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Note

High-performance liquid chromatography of plant membrane proteins

NADH-cytochrome b_5 reductase as a model

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Membrane proteins play important rôle in living cells. Their purification is difficult since detergents are needed for their solubilization. In this work, we have studied NADH-cytochrome b_5 reductase, a membrane flavoprotein, as a model. It is a plant microsomal flavoprotein which is assumed to play an important rôle in the oleate-desaturase activity[1]. In previous work[2] we purified the flavoprotein from potato tuber or maize seedling microsomes by using low-pressure chromatography; the successive steps of purification were chromatography on DEAE-Trisacryl and blue Ultrogel affinity columns, followed by gel filtration.

In the present study, we replaced the filtration step by a separation by fast protein liquid chromatography (FPLC) on an anion-exchange column (Mono Q). This technique yielded a pure flavoprotein which an high specific activity. The purified fraction was also subjected to high-performance liquid chromatography (HPLC) on a reversed-phase column. This work opens new perspectives for the study of other plant membrane proteins.

EXPERIMENTAL

The preparation of microsomal membranes and the solubilization of protein membranes by 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), a zwitterionic detergent, were performed according to Galle *et al.*[2]. The solubilized proteins were then successively chromatographed on a DEAE-trisacryl and a blue Ultrogel columns. The blue Ultrogel fraction, which contained NADH-cytochrome b_5 reductase activity, was then either chromatographed on a Sephadex G-75 column according to Galle *et al.*[2] or analyzed by FPLC or HPLC. The protein concentration was carried out according to Bradford[3].

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Separation on Mono Q column by FPLC

A FPLC system (Pharmacia) was employed with a Mono Q HR5/5 column (5 cm × 0.5 cm). The column was equilibrated with buffer A [1 m*M* EDTA, 3 m*M* sodium azide, 1 m*M* phenylmethylsulphonyl fluoride (PMSF), 10% glycerol, 10 m*M* β-mercaptoethanol, 10 m*M* Tris–HCl (pH 8.0), 1 m*M* CHAPS]. After rinsing with buffer A for 10 min, a gradient of 0–0.25 *M* sodium chloride in buffer A was performed in two steps; the percentage of buffer A containing 0.25 *M* sodium chloride was increased from 0 to 60% in 20 min, then to 100% in 5 min. Elution was performed with a flow-rate of 1 ml/min and 1-ml fractions were collected.

Separation by reversed-phase HPLC

The HPLC system (Waters) was used with a reversed-phase column (7.5 cm × 4.6 mm) (Ultrapore, Beckman). The column was equilibrated with buffer B [water, 0.1% trifluoroacetic acid (TFA), pH 2.0] containing 8% of acetonitrile. A 200-μl volume of filtered fraction (total microsomal fraction or blue Ultrogel fraction) was injected. The elution (flow-rate 1 ml/min) was performed with a linear gradient from 8 to 80% acetonitrile in buffer B during 80 min. The absorbance at 214 nm was membered since the activity of NADH-ferricyanide reductase was destroyed by the solvent. After evaporation of acetonitrile under nitrogen, the peaks were identified by sodium dodecyl sulphate (SDS)-acrylamide gel electrophoresis, performed according to the method of Chua[4].

RESULTS

Purification of NADH-cytochrome b_5 reductase by FPLC

When 1 ml of the blue Ultrogel fraction was chromatographed on a Mono Q column, one peak of NADH-cytochrome b_5 reductase was detected (Fig. 1). This peak was eluted at about 0.15 *M* sodium chloride. The absorbance at 280 nm indicates that other proteins are eluted before and after the peak of flavoprotein. Elec-

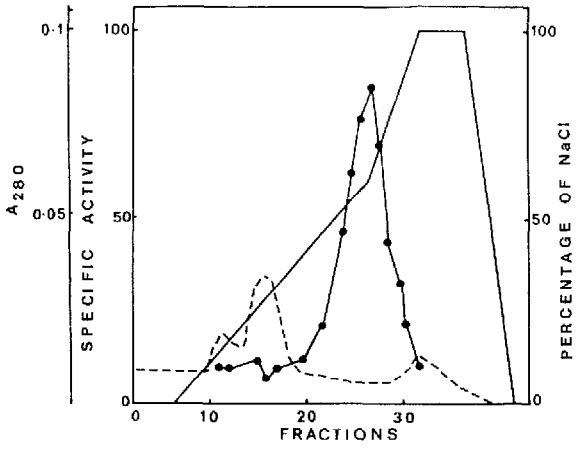

Fig. 1. FPLC elution profile on a Mono Q column. ●—●, NADH-ferricyanide reductase activity in nmol of ferricyanide reduced per min per 50 μl of fraction; ——, absorbance at 280 nm; ——, gradient of sodium chloride.

