Journal of Chromatography, 521 (1990) 325–334
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1973

Isolation of an outer membrane protein complex from *Borrelia burgdorferi* by *n*-butanol extraction and high-performance ion-exchange chromatography

KLAUS B. GONDOLF*, STEPHEN R. BATSFORD and ARNOLD VOGT
Department of Immunology, Institute for Medical Microbiology, Hermann-Herder Strasse 11, D-7800 Freiburg (F.R.G.)

ABSTRACT

Borrelia burgdorferi, the causative agent of Lyme disease, expresses two major membrane proteins, designated outer surface proteins A and B, which are of antigenic relevance, especially in the chronic phase of Lyme disease. Both proteins exhibit strain-related molecular weight variation. A method is described for obtaining these proteins from the bacterial membrane, without the use of detergents, by a combination of *n*-butanol extraction and cation-exchange chromatography on a Mono S fast protein liquid chromatographic column. This method yields up to five times larger amounts of the proteins in aqueous solution than previously described protocols, which applied ionic or non-ionic detergents. A comparison of extracts obtained by this method from different *Borrelia burgdorferi* strains is reported.

INTRODUCTION

The spirochete *Borrelia burgdorferi* is the causative agent of tick-borne Lyme disease [1,2]. This complex disorder, the most common vector-borne infection in the U.S.A. [3] and probably also Europe, encompasses a number of clinical symptoms. The primary manifestation is a skin efflorescence called erythema chronicum migrans (ECM). Weeks to months later neurological, dermatological and rheumatoid syndromes may develop (for a clinical review, see ref. 4).

Our interest has focused on two major borrelia proteins, outer surface proteins A and B (OspA and OspB), which vary in their electrophoretic mobility in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) between 30 000 and 32 000 dalton and 34 000 and 36 000 dalton, respectively, depending on the isolate examined [4]. They are surface-exposed membrane proteins [5,6] with a cationic charge [7]. Both proteins are encoded on a linear, extra-chromosomal plasmid [8] and their nucleic acid sequences have been published [9,10].

Because of the hydrophobic character of the Osps, the isolation procedures reported to date used detergents for membrane dissociation [11,12]. These techniques are subject to limitations because they gave a low yield in our hands and it is difficult to remove detergents from the protein solutions, restricting their use under *in vivo* and *in vitro* experimental conditions.

0021-9673/90/$03.50

We were able to avoid detergents by employing a combination of *n*-butanol as extraction medium and cation-exchange chromatography on a Mono S column for single-step purification and concentration.

EXPERIMENTAL

Spirochetes

The following strains were used: B31 (American type culture collection No. 35210), the original tick-isolate from Shelter Island, New York [1]; GeHo, Bo23, P-Er and Pko (the last was a kind gift from Dr. Preac-Mursic, Munich, F.R.G.), all isolated from ECM skin lesions; and Z37, Z118 and Z136, tick-isolates from the Freiburg area (these three strains were a kind gift from Dr. Pelz, Freiburg, F.R.G.). Cultures were grown in modified Barbour–Stoenner–Kelly (BSK II) medium [13] at 35°C for 4–5 days until the late logarithmic phase was reached.

The bacteria were harvested by centrifugation at 10 000 *g* for 20 min at 25°C and washed twice in phosphate-buffered saline (PBS) (pH 7.4) at 4°C. The pellet was resuspended in PBS and sonicated using a Branson (Danbury, U.S.A.) sonifier for 15 min in an ice-bath. The resulting suspension is refered to as "whole-cell lysate" in the following text.

n-Butanol extraction

The whole-cell lysate was centrifuged at 27 000 *g* for 90 min at 4°C and the resulting pellet was resuspended in PBS (1/50th of the original volume of the culture medium) and sonicated again for 15 s to aid suspension. Four parts of cold *n*-butanol (analytical-reagent grade; Merck, Darmstadt, F.R.G.) were added to five parts of the suspension and the mixture was stirred in an ice-bath. After 1 h the mixture was centrifuged at 27 000 *g* for 90 min at 4°C and a three-phase system resulted: an upper butanol phase, followed by a waxy interphase and finally an aqueous phase. The aqueous phase was carefully removed and dialysed extensively against 5 m*M* 2-(N-morpholino)ethanesulphonic acid (MES) (Sigma, Deisenhofen, F.R.G.), pH 6.0.

