HOKKAIDO UNIVERSITY

Instructions for use

Isolation and purification of cytochrome b_{555} from spadix tissue of *Lysichiton camtschatense* Schott

By

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Cytochrome b_{555} was purified to be electrophoretically homogeneous from spadix tissues of *Lysichiton camtschatense* Schott (Mizubasho, in Japanese). Peroxidase activity found in the tissues could be removed completely during the purification.

The absorption spectra of the cytochrome b showed peaks at 413 nm in the oxidized form, and 423.5, 527 and 555 nm with a pronounced shoulder at 560 nm in the reduced form.

The cytochrome b combined neither with carbon monoxide nor with cyanide, and the reduced cytochrome b was reoxidized easily by bubbling with air. It contained protoheme as the prosthetic group. The molecular weight was determined to be 27,000 by the gel filtration method.

The cytochrome b_{555} was reduced rapidly by sodium hydrosulfite and sodium borohydride, but not by ascorbate, cysteine, succinate nor NADH. The midpoint redox potential (E_0') of cytochrome b_{555} was −0.02 volt at pH 7.0 and 15°C.

Since the discovery by JAMES *et al.*[1,2] that the rapid oxidation processes characteristic of *Arum* spadix tissues are not influenced significantly by cyanide, there have been furnished a series of evidences to demonstrate that the respiration in spadices of *Symplocarpus*[3,4], *Philodendron* and *Paltandra*[5] is also insensitive to cyanide.

BENDALL and HILL[6] reported that a mitochondrial fraction from *Arum* spadix contained a normal cytochrome system with components *a*, *b* and *c*, and in addition a large amount of a cytochrome b_7. JAMES and ELLIOT[7] speculated that a cytochrome b_7 or flavoprotein participated as a terminal oxidase in the cyanide-insensitive respiration of *Arum* spadix. KUWAYAMA

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et al.[8~10] reported that the respiration of spadix tissues of *Lysichiton* (Mizu-basho, in Japanese) growing in field in Hokkaido, Japan, was not inhibited but positively stimulated by cyanide.

In the present paper, a method of isolation and purification of cytochrome b_{555} from *Lysichiton* spadix and properties of the purified preparations are described.

Materials and Methods

Lysichiton camtschatense SCHOTT (Mizubasho, in Japanese) was collected from the fields in Hokkaido, Japan, during April, May and June. Young spadix (the spadix was wrapped tightly in the spathe) and spadix prior flowering (just prior to the opening of the spathe) were mainly used[10]. Spadix slices were prepared from fresh spadix tissues, without central rachis.

Spectral determinations were made by a Hitachi EPS-3T recording spectrophotometer or a Hitachi Perkin-Elmer 139 UV–VIS spectrophotometer using cuvettes of 1 cm light path.

The content of cytochrome b_{555} in the fractions was calculated by the ratio of the difference in optical density of each fraction, ΔA_{555} (Red-Ox)-ΔA_{542} (Red-Ox), to the standard value of 19 cm^{-1} mM^{-1} obtained from the purified preparation.

The prosthetic group was determined by the method of SHICHI and HACKETT[11].

Peroxidase activity was determined by the method of SHICHI and HACKETT[12]. Specific activity was expressed in terms of the activity per minute per mg of protein.

Protein was estimated by the method of LOWRY *et al.*[13] using bovine serum albumin as a standard.

Molecular weight was determined by the method of ANDREWS[14]. The following proteins were used as standard; whale sperm myoglobin (mol. wt., 13,700), ovalbumin (45,000), and bovine serum albumin (67,000). Albumin and ovalbumin in column eluates were estimated by absorbance at 280 nm, myoglobin at 280 and 410 nm, and cytochrome b at 280 and 410 nm in the oxidized form and 555 nm in the reduced form.

The midpoint redox potential (E_0') was determined by the method of SHICHI and HACKETT[11] with some modifications. The ratio of reduced (b^{++}) to oxidized (b^{+++}) cytochrome b_{555} was calculated from the following formula.

$$\frac{b^{++}}{b^{+++}} = \frac{(\Delta A_{423nm} \text{ with } Fe^{++})}{((\Delta A_{423nm} \text{ with sodium hydrosulfite})-(\Delta A_{423nm} \text{ with } Fe^{++}))}$$

E'_0 of the pigment was determined from a plot of log (b^+/b^{+++}) *versus* log (Fe^+/Fe^{+++}) assuming that E'_0 of ferri-ferro oxalate system was 0.0 volt[15].

