Purification and properties of plant cytochrome b_5

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Microsomal cytochrome b_5 was 352-fold purified from potato tubers with a yield of 10.4%. To our knowledge, this is the first report relating the purification of higher-plant cytochrome b_5 . Its M_r (16700) and absorption spectrum are similar to those of animal and yeast cytochrome b_5 .

Linoleic acid is synthesized by a microsomal enzymic complex, the oleate desaturase, which is characteristic of plants. On the basis of homology to stearyl-CoA desaturase from animal tissues (Strittmatter *et al.*, 1974), it is assumed that this complex contains at least three components: NADH-cytochrome b_5 reductase, cytochrome b_5 and the desaturase (Kader, 1977; Murphy *et al.*, 1983). Cytochrome b_5 has been purified to homogeneity from animal (Spatz & Strittmatter, 1971) and yeast (Bertrand *et al.*, 1982; Yoshida *et al.*, 1983) microsomal fractions. In contrast, only a partial purification has been obtained in preliminary work on potato microsomal fraction (Christ *et al.*, 1982). The present paper shows the purification for the first time of a higher-plant cytochrome b_5 with the use of a zwitterionic detergent (CHAPS) as a solubilizing agent.

Experimental

Materials

Potato tubers (*Solanum tuberosum* L., var. Bintje) were kindly provided by Institut Technique de la Pomme de Terre, St.-Rémy l'Honoré, France. CHAPS was synthesized as described by Hjelmeland (1980). DEAE-Trisacryl and CM-Trisacryl were purchased from I.B.F. (Paris, France), and Sephadex G-75 was from Pharmacia (Uppsala, Sweden).

All the other reagents were analytical grade (Boehringer, Mannheim, Germany; Sigma Chemical Co., St. Louis, MO, U.S.A.; Rhône Poulenc, Lyon, France).

Abbreviation used: CHAPS, 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulphonate.

Methods

Preparation of microsomal pellets. All manipulations during the isolation procedure were performed at 4°C. Potato tubers were treated with Rindite in order to increase the microsomal content of cytochrome b_5 (Bonnerot *et al.*, 1985). Rindite (ethylene chlorohydrin/ethylene dichloride/carbon tetrachloride, 7:3:1, by vol.), currently used in agronomy for inducing germination, was used in its gaseous form. Peeled potato tubers (15 kg) were homogenized as described by Galle *et al.* (1984). Microsomal pellets were resuspended in buffer A [1 mM-EDTA/3 mM- NaN_3 /1 mM-phenylmethanesulphonyl fluoride/10 mM-2-mercaptoethanol/10% (v/v) glycerol/10 mM-Tris/HCl buffer, pH 8.0].

Solubilization of microsomal proteins. Solubilization of microsomal proteins (6 mg/ml) was allowed to proceed with gentle stirring for 30 min at 4°C with CHAPS (1.5–2 mg of detergent/mg of protein) as described by Bonnerot *et al.* (1984).

Centrifugation at 50000g for 120 min yielded a supernatant fraction, which was further purified.

Gel electrophoresis. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out as described by Chua (1980) with 15% acrylamide. Standards used were ovalbumin (M_r 45000), carbonic anhydrase (M_r 30000), soya-bean trypsin inhibitor (M_r 20100) and cytochrome c (M_r 12380).

Protein content. This was measured by the method of Bradford (1976), with bovine serum albumin as standard.

Cytochrome b_5 content. This was measured by difference spectrum by using a molar absorption coefficient of $160\text{ mm}^{-1}\cdot\text{cm}^{-1}$ between 409 and 423 nm as described by Bertrand *et al.* (1982) and Yoshida *et al.* (1983).

Results

Purification of cytochrome b_5

Step 1. This is the solubilization of proteins with CHAPS (Table 1).