Ion-exchange chromatography

All chromatographic procedures were carried out on a fast protein liquid chromatography (FPLC) system (Pharmacia LKB, Freiburg, F.R.G.).

A Mono S HR5/5 cation-exchange column (50 mm × 5 mm I.D.) was equilibrated with 5 m*M* MES buffer (pH 6.0) (starting buffer). The dialysed aqueous phase from the *n*-butanol extraction (see above; volume 20–50 ml with a total protein content of 2–15 mg) was applied to the column with a Superloop application system (Pharmacia LKB). The column was then washed with starting buffer until the absorbance at 280 nm returned to the baseline. The bound proteins were eluted with linear NaCl gradients in 5 m*M* MES (pH 6.0) (from 0 to 0.6 *M* in 25 ml and from 0.6 to 2 *M* in 10 ml) at a flow-rate of 1 ml/min.

Size-exclusion chromatography

This was performed on a Superose 12 HR10/30 column (300 mm × 10 mm I.D.), equilibrated with PBS or 8 *M* urea (Ultrapure; BRL, Gaithersburg, MD, U.S.A.) in PBS. A volume of 100–300 μl of protein solution (total protein content 300 μg) was

applied and chromatography was performed at a flow-rate of 0.5 ml/min with PBS and 0.3 ml/min when equilibrated with urea.

SDS-PAGE

The protein preparations were analysed by SDS-PAGE according to the procedure of Laemmli and Favre [14] at a constant current of 20 mA (separating gel T12.6%/C2.7%, stacking gel T5%/C2.7%[a]). Molecular mass standards (Pharmacia LKB) included the following: α-lactalbumin (14 100 dalton), soybean trypsin inhibitor (20 100 dalton), carbonic anhydrase (30 000 dalton), ovalbumin (43 000 dalton), bovine serum albumin (BSA) (67 000 dalton) and phosphorylase B (94 000 dalton). The gels were stained with Coomassie Briliant Blue R-250 (Sigma).

RESULTS

The protein patterns (whole-cell lysates) of the *Borrelia burgdorferi* strains chosen in this study are shown in Fig. 1. Strain B31, GeHo, Z37 and Z118 showed major bands, known to represent OspA and OspB [15], with electrophoretic mobilities of about 31 000 and 34 000 dalton, respectively, whereas the Osps of strains Bo23 and P-Er were in the range 32 000–35 000 dalton in SDS-PAGE. We found no prominent bands in the 31 000–35 000 dalton range in strains Pko and Z 136. The main bands of these strain were in the range 22 000 and 24 000 dalton, respectively, a region where all