Polyacrylamide gel electrophoresis was performed by the method of DAVIS[16], at a constant current of 3 mA per gel loaded with 15 μg of protein. After electrophoresis the gels were stained for 30 minutes in 1% amido black in 7.5% acetic acid and destained by 7% acetic acid.

Reagents were purchased commercially. DEAE-cellulose (ion exchange capacity of 0.8–0.9 m Eq/g) was purchased from Brown Co., and Sephadex were from Pharmacia.

Results

Isolation and Purification of Cytochrome b—All operations were carried out at 0–5°C unless otherwise indicated.

Step I : Batches of 1 kg (wet weight) of spadix slices were homogenized with 1 liter of 0.02 M phosphate buffer, pH 7.2, containing 0.1 mM EDTA, in a Waring blendor at the maximum speed for 2 minutes. Just before homogenization, 4 g of sodium ascorbate was added. The homogenate was filtrated with cheese-cloth. The filtrate was centrifuged at 3,000 × g for 15 minutes and the supernatant was gently stirred with approx. 10 volumes of cold acetone (−15°C). After standing 10 minutes, the mixture was filtrated through a filter paper (Toyo, No. 2) on a Buchner funnel. The residue on the funnel was washed by passing cold acetone a few times. Immediately, the residue was dried on P_2O_5 in a desiccator by evacuation. After complete drying, powdered residue was stored at room temperature *in vacuo*, until use.

Step II : 100 g of the powder was suspended in 2 liter of 0.01 M phosphate buffer, pH 7.0. After standing overnight at room temperature the mixture was centrifuged at 9,000 × g for 15 minutes. To the supernatant fluid, saturated ammonium sulfate solution was added to 30% saturation. After standing for 3 hours, precipitate was centrifuged off at 9,000 × g for 15 minutes. To the supernatant fluid, powdered ammonium sulfate was added to 90% saturation. After standing overnight the precipitate was collected by centrifugation at 9,000 × g for 15 minutes and dissolved in 400 mℓ of 0.01 M phosphate buffer, pH 7.0. The solution was centrifuged at 9,000 × g for 15 minutes and the supernatant was dialyzed 2 days against 0.05 M phosphate buffer, pH 7.0. The dialyzed solution was passed through a first DEAE-cellulose column (4 × 10 cm) equilibrated with 0.05 M phosphate buffer, pH 7.0. Cytochrome b_{555} was adsorbed on the top of the column as a dark red band, but cytochrome c passed through it. Then

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the column was washed with water, 0.05 M NaCl and 0.5 M NaCl successively. During the washing with 0.5 M NaCl the dark red band was eluted off the column. The dark red eluate was dialyzed overnight against 0.05 M phosphate buffer, pH 7.0. The dialyzed solution was passed through a second DEAE-cellulose column (1.4×7 cm). After the same treatment as above, a brown red eluate was obtained. The eluate was applied on a first Sephadex G–75 column (2.6×95 cm) equilibrated with 0.01 M phosphate buffer, pH 7.0, and the column was washed with the same buffer. A profile of chromatography of cytochrome b_{555} on the first Sephadex G–75 column is shown in Fig. 1. Peaks I and II in Fig. 1 were not considered as cytochrome fractions, therefore peak III was collected and used as cytochrome b_{555} fraction. A fraction of peak III in Fig. 1 was dialyzed
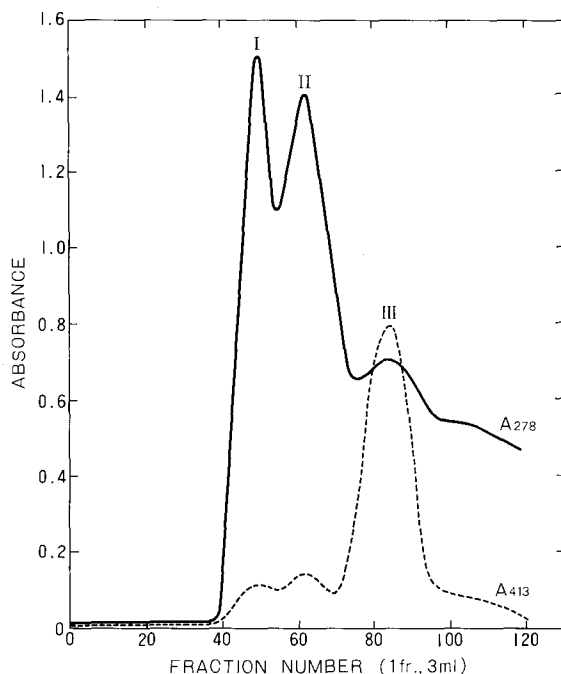

