen of *Plasmodium falciparum* (PF83/AMA-1), that has been expressed as a full-length baculovirus recombinant protein, PF83-7G8-1. The first step utilizes a new approach to high-performance ion-exchange chromatography (HPIEC) in which elution conditions are not only defined by charge, but also by hydrophobicity. HPIEC fractionation involves successive sodium chloride gradient anion-exchange elutions (A and B), where a change in the non-ionic detergent polyoxyethylenealkylether $C_{10}E_5$ concentration between elutions A and B (from 0.01% to 0.1% (w/v) respectively), results in a fraction that comprises from 2% to 9% PF83-7G8-1. Subsequent column immunoaffinity purification of this fraction on Q-Sepharose CL 4B-28G2dc1 mAb yields a PF83-7G8-1 preparation that is 56% pure. Rat mAb 28G2dc1 recognizes a C-terminal region that is conserved and cross reactive within the AMA-1 family, thus permitting recombinant and native full-length AMA-1 molecules from other species to be purified for molecular analysis. Immunological and molecular characterisation of the vaccine-related characteristics of purified PF83/AMA-1 are now underway.

INTRODUCTION

Malaria continues to be one of the major health problems of tropical regions. Over 40 percent of the world's population are considered at risk and, in sub-Saharan Africa alone, it is estimated that annually between 1–2 million children die from the disease. Morbidity and mortality associated with malaria are rising alarmingly as major control measures become less effective due in part to the rapid spread of resistance to chemotherapy [1]. The development of new means to combat the disease is

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imperative, and one approach is to identify parasite targets that may comprise vaccine components. A number of such targets have been identified, and rapid analysis of their potential and, where appropriate, development of these molecules as vaccines is essential. One promising vaccine molecule, PF83/AMA-1 (*P. falciparum* M_r 83 000 protein/apical membrane antigen-1), belongs to a family of quantitatively minor antigens that were originally identified in a M_r 66 000, conformation dependent, asexual blood stage merozoite antigen of the simian malaria *Plasmodium knowlesi* that was shown to be the target of inhibitory monoclonal antibodies (mAb), and that could induce protection in rhesus monkeys [2–6]. PF83/AMA-1 is a highly similar M_r 83 000 analogue of this antigen of limited variability [7–11] that has been identified in *P. falciparum*, the cause of the most severe human malaria. PF83/AMA-1 has been expressed as a full-length recombinant molecule (PF83-7G8-1) that remains associated with the cellular fraction in the eukaryotic baculovirus expression system (manuscript in preparation). We have developed a purification scheme for the preparation of recombinant PF83-7G8-1 comprising over 56% of total protein using high-performance ion-exchange chromatography (HPIEC) and immunoaffinity chromatography. Resin-based ion-exchange sorbents' main mechanism of interaction with proteins is electrostatic in nature, due to their chemical composition. In addition such ion-exchange packings may exhibit hydrophobic properties [12–15]. This may give rise to mixed-mode contributions to solute separations depending on the hydrophobic properties of the protein and the composition of the eluent, *i.e.*, salt concentration, type of salt, presence of organic solvents or detergents. The present protocol is, in part, based on the novel HPIEC strategy of Welling-Wester *et al.* [16] that achieves differential elution of integral membrane proteins by adjustment of the concentration of non-ionic detergents such as the polyoxyethylenealkylether $C_{10}E_5$. Further purification of HPIEC eluted recombinant material was obtained by passage through a Q-Sepharose CL 4B-28G2dc1 immunoaffinity column. Rat mAb 28G2dc1 recognizes, within the *Plasmodium* AMA-1 family of molecules, a highly conserved C-terminal region [17]. Recombinant material, purified as described, is currently being used for the further immunological and molecular characterisation of PF83/AMA-1.