Step 2. The solubilized proteins were applied to a DEAE-Trisacryl anion-exchange column (5 cm $\times$ 20 cm) equilibrated with buffer A containing 1 mM-CHAPS (buffer A+). The column was washed with buffer A+, and then with buffer A+ containing 0.1 M-NaCl. Cytochrome b_5 was eluted with buffer A+ containing 1 M-NaCl and chromatographed on a Sephadex G-75 column (5 cm $\times$ 70 cm) equilibrated with buffer A+ at a flow rate of 90 ml/h (Fig. 1). This gel filtration permits the separation of aggregated cytochrome b_5 (void volume) and non-aggregated cytochrome b_5 (fractions 71–92), which was further purified.

Step 3. The non-aggregated cytochrome b_5 was loaded on a DEAE-Trisacryl column

(2.5 cm $\times$ 15 cm) equilibrated with buffer A+. The buffer was then changed to a phosphate buffer P+ [1 mM-EDTA/3 mM- NaN_3 /1 mM-phenylmethanesulphonyl fluoride/10 mM-2-mercaptoethanol/1 mM-CHAPS/10% (v/v) glycerol/10 mM- KH_2PO_4 /NaOH buffer, pH 7.0]. When the column was equilibrated at this new pH, cytochrome b_5 was eluted with a linear gradient of 500 ml of buffer P+ containing 0.07 M-NaCl and 500 ml of buffer P+ containing 0.3 M-NaCl with a flow rate of 50 ml/h.

Two peaks were eluted (Fig. 2). Only the second (D2), which has the highest specific content, was further purified.

Step 4. Fraction D2 was diluted with buffer P+ and its pH adjusted to 5.9. This fraction was then applied to a CM-Trisacryl cation-exchange column (1 cm $\times$ 6 cm) equilibrated with buffer P+, pH 5.9. The elution was conducted with a linear gradient

Fig. 1. Elution profile on Sephadex G-75 column (step 2)

The concentrated solubilized fraction was chromatographed on a Sephadex G-75 column (5 cm $\times$ 70 cm) with buffer A+ at a flow rate of 90 ml/h; 10 ml fractions were collected. For details see the text. Absorbance at 280 nm (—) was monitored continuously, and cytochrome b_5 content (◆—◆) was measured in individual fractions.

Fig. 2. Elution profile on DEAE-Trisacryl column. The buffer was changed to buffer P+ (fraction 1), and the column (2.5 cm $\times$ 15 cm) was eluted (arrow) with a linear gradient of 500 ml of buffer P+ containing 0.07 M-NaCl and 500 ml of buffer P+ containing 0.3 M-NaCl at a flow rate of 50 ml/h. For details see the text. Absorbance at 280 nm (—) was monitored continuously. Cytochrome b_5 content (◆—◆) and conductivity (.....) were measured in collected 10 ml fractions.

Table 1. Purification of cytochrome b_5 from potato microsomal fraction
For experimental details see the text.

Step	Protein (mg)	Cytochrome b_5 (nmol)	Yield (%)	Specific content (nmol of cytochrome b_5 /mg)	Purification factor
1. Solubilized proteins	2270	257	100	0.11	1
2. Sephadex G-75 (fractions 71–92)	40.7	71	27.5	1.7	15
3. DEAE-Trisacryl (fractions 45–70: D2)	4.9	33.7	13	6.9	61
4. CM-Trisacryl (fractions 62–71: C1)	2.3	22.2	8.6	9.5	84
5. Sephadex G-75 (fractions 74–84)	0.67	26.9	10.4	39.8	352

of 150 ml of buffer P+ containing 0.05 M-NaCl and 150 ml of buffer P+ containing 0.2 M-NaCl at a flow rate of 50 ml/h. Cytochrome b_5 was eluted in a sharp peak (C1) (Fig. 3).

Step 5. Fraction C1 was then concentrated and its buffer changed to buffer A+ with a DEAE-Trisacryl column (1 cm $\times$ 10 cm) eluted by buffer A+ containing 1 M-NaCl.

The concentrated fraction was then chromatographed on a second Sephadex G-75 column (5 cm $\times$ 70 cm) equilibrated with buffer A+ at a flow rate of 70 ml/h. A single peak was detected (Fig. 4), and, after concentration on a DEAE-Trisacryl column (1 cm $\times$ 10 cm), a 352-fold-purified fraction was obtained. The overall yield was 10.4%, which is similar to that obtained for yeast cytochrome b_5 .