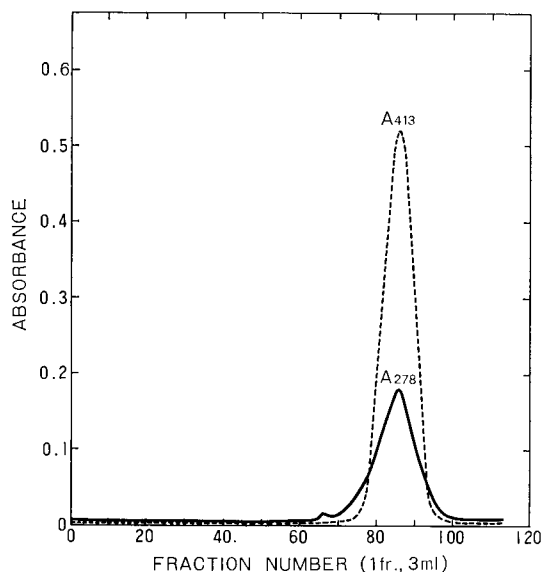**Fig. 1.** Chromatography of cytochrome b on the first Sephadex G–75 column.

Sephadex G–75 column (2.6×95 cm) was equilibrated with 0.01 M phosphate buffer, pH 7.0, and the column was washed with the same buffer.

——: Absorption at 278 nm.
⋯⋯: Absorption of cytochrome b at Soret band in oxidized form (A_{413}).

overnight against 0.05 M phosphate buffer, pH 7.0. The dialyzed solution was passed through a third DEAE-cellulose column (1.4 × 7 cm). After the same treatment as above, a brown red eluate was obtained. The eluate was applied on a second Sephadex G–75 column (2.6 × 95 cm). Fractions of a second Sephadex G–75 column treatment having more than 1.0 of a A_{413}/A_{278} value were collected as cytochrome b fraction. After dialysis against 0.05 M phosphate buffer, pH 7.0, this fraction was successively applied on a fourth DEAE-cellulose column (1.4 × 7 cm) and a third Sephadex G–75 column (2.6 × 95 cm) with the same method as above. Elution pattern of cytochrome b_{555} on a third Sephadex G–75 column is shown in Fig. 2.

Fractions in Fig. 2 having more than 2.0 of a A_{413}/A_{278} value were collected as cytochrome b fraction. Dialysis and final (fifth) DEAE-cellulose column treatment were performed. With the final DEAE-cellulose column, a red eluate was obtained. The red eluate was dialyzed against 0.05 M
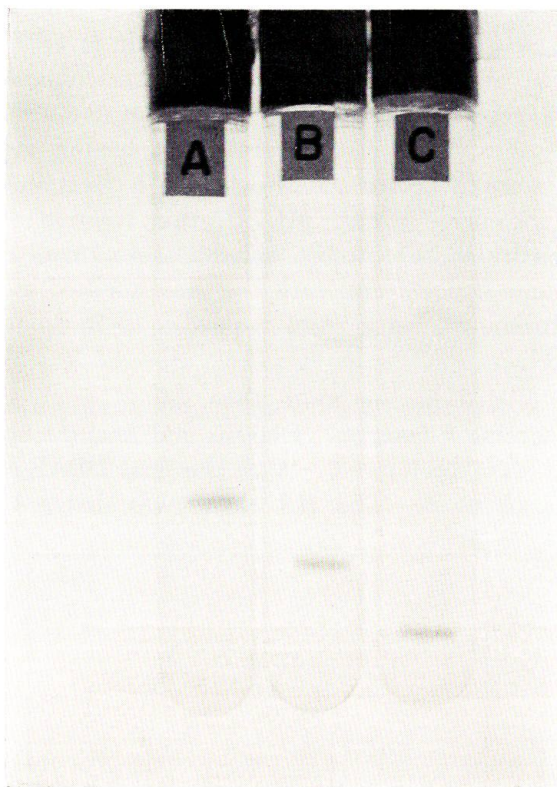

