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Note

Partial purification of microsomal proteolipid(s) from *Neurospora crassa* by high-performance liquid chromatography on silica gel

WENDY B. MACKLIN*,*
Department of Biological Sciences, Stanford University, Stanford, CA 94305 (U.S.A.)
LOREN PICKART
Virginia Mason Research Center, 1000 Seneca Street, Seattle, WA 98101 (U.S.A.)
and
DOW O. WOODWARD
Department of Biological Sciences, Stanford University, Stanford, CA 94305 (U.S.A.)
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Certain membranes, in particular myelin and mitochondria, contain intrinsic proteins of extreme hydrophobicity. Those hydrophobic proteins which are soluble in chloroform–methanol (2:1) have been operationally defined as proteolipids[1]. Although the amino acid composition of different proteolipids differs, a striking feature common to these proteins is their high content of hydrophobic and neutral amino acid residues[2]. In addition to hydrophobic interaction with complex lipids, at least one proteolipid, the myelin proteolipid, contains covalently bound fatty acids[3]. Proteolipids have been purified by chloroform–methanol extraction, followed by dialysis in chloroform–methanol to remove non-covalently bound lipid; however, proteolipids of molecular weight less than 12,000 are lost from the dialysis rentate by this procedure[3]. Thus, dialysis is inappropriate for purification of low-molecular-weight proteolipids. Solubilization and chromatography in sodium dodecyl sulfate (SDS) have also been used to purify hydrophobic subunits of enzyme complexes such as cytochrome oxidase[4] or cytochrome b[5]; however, the associated SDS is difficult to remove and can interfere with subsequent analysis. Improved fractionation procedures would facilitate the work in this field.

The microsomal fraction of *Neurospora crassa* contains a proteolipid(s) of approximately 6500 daltons as measured by SDS–urea gels[6]. The synthesis of the proteolipid is blocked by the mitochondrial protein synthesis inhibitor chloramphenicol, but not by the cytoplasmic protein synthesis inhibitor cycloheximide, suggesting mitochondrial synthesis of the microsomal proteolipid. Only a small fraction of cellular protein is synthesized in the presence of cycloheximide. The microsomal proteolipid can be specifically radioactively labeled under these conditions and initial solubilization of this labeled proteolipid can be achieved by extraction with chloroform–methanol. The extract contains microsomal lipids such as phosphatidyl choline,

* Present address: Department of Biochemistry, E. K. Shriver Center, 200 Trapelo Rd., Waltham, MA 02254, U.S.A.

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ergosterol, fatty acids and triglycerides plus a number of proteolipids[6]. The large amount of lipid in the extract and the low molecular weight of the proteolipid are major factors complicating further purification of the proteolipid. We report here the use of high-performance liquid chromatographic (HPLC) silica gel columns to obtain an approximately 174-fold purification of this chloramphenicol-sensitive, cycloheximide-insensitive proteolipid with respect to protein specific activity, with the concomitant removal of the major contaminating lipids. The methods described here may be applicable to the purification of other proteolipids.

METHODS

Radioactive labeling and extraction of N. crassa proteolipids

N. crassa was cultured, labeled and fractionated as described previously[6]. Briefly, cells were grown for 18 h at 30°C. Cultures were exposed to cycloheximide for 5 min and subsequently labeled for 20 min with [^{3}H]phenylalanine. The cells were ground twice in a mortar with sand in 0.25 *M* sucrose, 50 m*M* Tris, 1 m*M* EDTA pH 7.5, containing 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF) to inhibit protease activity. The microsomal fraction was isolated by density gradient centrifugation at the 0.6/1.3 *M* sucrose interface of discontinuous sucrose gradients. A crude proteolipid preparation, containing proteolipids and lipids was prepared by extraction of the microsomes with chloroform–methanol (2:1). For large-scale preparations of the proteolipid, approximately 20 mg labeled microsomal protein was mixed with 700 mg unlabeled microsomal protein and the proteolipid fraction was extracted with chloroform–methanol.