EXPERIMENTAL

Detergent extraction of recombinant PF83-7G8-1 from Sf9 cells and sample preparation for chromatography

Full-length PF83/AMA-1 was expressed in *Spodoptera frugiperda* (Sf9) cells [18]. Batch-to-batch variation in the efficiency of expression of the recombinant malarial protein (PF83-7G8-1) resulted in starting material in which PF83-7G8-1 constituted between an estimated 0.1% and 0.5% by weight of the total cell pellet. PF83-7G8-1 was associated with the plasma membrane of infected cells. Cells expressing PF83-7G8-1 were washed in phosphate buffered saline (PBS) and cell pellets (5 min, 100 *g*) were either rapidly frozen and stored at − 70°C or were immediately extracted ($4 \cdot 10^7$ cells ml^{-1}) for 1 h on ice in extraction buffer [50 m*M* Tris–HCl pH 8.0, 1% (w/v) $C_{10}E_5$ (Kwant–Hoog Vacolie Recycling and Synthesis, Bedum, Netherlands), 5 m*M* EDTA pH 8.0, 20 m*M* iodoacetamide, 0.8 mg ml^{-1} 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF) (Calbiochem, San Diego, CA, USA), 5 μl ml^{-1} aprotinin, 1 μg ml^{-1} pepstatin A, and 40 μg ml^{-1} chymostatin]. The cell extract was centrifuged (10 min, 10 000 *g*, 10°C), filtered through a 0.45 μm filter (Millex HA, Millipore, Bedford, MA, USA) and then either directly applied to the Mono Q column or stored at −70°C. Frozen extracts were centrifuged and refiltered prior to HPLC. Cell extracts contained between 5.6 and 8 mg ml^{-1} protein (BCA kit, Pierce, Oud Beijerland, Netherlands).

Immunoaffinity chromatography sample preparation was as follows: Mono Q 10/10 chromatography fractions containing PF83-7G8-1 were pooled and briefly dialyzed (3 h, 4°C) against 100 volumes of 20 m*M* Tris–HCl pH 7.6. Dialyzed samples were then either directly applied to the immunoaffinity column or stored at − 70°C.

Ion-exchange HPLC and immunoaffinity chromatography

Ion-exchange chromatography was performed using the micro-HPLC Smart System (Pharmacia-LKB, Uppsala, Sweden) with a Mono Q PC 1.6/5 (50 mm × 1.6 mm I.D.) column. Alternatively, a basic HPLC system with a GP-250 gradient programmer (Pharmacia-LKB) was used with a Mono Q 5/5 (50 mm × 5 mm I.D.) or a 10/10 (100 mm × 10 mm I.D.) column loaded with a 10-ml Superloop (Pharmacia-LKB). The gradient profile for the Mono Q 1.6/5 and Mono Q 5/5 columns consisted of an 8-min isocratic elution with a linear 12-min gradient from 20 m*M* Tris–HCl pH 7.6 (buffer I) to 0.5 *M* sodium chloride in the same buffer (buffer II). Buffers I and II also contained either 0.01%, or 0.1% (w/v) $C_{10}E_5$. Flow rates for the Mono Q 1.6/5 and 5/5 columns were 100 μl min^{-1} and 1 ml min^{-1}, respectively. The gradient volume used for the Mono Q 10/10 column was calculated in proportion to the relative Mono Q 5/5 and 10/10 column volumes to yield a 16-min isocratic elution followed by a linear 24-min gradient at a flow rate of 4 ml min^{-1}. All chromatography was performed at room temperature. Fraction volumes collected during elution were 100 μl (Smart system) and 4 ml (Mono Q 10/10). An initial buffer I to buffer II linear elution gradient containing 0.01% (w/v) $C_{10}E_5$ was followed by a second gradient containing 0.1% (w/v) $C_{10}E_5$ to elute the PF83-7G8-1.

