Purification and Some Properties of Cytochrome c_{550} from *Flavobacterium saccharophilum*

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Cytochrome c_{550} was purified to electrophoretic homogeneity by ion-exchange chromatography and gel filtration from a soluble fraction of *Flavobacterium saccharophilum*. The purified cytochrome c_{550} had a molecular weight of 12500 as determined by gel filtration and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Cytochrome c_{550} possessed absorption peaks at 407 nm in the oxidized form, and at 415, 521, 550 nm in the reduced form. Soluble D-glucoside 3-dehydrogenase reduced the purified cytochrome c_{550} with sugar, and this sugar was converted to the corresponding 3-keto sugar. The purified cytochrome c_{550} was autoxidizable. This shows that cytochrome c_{550} may be an *in vivo* electron acceptor for soluble D-glucoside 3-dehydrogenase.

Keywords cytochrome c_{550}; electron acceptor; soluble D-glucoside 3-dehydrogenase; *Flavobacterium saccharophilum*

We recently found the D-glucoside 3-dehydrogenase [EC 1.1.99.13] in *Flavobacterium saccharophilum* which is involved in the degradation of the antifungal agents, validamycins, as the trigger enzyme in the C–N bond cleavage of validoxylamine A.[2-4] The D-glucoside 3-dehydrogenases, which had flavin-adenine dinucleotide (FAD), nonheme iron and an acid-labile sulfide as prosthetic groups, were purified from a soluble and a membrane fraction of this organism.[3,4] The scheme of purification we developed was based on the observation of cytochrome in the carboxymethyl (CM)-Sepharose CL-6B eluant we obtained during soluble D-glucoside 3-dehydrogenase purification.[4]

In this paper we describe the purification and some properties of cytochrome c_{550} obtained from a soluble fraction of *F. saccharophilum*.

Materials and Methods

Microorganism The *F. saccharophilum* used throughout this work was isolated in our laboratory and kept on nutrient agar slants. Growth of the organism was as described in our previous paper.[3]

Preparation of Soluble Fraction The washed cells (200 g obtained from 16 l of culture) were suspended in 600 ml of 10 mM phosphate buffer (pH 7.0) and ruptured with a sonic oscillator (Tomy Model UR 2000P) for 10 min by cooling with ice. The cell debris was removed by centrifugation at 25000 × *g* for 15 min and the supernatant fraction was then centrifuged at 105000 × *g* for 1 h. The clear supernatant was called the "soluble fraction."

Purification of the Cytochrome c_{550} Ammonium sulfate was added to the soluble fraction to 60% saturation. The precipitate was dissolved in an aliquot of 10 mM phosphate buffer (pH 8.0) and then dialyzed against the buffer overnight. The dialyzate was passed through a diethylaminoethyl (DEAE)-Sephacel column (4 × 40 cm) previously equilibrated with 10 mM phosphate buffer (pH 8.0). Absorption at 415 nm of each fraction was measured spectrophotometrically. Cytochrome c fractions were collected, adjusted to pH 5.4 with 1 N acetic acid, and then dialyzed against 10 mM acetate buffer (pH 5.4). The dialyzate was put onto a CM-Sepharose CL-6B column (2.5 × 40 cm) previously equilibrated with the same buffer. Nonadsorbable protein contaminants were washed out with this buffer and the cytochrome c was eluted with 25 mM acetate buffer (pH 5.4). Cytochrome c_{550} was eluted earlier than D-glucoside 3-dehydrogenase. Cytochrome c fractions were combined and concentrated by ultrafiltration (Amicon YM-5). The solution was put onto a column of Sephadex G-75 (1.5 × 95 cm) equilibrated with 50 mM phosphate buffer (pH 7.0). The cytochrome c fractions were combined and concentrated by ultrafiltration.

Protein Measurement Protein was measured as described by Lowry *et al.*,[5] with bovine serum albumin as a standard.