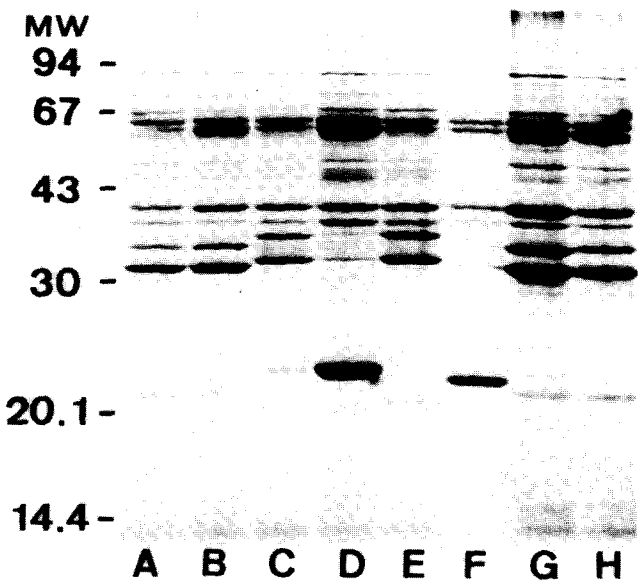

Fig. 1. SDS-PAGE of whole-cell lysates of different strains of *Borrelia burgdorferi* under non-reducing conditions. The protein load per lane was 5 μg. Lanes: A = B31; B = GeHo; C = Bo23; D = Pko; E = P-Er; F = Z136; G = Z37; H = Z118; MW = molecular mass markers (kilodalton, kD).

[a] C = g N,N'-Methylenebisacrylamide (Bis)/%T; T = (g acrylamide + g Bis)/100 ml solution.

the other strains tested showed only minor bands. Two other significant bands, 41 000 dalton (flagellin) and 65 000 dalton were uniform in all strains examined.

Extraction of 700 mg of borrelia (wet weight from 1 l of culture medium) with butanol resulted in an aqueous phase with a protein content between 0.3 and 0.6 mg/ml (total volume 20 ml), depending on the strain examined. The butanol phase did not contain protein when tested in SDS-PAGE and we did not examine the lipid components of this compartment further. SDS-PAGE of the interphase (not shown) revealed a protein pattern similar to that found in the whole-cell lysate (Fig. 1), apart from the proteins present in the aqueous phase, which were markedly reduced in this fraction.

Fig. 2 shows the content of the aqueous phase after dialysis against 5 m*M* MES (pH 6.0). As already seen when analysing the whole-cell lyates, the electrophoretic mobility of both OspA and OspB also varied in the *n*-butanol extraction within the different strains and was between 31 000–32 000 and 34 000–35 000 dalton, respectively. We found an identical pattern and size distribution of the Osps in the *n*-butanol extracts and the whole lysate in six of the eight strains examined. Strain Pko differed from all the others tested, as analysis of the whole-cell lysate (Fig. 1, lane D) and the *n*-butanol extract (Fig. 2, lane D) revealed at most only traces of Osps, the main band being a protein in the region of 22 000 dalton.

In contrast to the findings with strain Pko, we could extract both Osps (31 000 and 34 000 dalton) from strain Z136, which, like Pko, showed only faint bands in the Osp range and a major band at 21 000 dalton on analysis of whole-cell lysates (Figs. 1 and 2, lane F). Interestingly, the extract of this latter strain contained only minor amounts of the 21 000 dalton protein (Fig. 2, lane F).

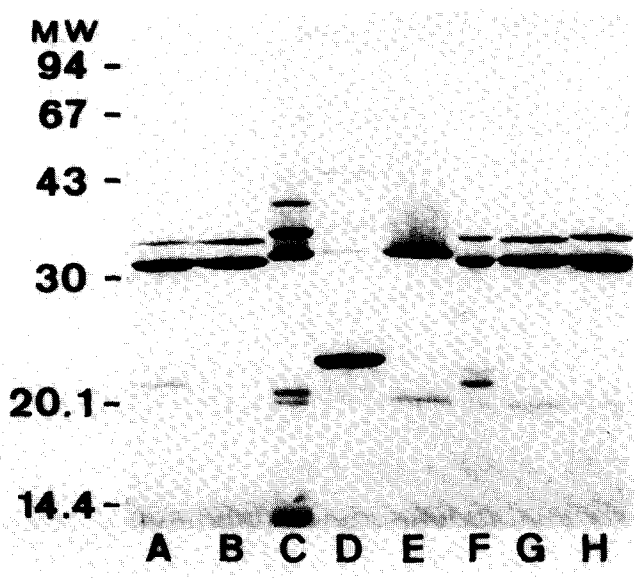

Fig. 2. SDS-PAGE of fractions obtained from *n*-butanol extraction of different strains of *Borrelia burgdorferi*. Running conditions, protein load and strains analysed as in Fig. 1.