Fig. 2. Chromatography of cytochrome b on the third Sephadex
G–75 column.
Sephadex G–75 column (2.6 × 95 cm) was equilibrated with
0.01 M phosphate buffer, pH 7.0, and the column was washed
with the same buffer.
——: Absorption at 278 nm.
······: Absorption of cytochrome b at Soret band in oxidized
form (A_{413}).

Fig. 3. Polyacrylamide gel electrophoresis of purified cytochrome *b*.

Polyacrylamide gel electrophoresis was performed by the method of Davis (16), at a constant current of 3 mA per gel. Final DEAE-cellulose column eluate (protein content, 15 μg per tube) was used. Time of electrophoresis, A) 60 min., B) 75 min., C) 90 min..

phosphate buffer, pH 7.0. It was ascertained by the polyacrylamide gel electrophoresis that the red eluate was not contaminated with other proteins (Fig. 3).

A summary of the purification procedure is presented in Table 1.

Absorption Spectra—The absorption spectra of the purified cytochrome *b* in 0.05 M phosphate buffer, pH 7.0, are shown in Fig. 4. The oxidized form has a sharp peak at 413 nm, and smaller bands at 360, 533 and 564 nm, in addition to the characteristic protein band at 278 nm. The reduced form has major absorption peaks at 423.5, 527 and 555 nm, and α-band at 555 nm is asymmetric with a pronounced shoulder at 560 nm· The spectra of the

TABLE 1. Summary of purification procedure

Protein contents were determined by the method of Lowry *et al.*[13]. Cytochrome b_{555} contents were estimated spectrophotometrically.

Fraction	Total Protein (mg)	Total Cyt. *b* (μ moles)	μ moles Cyt. *b* /g Protein
Filtrate	54,000	—	—
Sup. from Powder Mixture	26,900	(0.945)[1]	(0.035)[1]
Ammonium Sulfate, 30–90%	13,500	(0.681)[1]	(0.051)[1]
First DEAE-Cellulose Eluate	550	0.204	0.37
Second DEAE-Cellulose Eluate	120	0.19	1.58
Third DEAE-Cellulose Eluate	19.8	0.18	9.1
Final DEAE-Cellulose Eluate	4.7	0.17	36.2

1) This value was an apparent value, since these fractions contained cytochrome *c*.
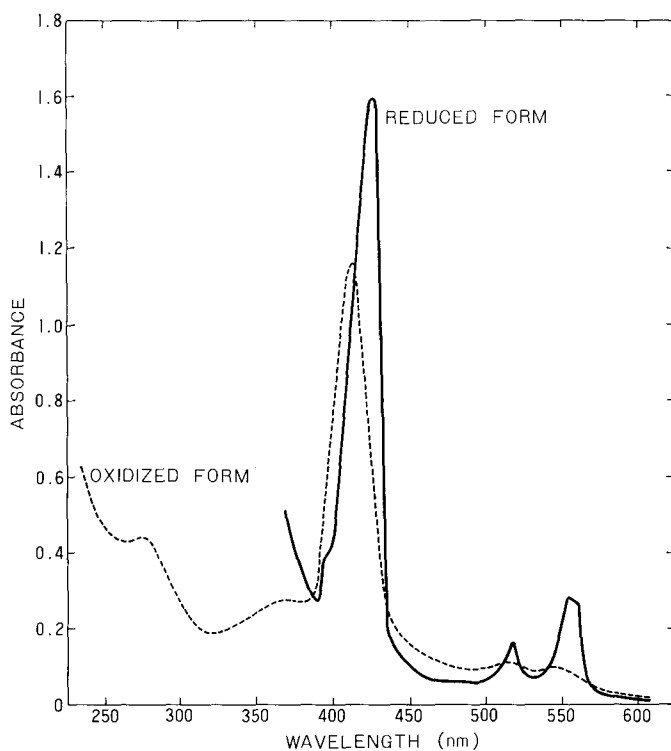
Fig. 4. Absolute absorption spectra of purified cytochrome *b*.