Chromatographic separations

The proteolipid preparation was analyzed for lipids and proteolipids by thin-layer chromatography (TLC) on silica gel HR (Merck, Darmstadt, G.F.R.)[6]. The sample was separated by successive development in chloroform–methanol–glacial acetic acid–water (65:25:8:4) and then light petroleum (b.p. 35–60°C)–diethyl ether–glacial acetic acid (75:25:1). Lipids were visualized with iodine vapor; protein was visualized by ninhydrin reactivity. Bands were scraped from the plate into counting vials and radioactivity was determined by counting in toluene-liquifluor (New England Nuclear, Boston, MA, U.S.A.).

Analysis by paper chromatography utilized a tank saturated with *tert.*-amyl alcohol–methyl ethyl ketone–water (3:1:1). The chloroform–methanol-soluble sample was applied to Whatman paper and developed for 12 h. The paper was analyzed for protein and for radioactivity.

Separation of the crude proteolipid preparation was attempted by application of the fraction to Sephadex LH-20 or LH-60 columns (60 × 2 cm) equilibrated in chloroform–methanol (2:1). The columns were eluted at 9 ml/h for 24 h. Fractions were collected and analyzed for absorbance at 280 nm and for radioactivity.

Preparative HPLC separation was performed on the crude proteolipid preparation. Silica gel columns were packed with 5-μm LiChrosorb (Altex Labs., Berkeley, CA, U.S.A.). The HPLC instrument used was a ISCO Model 384 (Omaha, NE, U.S.A.). A 1-ml volume of crude proteolipid extract was injected onto a preparative silica column (25 × 1 cm) equilibrated with benzene–ethanol (95:5). A gradient was applied for 20 min which raised the final percentage of ethanol to 66%.

Other procedures

Protein was determined by the method of Lowry *et al.*[7] as adapted by Lees and Paxman[8], using bovine serum albumin as the standard.

Amino acids were determined after acid hydrolysis of the partially purified proteolipid. Protein was hydrolyzed in 6 *N* hydrochloric acid at 110°C for 72 h. Amino acids were separated on silica gel plates (EM Labs.) with ethanol–water (7:3) and visualized with ninhydrin.

RESULTS AND DISCUSSION

A crude microsomal proteolipid fraction was prepared by extraction of [^{3}H]phenylalanine-labeled microsomes with chloroform–methanol. Approximately 1.9% of the total microsomal protein was solubilized by chloroform–methanol. The crude proteolipid fraction was initially separated by chromatography on a Sephadex LH-20 column (Fig. 1). Two major radioactive peaks were obtained and further analyzed using a Sephadex LH-60 column. The radioactive fractions I and II migrated as single peaks and were separated from non-radioactive proteins on the LH-60 column. Analysis of peaks I and II after chromatography on LH-60 was attempted by paper chromatography (Fig. 2). The two fractions did not appear to be significantly different, nor particularly pure. Both fractions still contained non-radioactive ninhydrin-positive spots and most of the radioactive material remained at the origin. It is possible that they could represent a single radioactive protein which migrates as multiple peaks in LH-20 and LH-60 depending on association with lipids or on its state of aggregation. As a consequence of their chemical nature, the chromatographic behavior of proteolipids is somewhat unusual. The myelin proteolipid, for example, which on the basis of several criteria is considered to consist of a single protein[2], produces multiple peaks upon silica gel chromatography in chloroform–methanol[9]. These peaks have identical amino acid compositions and certain of them can be interconverted, suggesting that these multiple peaks may contain the same protein