In strain P-Er the upper band (interpreted as OspB), which was clearly visible in the whole-cell lysate, was not extractable by *n*-butanol (Figs. 1 and 2, lane E). Strain Bo23 differed from the others in that butanol extraction yielded three additional proteins (41 000 dalton, a double band at 20 000 dalton and a 12 000 dalton protein; Fig. 2, lane C).

The crude aqueous phase was heavily contaminated with nucleic acids and with minor amounts of higher-molecular-weight proteins (60 000, > 100 000 dalton). For further purification and simultaneous concentration, the preparation was applied to a Mono S cation-exchange column after dialysis against the starting buffer (5 m*M* MES, pH 6.0). If the preparations were applied to the ion-exchange column without dialysis or without removing all the *n*-butanol, the proteins would not bind to the Mono S matrix.

Fig. 3 shows an example of the elution profile obtained by ion-exchange chromatography. The first, broad peak of non-binding material (fractions 0–15) contained mainly nucleic acids ($A_{280}/A_{260} \approx 0.55$) and also some contaminating proteins (Fig. 4, lane NB).

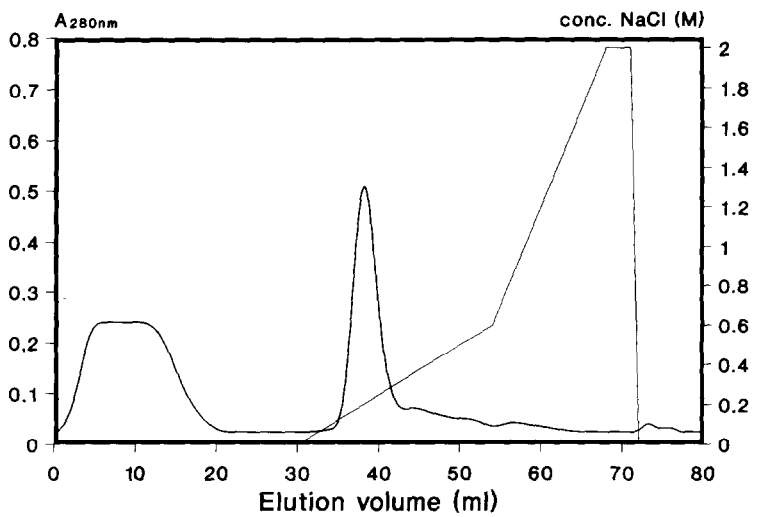

Fig. 3. Cation-exchange FPLC of *n*-butanol extract of strain GeHo on a Mono S cation-exchange column; 10 mg of protein in a volume of 20 ml were applied. Starting buffer, 5 m*M* MES (pH 6.0); gradient, 0–2 *M* NaCl; flow-rate, 1 ml/min; chart speed, 1 cm/ml.

The main protein peak was eluted from the column at about 0.2 *M* NaCl when a linear salt gradient was applied (Fig. 3). As an example of the protein pattern of this peak, the SDS-PAGE of the eluted fractions from ion-exchange chromatography of strain GeHo is shown in Fig. 4.

To elucidate the aggregation status of the fractions obtained from ion-exchange chromatography, we applied the 0.2 *M* peak fraction (after dialysis) to a Superose 12 size-exclusion chromatographic column, which had been calibrated with immunoglobulin (Ig) M (900 000 dalton), horse spleen ferritin (450 000 dalton), IgG (150 000