The spectra were measured in 0.05 M phosphate buffer, pH 7.0, at 20°C.
······: Oxidized form.
——: Reduced form (sodium hydrosulfite).

oxidized and the reduced pigments are not altered by bubbling with CO, nor 1 mM of cyanide at neutral pH, indicating that this b-type cytochrome does not combine with these agents. The reduced cytochrome b was reoxidized easily by bubbling with air.

Identification of Prosthetic Group—Treatment of the cytochrome with alkaline pyridine gives a reduced hemochrome with absorption maxima at 419, 526 and 557 nm. So, it is concluded that the cytochrome contains protoheme as a prosthetic group.

Molecular Weight—The molecular weight of the cytochrome b_{555} was estimated by gel filtration on a column of Sephadex G–100 by the method of ANDREWS[14]. As a result, the molecular weight of the cytochrome b was calculated to be 27,000.

Peroxidase Activity—Peroxidase activity of this preparation was measured, because peroxidase also contains protoheme and has an absorption spectra similar to that of cytochrome b_{555}. During early steps of the purification procedure, some peroxidase activity persisted in the preparations, but no peroxidase activity was detected in the final preparation (Table 2).

TABLE 2. Changes in peroxidase activity during
purification of the cytochrome b

Peroxidase activity was determined by the
method of Shichi and Hackett[12].

Fraction	Activity (ΔA_{460}/min./mg. prot.)
Sup. from Powder Mixture	1.35
First DEAE-Cellulose Eluate	4.41
Third DEAE-Cellulose Eluate	0.15
Final DEAE-Cellulose Eluate	0

Reduction of Cytochrome b_{555}—Of several reductants, sodium hydrosulfite and sodium borohydride reduced cytochrome b_{555} rapidly, but ascorbic acid, cysteine, succinate and NADH did not.

Midpoint Redox Potential—In ferric-ferrous oxalate buffer systems, the extent of reduction of the cytochrome b_{555} was determined as a function of the Fe^{++}/Fe^{+++} ratio. Consequently, the E_0' value of the cytochrome b was calculated to be −0.02 volt at pH 7.0 and at 15°C, assuming the E_0' value of ferric-ferrous oxalate system to be 0.0 volt[15].

Discussion

The b-type cytochrome has a sharp peak at 413 nm in the oxidized

form and major absorption peaks at 423.5, 527 and 555 nm (with a shoulder at 560 nm) in the reduced form. An asymmetric α-band of the cytochrome b_{555} preparation in the reduced form may suggest that the preparation contained other cytochrome components. In this preparation, however, any other cytochrome component than the cytochrome b_{555} was not detected by electrophoretic analysis (Fig. 3) and by chromatography using a DEAE-cellulose column and a Sephadex G–75 column (Figs. 1 and 2). In this connection, it was reported by OKADA and OKUNUKI[18] that highly purified cytochrome b_{555} from housefly showed an asymmetric α-band and the asymmetry was one of characteristics of the b-cytochrome. The purified cytochrome b_{555} from *Lysichiton* may be a single cytochrome like housefly cytochrome b_{555}.

The reduced cytochrome b_{555} was reoxidized and the reoxidization was not inhibited by cyanide, while peroxidase activity found in the initial purification steps was greatly inhibited by cyanide.

Molecular weight of the cytochrome b_{555} was estimated to be 27,000 by the gel filtration. This value does not agree with those of cytochrome b_5-like hemoproteins from various sources[18,20~22], viz. 13,500 to 16,900. There was a possibility that a partial polymerization of the monomeric cytochrome might occur during ultracentrifugation of protein in high concentration. As for the polymerization, GOLDBERGER *et al.*[19] reported that the isolated cytochrome b tends to aggregate.

Comparing with cytochrome b_5-like hemoproteins from various sources (Table IV in ref. 18), the cytochrome b_{555} from *Lysichiton* spadix tissues considerable resembles these hemoproteins especially cytochrome b_{555} from mung bean seedlings with respect to chemical and physical properties except for molecular weight.

In conclusion, there is a possibility that the cytochrome b_{555} investigated here is functioning as a terminal oxidase in the cyanide-insensitive repiration of *Lysichiton* spadix.

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