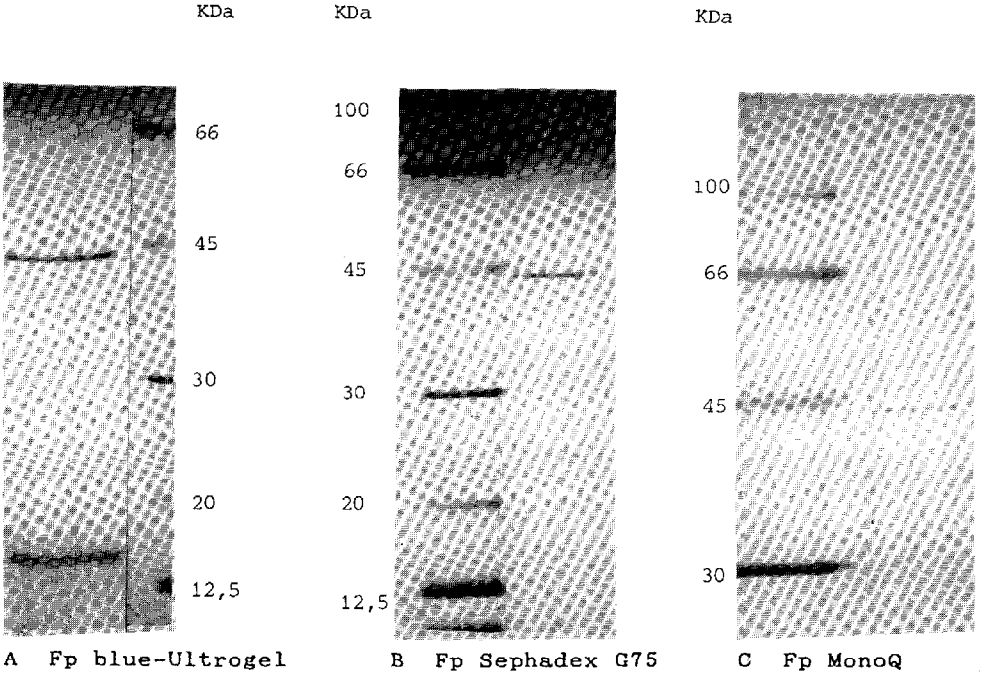

Fig. 2. Electrophoretic analysis on SDS-polyacrylamide gel of NADH-ferricyanide reductase from different steps of the purification: (A) blue Ultrogel column; (B) Sephadex G-75 column; (C) FPLC on Mono Q column.

trophoresis in SDS-polyacrylamide gel yielded a single band which migrated before ovalbumin (MW 45000) (Fig. 2C). The contaminating proteins present in the blue Ultrogel fraction (Fig. 2A) have disappeared. A similar band was observed when a Sephadex G-75 column was used instead of FPLC (Fig. 2B). The molecular mass of the NADH-cytochrome b_5 reductase obtained with the Mono Q column is 44 000 ± 1000 similar to that of the flavoprotein obtained with Sephadex G-75[2]. It is of interest that a similar molecular mass has been obtained for NADH-cytochrome b_5 reductase from animals[5,6].

Comparison of NADH-cytochrome b_5 reductase obtained with Sephadex G-75 or Mono Q column

After chromatography of the blue Ultrogel fraction by FPLC on the Mono Q column, 15% of the initial activity was recovered; a lower yield was obtained when Sephadex G-75 chromatography was performed (Table I). The specific activity of the Mono Q fraction was also higher (628 μmol per min per mg of protein). When 1 ml of the blue Ultrogel fraction was chromatographed, the active fraction was recovered in the same volume as that of the injected fraction; in contrast, use of the Sephadex G-75 column introduced a dilution of 34%.