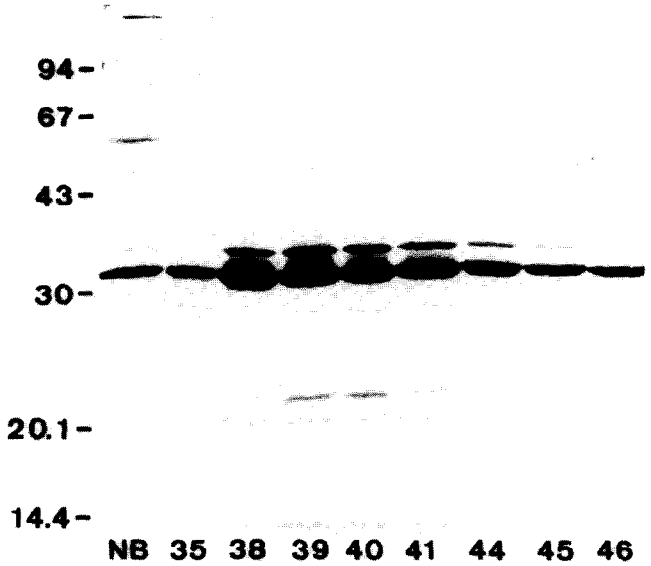

Fig. 4. SDS-PAGE of fractions from ion-exchange chromatography (Fig. 3; strain GeHo). Running conditions as in Fig. 1. The lane numbers refer to the eluted fractions from ion-exchange chromatography (Fig. 3). NB = material that did not bind to the matrix of the cation exchanger. The protein load was between 2.5 (lane NB) and 20 μg (lane 38) per lane.

dalton) and BSA (67 000 dalton). The fraction eluted as one peak shortly after IgM (Fig. 5). The same result was obtained when size-exclusion chromatography was conducted with 8 *M* urea in PBS as the buffer.

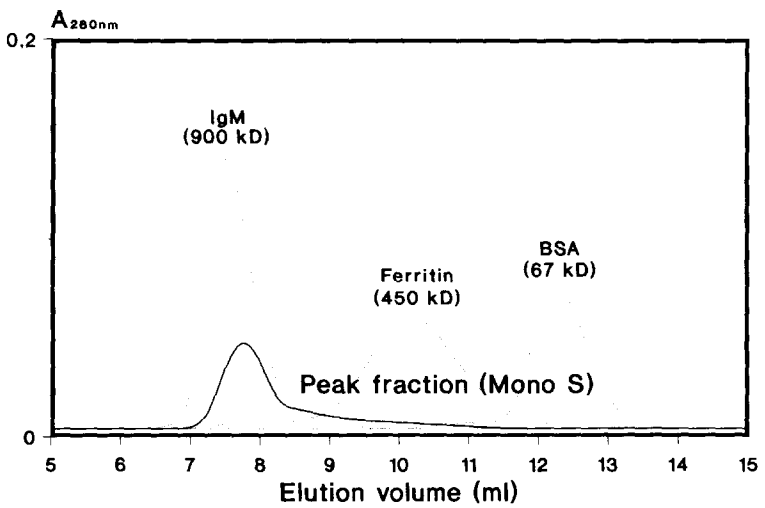

Fig. 5. Size-exclusion chromatography of the main peak (obtained from ion-exchange chromatography; fraction 29, Fig. 3) of strain GeHo. The buffer was PBS at a flow-rate of 0.5 ml/min. The superimposed curves are human IgM (900 000 dalton), horse spleen ferritin (450 000 dalton) and BSA (67 000 dalton).