Immunoaffinity resin (8 mg ml^{-1} mAb) was prepared by coupling [19] purified rat mAb 28G2dc1 (IgG2a isotype) to cyanogen bromide activated Q-Sepharose CL 4B (QS-28G2dc1) (Pharmacia LKB). A chromatography column containing 250 μl QS-28G2dc1 was equilibrated with 5 column volumes of buffer [150 m*M* NaCl, 5 m*M* EDTA pH 8.0, 0.1% (w/v) $C_{10}E_5$, 20 m*M* Tris–HCl pH 7.6, (150 m*M* NECT)]. Pooled and dialysed Mono Q 10/10 fractions containing PF83-7G8-1 were passed over the column at a flow rate of 0.5 ml min^{-1}. Fractions passing through were subsequently monitored for the presence of unbound PF83-7G8-1 by immunoblot; it appeared that saturation levels for the column were not reached. The column was washed with 15 column volumes each of 150 m*M* NECT, 500 m*M* NECT (NaCl concentration 500 m*M*), and 150 m*M* NECT. PF83-7G8-1 was eluted with Pierce Elution Buffer pH 2.8 (Pierce) into vials containing 1 *M* Tris–HCl pH 8 to rapidly neutralize the pH.

SDS-PAGE, immunoblotting, and scanning laser densitometry

Chromatography fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions by mixing with equal volumes of sample buffer, heating and electrophoresing essentially as previously described [20]. Duracryl (Millipore) was used to prepare gels for silver staining (Gelcode Color Silver Staining Kit, Pierce) and 10% polyacrylamide gel was used for electroblots onto nitrocellulose. The migration of prestained molecular mass markers (Gibco BRL, Bethesda, MD, USA) is noted alongside all figures. Immunoblots were stained with pooled rat mAbs, 28G2dc1 and 58F8dc1 (rat mAb 58F8dc1, isotype IgG2a, reacts with the extreme N-terminal region of PF83/AMA-1 [17], both incorporated at 5 μg ml^{-1}. All immunoblots were developed using alkaline phosphatase conjugated reagents (Pierce). The relative proportions of fractionated components were determined by scanning laser densitometry on silver stained gels using an UltraScan XL (Pharmacia LKB). Backgrounds were subtracted after being determined on adjacent tracks to which no sample had been added.

RESULTS AND DISCUSSION

Welling-Wester *et al.* [16] have reported the selective elution of Sendai virus integral membrane proteins from an anion-exchange column through a two-step elution involving manipulation of the concentration of non-ionic detergent ($C_{10}E_5$). We anticipated that this technique would also be applicable to the separation of PF83/AMA-1, a molecule which, in its mature, full length native form is an M_r 83 000, non-glycosylated protein composed of 34% non-polar and hydrophobic amino acids and contains a

predicted trans-membrane region. Initial experimentation (not shown) determined that the efficiency of PF83/AMA-1 extraction with 1.0% (w/v) $C_{10}E_5$ was comparable to that obtained with 1.0% (w/v) TX-100, the detergent we had previously used for AMA-1 extraction. PF83-7G8-1 could be eluted in a sodium chloride anion-exchange gradient containing 0.1% $C_{10}E_5$ (Fig. 1). It was detected by immunoblot as a M_r 90 000 protein of fraction 10. The observed increase in molecular mass over native PF83 is the result of N-linked glycosylation in the baculovirus system [18]. The additional bands in Fig. 1B, fractions 8, 9, and 10 at approximately M_r 55 000–60 000 are attributable to weak cross-reactions with Sf9 cell protein(s) present in large amounts (Fig. 1A fractions 8–10), as they were also detected on immunoblots of non-recombinant Sf9 preparations. Immunoblot analysis of the eluant during sample loading and isocratic elution did not reveal unbound PF83-7G8-1 (data not shown). In Fig. 2 the effect of changing detergent concentrations on the elution of PF83-7G8-1 is revealed. Although ten times less sample is loaded in tracks 8–12 (0.01% $C_{10}E_5$) than in tracks B8–B12 (0.1% $C_{10}E_5$) the total protein levels per track are comparable (panel A), showing that the bulk of the total protein is eluted during the initial, hydrophilic, 0.01% $C_{10}E_5$ elution. PF83-7G8-1 is predominantly