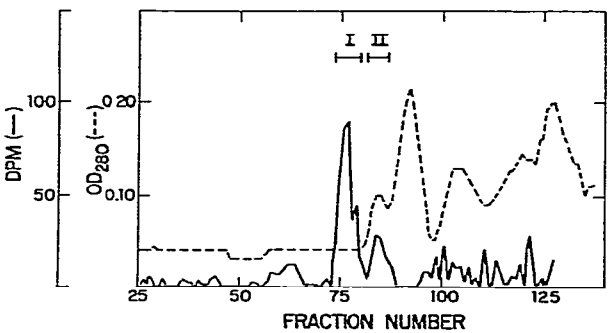

Fig. 1. Fractionation of chloroform–methanol extract of *N. crassa* microsomes on Sephadex LH-20 column. Microsomes were prepared from cells radioactively labeled with [^{3}H]phenylalanine in the presence of cycloheximide. 2.75 mg microsomal protein (0.6 ml) were extracted in 15 ml chloroform–methanol (2:1). After centrifugation to remove unextracted material, the supernatant was concentrated under nitrogen to approximately 2 ml and applied to a Sephadex LH-20 column equilibrated with chloroform–methanol (2:1). The column was eluted at 9 ml/h. Fractions (1 ml) were collected and analyzed for protein by absorbance at 280 nm and for radioactivity.

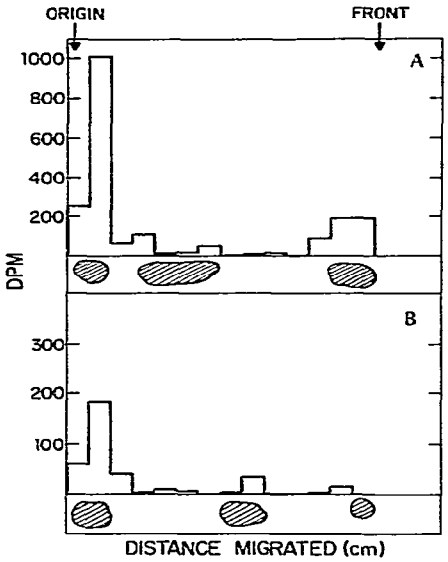

Fig. 2. Analysis of Sephadex LH-20–LH-60 column fractions I and II by paper chromatography. Sephadex LH-20 fractions I and II were re-chromatographed on a Sephadex LH-60 column and then concentrated under nitrogen, applied to Whatman paper and chromatographed. Crosshatched regions represent ninhydrin-positive spots. A, LH-20 fraction I; B, LH-20 fraction II.

which interacts with the silica gel differently depending on associated lipids or its state of aggregation.

In the present study, the usefulness of chromatography on LH-20 or LH-60 columns was limited to an analytical assessment of the microsomal proteolipid. Although some purification of the radioactive proteolipid was possible by chromatography on LH-20 and LH-60 columns, in general, the procedures were unsatisfactory for purification of the microsomal proteolipids. The radioactive proteolipid was not completely separated from the non-radioactive material and the recovery of material from the columns was quite poor as a consequence of non-specific adsorption of radioactive material to the columns. Once the proteolipid fraction was taken to dryness for further separation by TLC or paper chromatography, re-extraction of the protein was virtually impossible. Furthermore, the TLC or paper chromatographic systems tried by us resulted in poor resolution of the proteolipid. Thus, these chromatographic methods were inadequate for purification of the proteolipid.

A different approach to separation of the crude proteolipid preparation was attempted by analytical and preparative HPLC utilizing silica gel columns. A number of solvents were used for proteolipid fractionation in the HPLC system. Solvent systems employing chloroform–methanol or chloroform–methanol–acetic acid in various proportions did not resolve the proteolipid significantly on silica gel columns nor did use of a stronger acid, such as hydrochloric acid, with the chloroform–methanol. The most successful solvent system consisted of an initial solvent of benzene–ethanol (95:5) and a gradient of ethanol to a final concentration of 66%. The major radioactive fraction eluted as a sharp peak at benzene–ethanol (*ca.* 42:58) (Fig. 3). Direct counting of the silica gel from the columns indicated less than 1% of the proteolipid radioactivity was retained in the columns.