In conclusion, with Mono Q chromatography as the last step of the purification, we obtained a pure and non-diluted flavoprotein fraction with a higher specific activity of NADH-cytochrome b_5 reductase. Another interesting point is that, with

TABLE I

COMPARISON OF THE NADH-CYTOCHROME b_5 REDUCTASE PURIFIED ON MONO Q AND SEPHADEX G-75 COLUMNS

The values are expressed relative to the blue Ultrogel fraction.

	Fraction	
	Mono Q	*Sephadex G-75*
% of total NADH-FeCN reductase recovered	15	10
Final specific activity*	628	401
Augmentation of volume (%)	0	34.2

* Expressed as μmol of ferricyanide reduced per min per mg of protein.

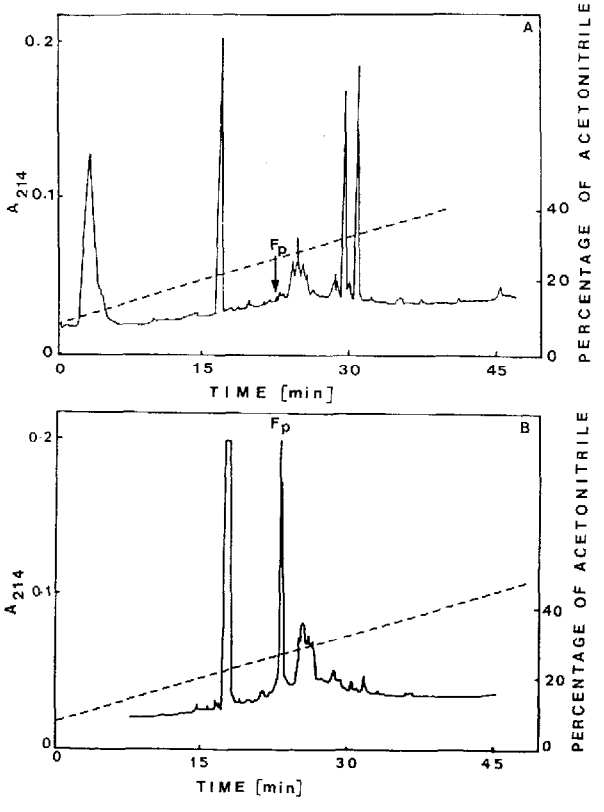

Fig. 3. HPLC elution profile on a reversed-phase column. (A) Microsomal fraction; (B) blue Ultrogel fraction. Fp = NADH-cytochrome b_5 reductase. ——, Absorbance at 214 nm; – – –, gradient of acetonitrile.

FPLC, the experiment lasts about 30 min and 30 fractions have to be assayed, whereas a 16 h are needed for Sephadex G-75 and 120 fractions have to be studied.

Purification of NADH-cytochrome b_5 reductase by reversed-phase HPLC

When the microsomal fraction was injected into the reversed-phase column different peaks were observed (Fig. 3A). When the blue Ultrogel fraction was injected, the first peak at 17 min corresponded to β-mercaptoethanol (Fig. 3B). A second major peak was eluted by 27% acetonitrile. The other peaks corresponded to salts. Electrophoresis showed that the major peak corresponded to the flavoprotein, with a molecular mass of 44000. However, under those conditions, the reductase activity was destroyed by the solvent. It can be concluded that, in three steps of chromatography, a fraction highly enriched in flavoprotein was obtained, although this protein is a minor component of microsomal membranes.

CONCLUSION

The present work shows that FPLC performed with an anionic column in the presence of a zwitterionic detergent yields active and purified NADH-cytochrome b_5 reductase. To the best of our knowledge, this is the first time that this technique, which is fast, highly resolutive and non-diluting, has been used for this purpose. We think that this combination of FPLC and a zwitterionic detergent, which maintains the functional activity of the proteins, could be used for the purification of other membrane proteins. HPLC with a reversed-phase column, although inactivating proteins, could give additional information since chemical and immunochemical studies could be performed.

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