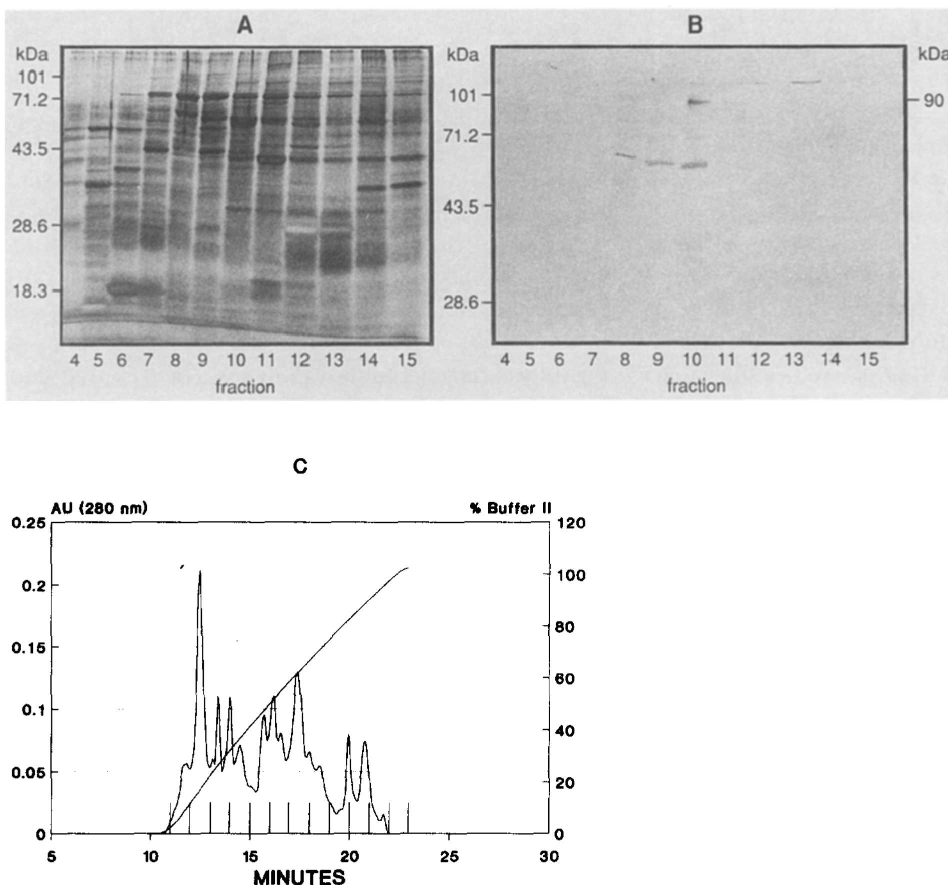

Fig. 1. SDS-PAGE silver stain with 10% Duracryl (A) and pooled mAb immunoblot analysis (B) of a Smart system anion-exchange elution of total PF83-7G8-1/Sf9 cell extract (400 μg) using a Mono Q PC 1.6/5 column. After isocratic elution retained proteins were fractionated with a sodium chloride gradient containing 0.1% (w/v) $C_{10}E_5$. Fractions 4–15 (100 μl) were prepared for SDS-PAGE and divided equally for silver stain and immunoblot. (C) Elution profile (vertical lines delineate consecutive fractions 4–15 that were collected for analysis in A and B).