Electrophoresis Polyacrylamide gel electrophoresis (PAGE) was performed by the method of Davis.[6] Sodium dodecyl sulfate-PAGE (SDS-PAGE) was performed by the method of Weber and Osborn.[7]

Determination of Molecular Weight The molecular weight of the purified cytochrome c_{550} was determined by SDS-PAGE and gel filtration on a column of Sephadex G-75 (1.5 × 95 cm) by the method of Andrews.[8]

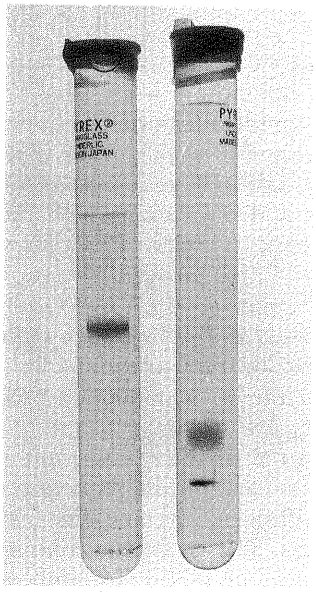
Identification of the Enzymatic Reaction Product The enzymatic reaction product with soluble D-glucoside 3-dehydrogenase and purified cytochrome c_{550} was identified as described in our previous paper.[4,9]

Results and Discussion

The purification procedure of the cytochrome c_{550} from *F. saccharophilum* is summarized in Table I. The final

TABLE I. Summary of Purification of Soluble Cytochrome c_{550} from *F. saccharophilum*

Fraction	Total protein (mg)	A_{280}	A_{415}	A_{415}/A_{280}
Soluble fraction	7620	38710	384	0.010
Ammonium sulfate fraction	5450	10800	127	0.012
DEAE-Sephacel	219	324	32.8	0.101
CM-Sepharose CL-6B	2.7	2.5	5.7	2.28
Sephadex G-75	1.1	0.9	5.0	5.56



(A) (B)

Fig. 1. Native and SDS-PAGE of the Purified Cytochrome c_{550}

Approximately 10 μg of purified cytochrome c_{550} was electrophoresed on 7.5% polyacrylamide gel (A) and 10% polyacrylamide gel containing 0.1% SDS (B). The gels were stained with Coomassie brilliant blue and destained with a mixture of 20% methanol and 7% acetic acid.

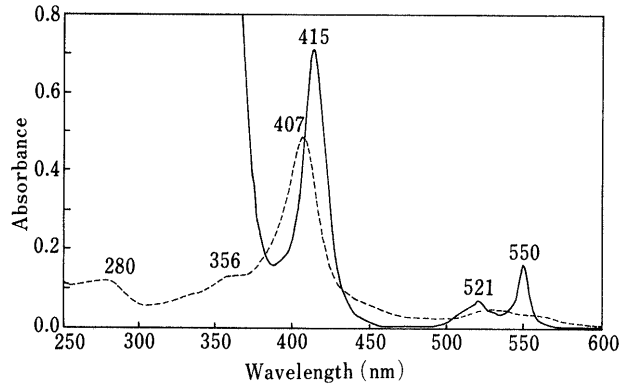


Fig. 2. Absorption Spectra of the Purified Cytochrome c_{550}

The sample cuvette contained 0.16 mg of the purified cytochrome c_{550} in 1.0 ml of 50 mM phosphate buffer (pH 7.0). ------, ferricyanide-oxidized form; ——, reduced form with sodium dithionite.