The specific content (39.8 nmol of cytochrome b_5 /mg of protein) is closely similar to those obtained for animal or yeast cytochrome b_5 .

Properties of cytochrome b_5

Homogeneity. Electrophoresis of the concentrated fraction shows a major band with some contaminants (Fig. 5).

Physical properties. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicates an M_r value of 16600 (Fig. 5). Calibration of the Sephadex G-75 column gives an M_r of 16800.

The average M_r value (16700) is similar to the M_r reported for animal and yeast cytochrome b_5 .

The isoelectric point is between 5.9 and 7, as indicated by binding to ion-exchange columns (DEAE-Trisacryl and CM-Trisacryl).

Fig. 3. Elution profile on CM-Trisacryl column The column (1 cm $\times$ 6 cm) was eluted with a gradient of 150 ml of buffer P+ containing 0.05 M-NaCl and 150 ml of buffer P+ containing 0.2 M-NaCl at a flow rate of 50 ml/h. For details see the text. Absorbance at 280 nm (—) was monitored continuously. Cytochrome b_5 content (◆—◆) and conductivity (.....) were measured in 10 ml fractions.

Fig. 4. Elution profile on Sephadex G-75 column (step 5) The concentrated C1 fraction was chromatographed on a Sephadex G-75 column (5 cm $\times$ 70 cm) with buffer A+ at a flow rate of 70 ml/h; 10 ml fractions were collected. For details see the text. Cytochrome b_5 content (◆—◆) was measured in individual fractions.

Fig. 5. Electrophoretogram of purified cytochrome b_5 (step 5) on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

For details see the text. (a) Cytochrome b_5 (5 μ g); (b) standard proteins (from top to bottom: ovalbumin, M_r 45000; carbonic anhydrase, M_r 30000; trypsin inhibitor, M_r 20100; cytochrome c, M_r 12380).

Fig. 6. Low-temperature spectrum of reduced purified cytochrome b_5 (step 5)
The preparation was reduced by $\text{Na}_2\text{S}_2\text{O}_4$.

Absorption spectrum. The low-temperature absorption spectrum of reduced cytochrome b_5 (Fig. 6) shows two peaks in the α -region (552 and 558 nm), four peaks in the β -region (532, 524, 514 and 509 nm) and a major peak at 422 nm, with a shoulder at 400 nm that seems to be a contaminant.

The spectrum of the reduced preparation in the presence of CO does not show any peak at 450 nm. This proves that the purified fraction is indeed cytochrome b_5 (and not cytochrome P -450).

Discussion

The major difficulties in this work were the very low specific content of cytochrome b_5 in plant microsomal preparations (Rich & Bendall, 1975) and the amphipathic nature of this protein, which is embedded in the membrane.

These properties make any buffer change or concentration through dialysis harmful: the protein has high affinity for all kinds of dialysis membrane. Any concentration against poly(ethylene glycol) or sucrose or similar materials would change the ratio protein/detergent and result in an irreversible aggregation.

We therefore preferred dilutions of solutions rather than dialysis to lower ionic strength, and chromatography on a small DEAE-Trisacryl column rather than concentration against poly(ethylene glycol) for concentration steps.

CHAPS was chosen as solubilizing agent after comparison with cholate and Triton X-100 (Bonnerot *et al.*, 1984).

It is worth noting that the buffer change was also performed on an ion-exchange column and that, for the first time, cytochrome b_5 was bound to a cation-exchange column. In conclusion, the present paper reports the first purification of cytochrome b_5 from higher-plant microsomal fraction. The isolation procedure involves gel-filtration steps and chromatography on anion-exchange and cation-exchange columns.

A fraction with a high specific content (39.8 nmol of cytochrome b_5 /mg of protein) was obtained, which allows further studies on this membrane protein.

This purification of cytochrome b_5 and the purification of NADH-cytochrome b_5 reductase (Galle *et al.*, 1984) should allow us to reconstitute the NADH-cytochrome c reductase, i.e. the complex flavoprotein-cytochrome b_5 , which is the first step of the oleate-desaturation process.

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