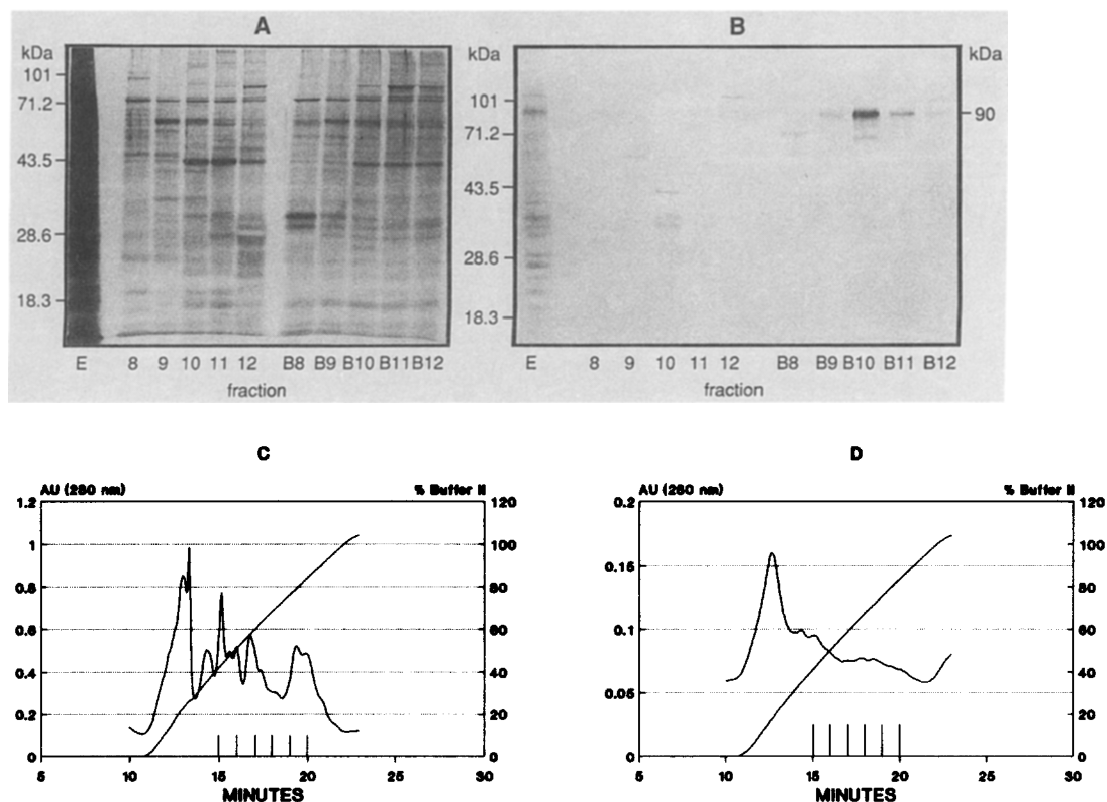

Fig. 2. Two-step $C_{10}E_5$ anion-exchange chromatography of PF83-7G8-1/Sf9 extract. Following isocratic elution a Smart system Mono Q PC 1.6/5 column loaded with 2.4 mg total extract was sequentially eluted with 0–0.5 *M* sodium chloride gradients containing 0.01% $C_{10}E_5$ (fractions 8–12, 100 μl each) and 0.1% $C_{10}E_5$ (fractions B8–B12, 100 μl each). 80 μg of total cell extract (track E) are shown alongside 5-μl samples from fractions 8–12 and 50-μl samples from fractions B8–B12 after silver stained SDS-PAGE with 10% Duracryl (A) and pooled mAb immunoblot (B). Elution profiles with 0.01% $C_{10}E_5$ (C) and 0.1% $C_{10}E_5$ (D) are marked with vertical lines to delineate consecutive fractions 8–12 (C) and B8–B12 (D) that were collected for analysis.

eluted in fraction B10 of the 0.1% $C_{10}E_5$ elution (panel B). Even when volumes of fractions 8–12 equivalent to those used for B8–B12 were analysed by immunoblot, no PF83-7G8-1 was detectable (not shown), demonstrating the selective elution of PF83-7G8-1 achieved under the higher detergent concentration. The elution profiles for anion-exchange chromatography with 0.01% $C_{10}E_5$ (panel C) and 0.1% $C_{10}E_5$ (panel D) confirm that the major protein elution occurs under low detergent conditions.

The conditions which had been defined using the Mono Q PC 1.6/5 column were predictive for scale-up to Mono Q 5/5 (data not shown) and then Mono Q 10/10 columns. The results shown in Fig. 3 derive from a Mono Q 10/10 fractionation of an PF83-7G8-1/Sf9 cell extract. Fractions were analyzed by immunoblot, and fractions eluted during the 0.1% $C_{10}E_5$ run which contained PF83-7G8-1 were pooled (28 ml) and dialyzed. Analysis of this pooled material by silver stain (Fig. 3A, track 1) and immunoblot (Fig. 3B, track 1) identified full-length PF83-7G8-1 (a), and truncated products at M_r 81 000 (b) and 72 000 (c). PF83-7G8-1 constituted 9% of this material. In other preparations the proportion of the pooled material represented by PF83-7G8-1 varied from between 2% and 9% of the pool in a manner directly dependent on the efficiency with which it was expressed in the starting material. The PF83-7G8-1 dialyzed fraction pool was then passed through a 250-μl QS-28G2dc1 immunoaffinity column. Rat mAb 28G2dc1 cross-reacts with the C-terminal