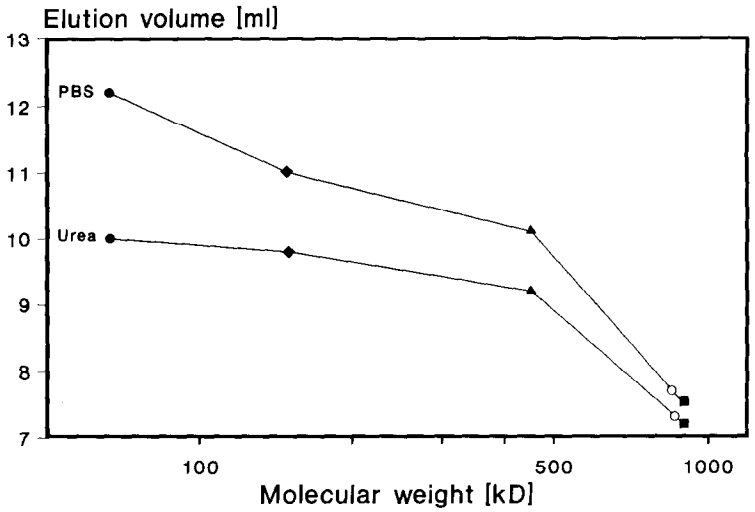

Fig. 6. Plot of elution volume against molecular mass for size-exclusion chromatography on Superose 12 using PBS or urea as buffers. Marker substances were (■) IgM (900 000 dalton), (▲) horse spleen ferritin (450 000 dalton), (◆) IgG (150 000 dalton) and (●) BSA (67 000 dalton). The open circles represent the elution volumes of *n*-butanol extracts, obtained by ion-exchange chromatography (fraction 29 in Fig. 3).

In Fig. 6 the results of the size-exclusion chromatographic runs are presented as elution volume of the marker substances *versus* the logarithm of their molecular mass. The elution behaviour of the 0.2 *M* peak (Mono S) on a Superose 12 column was similar in both PBS and urea and allows the assignment of a molecular mass of about 850 000 dalton.

SDS-PAGE of the eluted peaks from size-exclusion chromatography showed the same protein profile in both experiments as in the fractions applied (data not shown). These results indicate that the proteins extracted by *n*-butanol form a stable aggregate under physiological conditions and also when denaturated by urea.

DISCUSSION

The use of *n*-butanol for the extraction of proteins from microsomes and mitochondrial membranes was introduced by Morton in 1950 [16]. The procedure was subsequently used by other workers to allow the separation of a number of enzymes and other proteins from lipoprotein complexes. The method has also been used successfully for the purification of water-soluble proteins (for a review, see ref. 17).

The ability of organic solvents to disrupt lipoprotein complexes in aqueous media and to release undenatured protein may be ranked as follows: *n*- and isobutanol are most effective, followed by 2-butanol, cyclohexanol and 2-methyl-2-butanol; all other organic solvents tested were ineffective [17].

The unique effect of *n*-butanol may be attributed to a marked lipophilic property concomitant with hydrophilic characteristics. This combination causes the *n*-butanol molecules to orientate between lipid and water, produces a detergent-like action and allows the *n*-butanol molecules to compete with membrane phospholipids for the

hydrophobic domains of proteins. It has been reported that many of the enzymes extracted with *n*-butanol were water soluble and active after the procedure, indicating that this method causes only slight denaturation of proteins (for a more detailed description of the principle and references, see ref. 17).

Efforts have been made to develop a selective extraction method for the outer surface proteins of *Borrelia burgdorferi* and other spirochetes [11,12,18,19]. In all the published methods either ionic or non-ionic detergents were applied to disrupt the lipid layer of the outer membrane of the organism and to bring the proteins of interest into solution. In the preparation of *Borrelia burgdorferi* outer membrane proteins, the concentration of the detergent is a critical parameter: a modest increase drastically diminishes the selectivity of the method by solubilizing increasing amounts of other proteins [20].

In our hands, the methods described [11,12,18,19] gave a yield of 1–3 mg of Osps from about 700 mg of bacteria (wet weight from 1 l of BSK II medium) and rapid precipitation occurred on reduction of the detergent concentration, so that the preparations were not suitable for many experimental purposes. *n*-Butanol extraction is a highly reproducible alternative, yielding up to five times higher amounts of Osps under similar culture conditions.

The purification step on the FPLC cation-exchange column removed nucleic acids, which were a major contaminant and which accounted for most of the first peak in Fig. 3. Lane NB in Fig. 4 shows some protein contaminants that were also removed by this step. The amount of these contaminants is not readily assessable in Fig. 4, as lane NB contains only about one tenth of the amount of protein applied to lanes 38 and 39. Another important effect of the chromatographic procedure was concentration of the protein (from 0.3–0.6 mg/ml total protein in the crude *n*-butanol extract to 6–9 mg/ml in the major protein peak after chromatography). Hence it was possible to circumvent concentration, *e.g.*, by ultrafiltration, which in the case of cationic proteins often leads to undesirable losses of proteins.