The radioactive fraction from the silica gel column was analyzed by TLC which indicated that this HPLC system freed the proteolipid extract of ergosterol, triglycerides and free fatty acids, although the proteolipid fraction was still contaminated with some phospholipid (Fig. 4). The mobility of the partially purified proteolipid in this TLC system was similar to, although broader than that of the major radioactive component of the crude extract[6]. After acid hydrolysis of the proteolipid, amino acids were analyzed by TLC in an amino acid separating system. No free amino acids were detectable prior to hydrolysis. On the basis of protein specific activity, the partially purified proteolipid was purified 2.5-fold from the crude proteolipid preparation and 174-fold from the original microsomal fraction (Table I). Furthermore, 85% of the contaminating lipids in the chloroform–methanol extract were also removed by this HPLC procedure, leaving only phospholipid as the major contaminant. Since more than 90% of the chloroform–methanol extract was lipid, the actual purification of the proteolipid was significantly higher than 174-fold.

The use of silica gel chromatography for fractionation of various lipid classes is well established. Silica gel columns containing a finely grained silica gel (5 μm) have been used to fractionate growth-promoting polypeptides which have substantial

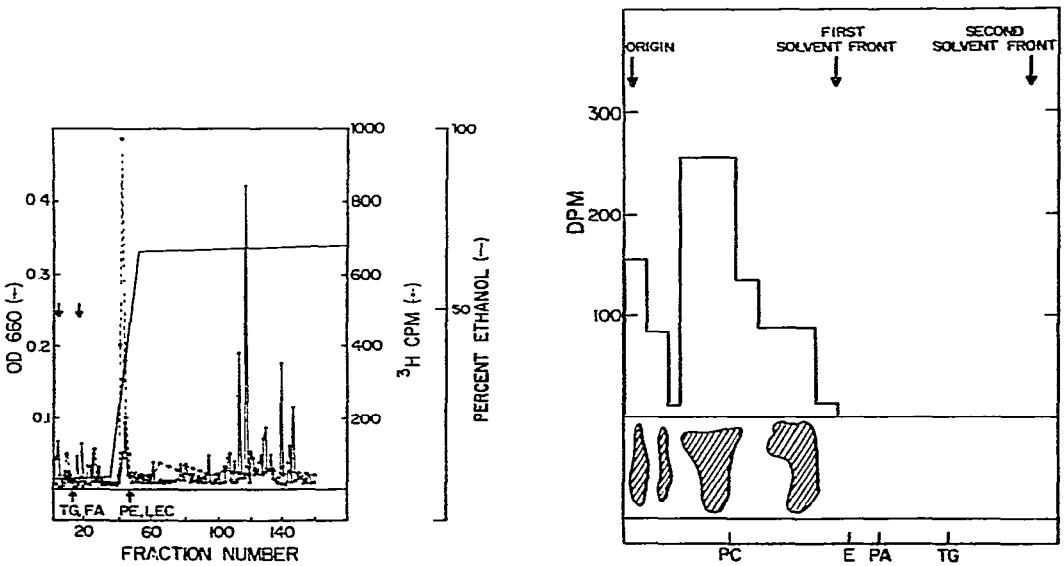

Fig. 3. Silica gel HPLC fractionation of chloroform–methanol extract of *N. crassa* microsomes. Two aliquots of 350 μg protein each were injected onto the column. The two arrows indicate times of sample injection onto the column. The column was originally equilibrated with benzene–ethanol (95:5) and was eluted with a 20-min gradient of 5–66% ethanol. Solid line represents the gradient. Fractions were analyzed for protein (circles), radioactivity (squares) and lipid content. Lipid standards: phosphatidyl ethanolamine (PE), phosphatidyl choline (LEC), triglycerides (TG) and fatty acids (FA).