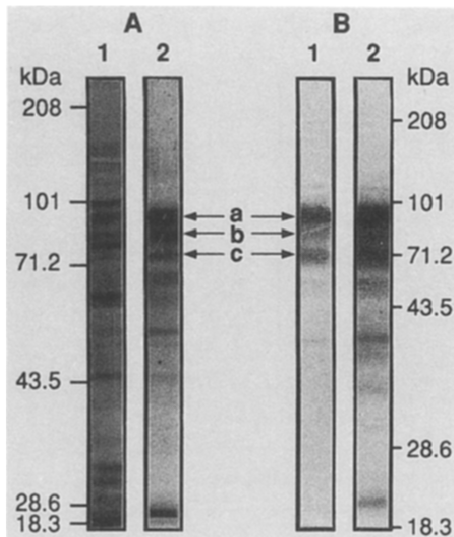

Fig. 3. Combined HPLC anion-exchange and immunoaffinity purification of PF83-7G8-1. 20 ml (6 mg ml^{-1}) of total PF83-7G8-1/Sf9 extract was fractionated by Mono Q 10/10 two-step detergent gradient elution at 4 ml min^{-1}. (A) (silver stained SDS-PAGE with 7.5% Duracryl) and (B) (immunoblot with pooled mAbs) show samples from pooled Mono Q 10/10 derived fractions containing PF83-7G8-1 before (tracks 1) and after (tracks 2) affinity purification on a Q-Sepharose CL 4B-28G2dc1 column. Arrows: (a) M_r 90 000; (b) 81 000; (c) 72 000.

region of all the molecules within the *Plasmodium* AMA-1 family studied so far and therefore provides a universal method for the purification of AMA-1 molecules from different *Plasmodium* species. The QS-28G2dc1 column was washed in a series of NECT buffers and PF83-7G8-1 was eluted with a glycine buffer, pH 2.8 (Fig. 3). By scanning laser densitometry of silver stained gels it was estimated that in the eluted fraction PF83-7G8-1 constituted 56% of total protein. This contrasts with similar immunoaffinity purification procedures using cell extracts directly, without an intervening HPIEC fractionation, where PF83-7G8-1 constituted approximately 10% of the eluted material (data not shown). The QS-28G2dc1 immunoaffinity column was rapidly re-equilibrated and reusable.

CONCLUSIONS

We have developed a two-step purification scheme that we anticipate will be of general application for molecules of the AMA-1 family of malarial proteins. The first step uses an approach to HPIEC enabling differential elution of integral membrane proteins based on their hydrophobic nature and the effect of non-ionic detergents on their solubility. This technique was initially developed with Sendai virus integral membrane proteins, and this is the first report to show a wider application for the method. The second step utilizes a QS-28G2dc1 immunoaffinity column to purify a recombinant PF83/AMA-1 molecule, PF83-7G8-1, to 56% of the total protein. MAb 28G2dc1 recognizes a C-terminal region that is conserved within the family of AMA-1 molecules of malarial parasites so far analysed. Immunological and molecular characterisation of PF83-7G8-1, made possible by this purification regime, is currently underway.

ACKNOWLEDGEMENTS

We wish to thank M. Dubbeld and J. Wubben for excellent technical assistance. We are also grateful to M.A. Braaksma and B.R.K. Douma for their willing assistance in the use of the Smart system at the Rijksuniversiteit, Groningen (Netherlands). Grant support was from the United States Agency for International Development (co-operative agreement DPE-5979-A-00-0042-00) and the STD-3 programme of the European Communities (TS3*-CT92-0147). DN was supported in part by a Graduate Research Assistantship Award from the UMAB Designated Research Initiative Fund.

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