It is well known that different strains of *Borrelia burgdorferi* species vary in their expression of Osps [21,22] and that even during cultivation shifts in protein expression may occur [23,24]. We selected some strains with distinct Osp profiles and examined whether the patterns found in SDS-PAGE of whole-cell lysates were identical with those seen in *n*-butanol extracts.

This was the case with most of the strains tested, but in two instances (P-Er and Z136) we obtained a protein profile in *n*-butanol extracts that differed considerably from that expected from whole cell analysis in SDS-PAGE. The most probable explanation for the apparent difference between whole-cell lysate and the *n*-butanol extract of strain Z136 (Figs. 1 and 2, lane F) is the presence of only minor amounts of Osps in this strain, so that the bands are barely visible in whole-cell lysate analysis, but are readily demonstrable when enriched by *n*-butanol extraction. A strong argument for this hypothesis is the fact that in immunoblotting it is possible to demonstrate binding of a monoclonal antibody (raised against OspA of strain B31) when analysing whole-cell lysates of Z136 (data not shown).

In strain P-Er, which did not reveal an OspB in *n*-butanol extracts (Fig. 2, lane E), either the 34000 dalton protein seen in SDS-PAGE of whole-cell lysates (Fig. 1, lane E) could represent an OspB with an altered structure that resisted extraction, or the band seen in the SDS-PAGE analysis of the whole-cell lysate could represent an unrelated protein of similar size.

Our results suggest that *n*-butanol may be a strong inducer of protein aggregation, probably by hydrophobic interaction and Van der Waals forces. Hence another possible explanation for the fact that the 35 000 dalton protein is not found in the *n*-butanol extract of strain P-Er and for the low yield of the 21 000 dalton protein in strain Z136 is aggregation to other proteins and subsequent separation into the interphase.

Several workers have reported that OspA and OspB [7,25] and the 22 000 dalton protein [25] are cationic when analysed by two-dimensional electrophoresis. The isoelectric points of OspA and OspB, as calculated from the amino acid sequences, are 9.5 and 9.6, respectively [9]. These findings are strongly supported by our results with ion-exchange chromatography, although the extent to which the individual proteins contribute to the positive charge of the complex found after *n*-butanol extraction cannot be decided.

There is no information about the native aggregation status of the Osp available. As prepared here, the outer surface proteins of *Borrelia burgdorferi* occur as a stable complex of about 800 000–900 000 dalton. The fact that the aggregate is not disintegrated by urea strongly suggests that the complexes are formed by hydrophobic interactions. So far an enhanced aggregation tendency of proteins after *n*-butanol extraction has not been reported; the question of whether the complex reflects the native aggregation state of the proteins or whether it is the result of the extraction method remains unanswered.

The major components which were co-extracted with the Osps were proteins in the range 20 000–23 000 dalton. It is not known whether these proteins are membrane associated. Bundoc and Barbour [24] reported recently that after cloning of a single strain of *Borrelia burgdorferi* by limiting dilution, they obtained clones which produced proteins of 18 500 or 21 000 dalton. These proteins were surface exposed and their production was coincidental with a decreased or absent production of OspB. Further, the smaller proteins shared a cross-reacting epitope with outer surface protein B when tested with OspB-reactive monoclonal antibodies.

The 22 000 dalton protein that we could extract from strain Pko was designated "pC" by Wilske *et al.* [21] and has been found to be a preferential target of the immune response in patients with Lyme disease. Other workers [25] have reported that a protein of 22 000 dalton is surface exposed and that the N-terminal amino acid sequence bears a significant homology to the deduced sequence of OspA, reported by Bergström *et al.* [9]. These data could indicate that the 22 000 dalton protein is a member of the Osp family and is expressed on the surface, although at present it is not known whether all the proteins in the range 20 000–23 000 dalton found in different strains are variants of one protein or members of one protein family.