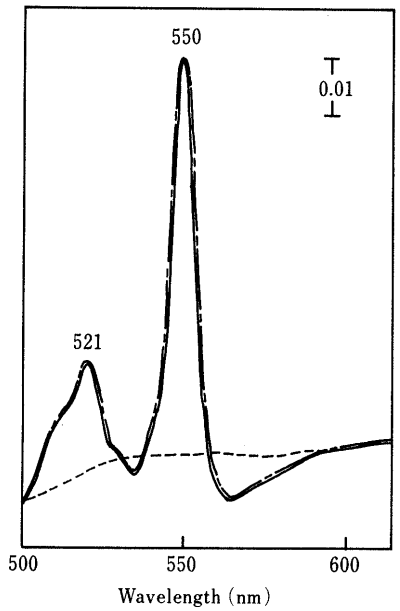


Fig. 3. Reduction of Cytochrome c_{550} by Soluble D-Glucoside 3-Dehydrogenase with Methyl α-D-Glucoside

Ten microliters of 0.9 mg/ml soluble D-glucoside 3-dehydrogenase was added to the reaction mixture (total 1.0 ml) of 0.2 mg ferricytochrome c_{550} and 10 mM methyl α-D-glucoside (sample cuvette alone). ------, before addition of the enzyme; ——, after addition of the enzyme; —·—, subsequent addition of sodium dithionite.

preparation gave a single band by PAGE and SDS-PAGE (Fig. 1). The molecular weight of the cytochrome c_{550} was 12500 by gel filtration with a Sephadex G-75 column and SDS-PAGE, indicating that the cytochrome c_{550} is a monomer.

Spectrophotometric measurement in this study was done with a Union Giken SM-401 recording spectrophotometer. The spectrum of the oxidized state of the purified cytochrome showed a peak at 407 nm (Fig. 2). In the reduced state, the absorbance maximum shifted to 415 nm and maxima at 521 and 550 nm were observed. Heme in the cytochrome was not extractable with acid–acetone and its pyridine ferrohemochrome showed absorption peaks at 414, 520, and 549 nm. Therefore, the cytochrome may be concluded to be a C-type cytochrome. The purified cytochrome c_{550} was autoxidizable.

As shown in Fig. 3, when methyl α-D-glucoside was used as an electron donor, cytochrome c_{550} was reduced rapidly in a reaction system containing soluble D-glucoside 3-dehydrogenase. This reduction by a substrate was not changed by the subsequent addition of dithionite. When purified soluble D-glucoside 3-dehydrogenase was incubated with methyl α-D-glucoside and purified cytochrome c_{550}, methyl α-D-3-ketoglucoside was formed as indicated by gas chromatography-mass spectrometric (GC-MS) analysis (data not shown). These results show that soluble cytochrome c_{550} may be an *in vivo* electron acceptor for soluble D-glucoside 3-dehydrogenase.

In the previous paper,[4] however, we reported that the purified membrane-bound cytochrome c_{551}, which had a molecular weight of 180000 (an oligomer of 15500-molecular-weight subunits),[9] was reduced by soluble D-glucoside 3-dehydrogenase and its substrate. On the other hand, Hirata and Fukui[10] isolated a cytochrome c_{552} from a soluble fraction of *Agrobacterium tumefaciens* that acts as an electron acceptor for a D-glucoside 3-dehydrogenase of this organism. Therefore, further investigation is needed to elucidate the role of soluble cytochrome c_{550} in the 3-keto sugar forming system.

References and Notes

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2) N. Asano, M. Takeuchi, K. Ninomiya, Y. Kameda and K. Matsui, *J. Antibiot.*, **37**, 859 (1984).
3) M. Takeuchi, K. Ninomiya, K. Kawabata, N. Asano, Y. Kameda and K. Matsui, *J. Biochem.* (Tokyo), **100**, 1049 (1986).
4) M. Takeuchi, N. Asano, Y. Kameda and K. Matsui, *Agric. Biol. Chem.*, **52**, 1905 (1988).
5) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
6) B. J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, 404 (1964).
7) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
8) P. Andrews, *Biochem. J.*, **96**, 595 (1965).
9) M. Takeuchi, N. Asano, Y. Kameda and K. Matsui, *J. Biochem.* (Tokyo), **103**, 938 (1988).
10) H. Hirata and S. Fukui, *J. Biochem.* (Tokyo), **63**, 780 (1968).