Fig. 4. TLC analysis of microsomal proteolipid after partial purification by silica gel HPLC. After chromatography on silica gel HPLC, the proteolipid preparation was concentrated under nitrogen, and applied to silica gel HR thin layer plates (Merck). Plates were successively developed in chloroform–methanol–glacial acetic acid–water (65:25:8:4) and then light petroleum (b.p. 35–60°C)–diethyl ether–glacial acetic acid (75:25:1). Crosshatched area represents ninhydrin-positive spots. Plates were scraped and analyzed for radioactivity. Sample lipids and lipid standards were visualized with iodine. Standards were phosphatidyl choline (PC), ergosterol (E), palmitic acid (PA) and triolein (TG).

TABLE I

PURIFICATION OF CHLORAMPHENICOL-SENSITIVE, CYCLOHEXIMIDE-INSENSITIVE PROTEOLIPID FROM *N. CRASSA*, BY HPLC ON SILICA GEL

The purification described here is relative to protein only. The increase in purity is significantly higher relative to total weight, since lipids constitute more than 90% (w/w) of the chloroform–methanol microsomal extract and more than 85% of these lipids were removed by HPLC.

Proteolipid preparation	*Protein (mg)*	*Protein (cpm/mg)*
Microsomal fraction	720	–
Chloroform–methanol extract from microsomes	13.4	930
Radioactive peak from silica gel HPLC	4.2	2330
Increase in purity	174-fold relative to the microsomal fraction	2.5-fold relative to the chloroform–methanol extract from microsomes

hydrophobic character[10]. More recently, various types of silica gel columns and especially reversed-phase silica gel columns have been used to fractionate a variety of polypeptides and proteins, such as interferon and mitochondrial proteolipids which had previously been resistant to isolation[11–14]. The separation described in this paper increased the protein specific activity of the labeled proteolipid by approximately 174-fold. In addition, a major purification of the chloroform-methanol extract was obtained by removal of lipid contaminants. The results described in this paper may be applicable to a variety of other types of biological proteins of extreme hydrophobic character such as serum lipoproteins or the hydrophobic subunits of enzymes such as cytochrome oxidase or cytochrome *b*.

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REFERENCES

1 J. Folch and M. Lees, *J. Biol. Chem.*, 191 (1951) 807.
2 M. B. Lees, J. D. Sakura, V. S. Sapirstein and W. Curatolo, *Biochim. Biophys. Acta*, 559 (1979) 209.
3 J. Folch-Pi and P. J. Stoffyn, *Ann. N.Y. Acad. Sci.*, 195 (1972) 86.
4 R. O. Poyton and G. Schatz, *J. Biol. Chem.*, 250 (1975) 752.
5 H. Weiss and B. Ziganke, in Th. Bucher (Editor), *Genetics and Biogenesis of Chloroplasts and Mitochondria*, North-Holland, Amsterdam, 1976, p. 259.
6 W. B. Macklin, D. J. Meyer, D. O. Woodward and S. K. Erickson, *Nature (London)*, 269 (1977) 447.
7 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
8 M. B. Lees and S. Paxman, *Anal. Biochem.*, 47 (1972) 184.
9 M. Matsumoto, R. Matsumoto and J. Folch-Pi, *J. Neurochem.*, 11 (1964) 829.
10 L. Pickart and M. Thaler, *Prep. Biochem.*, 5 (1975) 397.
11 W. S. Hancock, C. A. Bishop and M. T. W. Hearn, *FEBS Lett.*, 72 (1976) 139.
12 J. E. Rivier, *J. Liquid Chromatogr.*, 1 (1978) 343.
13 M. Rubenstein, S. Rubenstein, T. C. Familletti, R. S. Miller, A. A. Waldman and S. Pestka, *Proc. Nat. Acad. Sci. U.S.*, 76 (1979) 640.
14 G. A. Blondin, *Biochem. Biophys. Res. Comm.*, 90 (1979) 355.