The extraction procedure presented in this paper could provide a useful tool for the further study of antigenic variations in *Borrelia burgdorferi* and perhaps facilitate the classification of Borrelia strains. Studies with soluble outer surface proteins may allow new insights into the pathogenesis of Lyme disease, particularly the late manifestations.

ACKNOWLEDGEMENT

This work was supported by the Bundesministerium für Forschung und Technologie (BMFT 01 VM 8612).

REFERENCES

1 W. Burgdorfer, A G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt and J. P. Davis, *Science (Washington, D.C.)*, 216 (1982) 1317.
2 A. C. Steere, R. L. Grodzicki, A. N. Kornblatt, J. E. Craft, A. G. Barbour, W. Burgdorfer, G. P. Schmid, E. Johnson and S. E. Malawista, *N. Engl. J. Med.*, 308 (1983) 733.
3 Lyme disease—Connecticut, *Morbidity and Mortality Weekly Report*, U.S. Public Health Services, Vol. 37, 1988, p. 1.
4 A. C. Steere, *N. Engl. J. Med.*, 321 (1989) 586.
5 A. G. Barbour, S. L. Tessier and W. J. Todd, *Infect. Immun.*, 41 (1983) 795.
6 A. G. Barbour, S. L. Tessier and S. F. Hayes, *Infect. Immun.*, 45(1984) 94.
7 J. L. Benach, J. L. Coleman and M. G. Golightly, *J. Immunol.*, 140 (1988) 265.
8 A. G. Barbour and C. F. Garon, *Science (Washington, D.C.)*, 237 (1987) 409.
9 S. Bergström, V. G. Bundoc and A. G. Barbour, *Mol. Microbiol.*, 3 (1989) 479.
10 R. Wallich, U. E. Schaible, M. M. Simon, A. Heiberger and M. D. Kramer, *Nucleic Acids Res.*, 17 (1989) 8864.
11 P. A. Barstad, J. E. Coligan, M. G. Raum and A. G. Barbour, *J. Exp. Med.*, 161 (1985) 1302.
12 J. L. Coleman and J. L. Benach, *J. Infect. Dis.*, 155 (1987) 756.
13 A. G. Barbour, *Yale J. Biol. Med.*, 57 (1984) 71.
14 U. K. Laemmli and M. Favr, *J. Mol. Biol.*, 80 (1973) 575.
15 T. R. Howe, L. W. Mayer and A. G. Barbour, *Science (Washington, D.C.)*, 227 (1984) 645.
16 R. K. Morton, *Nature (London)*, 166 (1950) 1092.
17 R. K. Morton, *Methods Enzymol.*, 1 (1955) 40.
18 R. C. Johnson, M. S. Wachter and D. M. Ritzi, *Infect. Immun.*, 7 (1973) 249.
19 E. C. Klaviter and R. C. Johnson, *Acta Trop.*, 36 (1979) 123.
20 J. L. Coleman, J. L. Benach, G. Beck and G. S. Habicht, *Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A.*, 263 (1986) 123.
21 B. Wilske, V. Preac-Mursic, G. Schierz and K. V. Busch, *Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A.*, 263 (1986) 92.
22 A. G. Barbour, R. A. Heiland and T. R. Howe, *J. Infect. Dis.*, 152 (1985) 478.
23 T. G. Schwan and W. Burgdorfer, *J. Infect. Dis.*, 156 (1987) 852.
24 V. G. Bundoc and A. G. Barbour, *Infect. Immun.*, 57 (1989) 2733.
25 B. J. Luft, W. Jiang, P. Munoz, R. J. Dattwyler and P. D. Gorevic, *Infect. Immun.*, 57 (1989) 3637.