Purification and some properties of a *b*-type cytochrome from *Sclerotinia libertiana*

Sofar, attempts to extract the *b*-type cytochromes other than cytochrome b_2[1] in a truly water-soluble state have been uniformly unsuccessful, although some of them have been obtained in an apparently water-soluble state with the aid of detergents such as sodium cholate[2–4]. This may suggest either that the *b*-type cytochromes are essentially water-insoluble haemoproteins, or that they may exist in the cell firmly bound to some hydrophobic substance such as lipid. Experiments reported here show that a *b*-type cytochrome can be obtained in a truly water-soluble state from a certain fungus which has been known to contain a lipase of extraordinary high activity. This finding seems to favour the view that at least a certain form of *b*-type cytochrome exist in the cells in a lipid-bound form.

The material used for the extraction of the cytochrome was a fungus *Sclerotinia libertiana* which shows a strong absorption band of a *b*-type cytochrome. This fungus has been known to produce a very strong lipase, and its lyophilized powder has recently become available commercially in Japan as a preparation of lipase (under the name of Lipase "Saiken"*). Noteworthy is the fact that this preparation shows almost no proteolytic activity.

The optimum pH for the lipase of the fungus lies at 6–8 (see ref. 5). This pH range was also found to be most suitable for the extraction of the cytochrome. The lyophilized fungal powder was suspended in 0.2 *M* sodium phosphate buffer, pH 7.0, and left overnight at room temperature (15°–20°). (A suitable ionic strength of the extracting medium was found to be 0.3 or higher.) The suspension was filtered through a Büchner funnel with the aid of Celite, and the resulting filtrate was dialysed against tap water overnight. The dialysed solution was passed through an aluminium oxide column which had been buffered with 0.2 *M* ammonium phosphate and washed with water. The cytochrome adsorbed on the column was washed with water and then eluted with 1.0 *M* ammonium phosphate buffer, pH 7.0. To the eluate was added solid $(NH_4)_2SO_4$ to 90 % saturation, the resulting precipitate was discarded by centrifugation, and the supernatant was dialysed against tap water for one day. After repeating

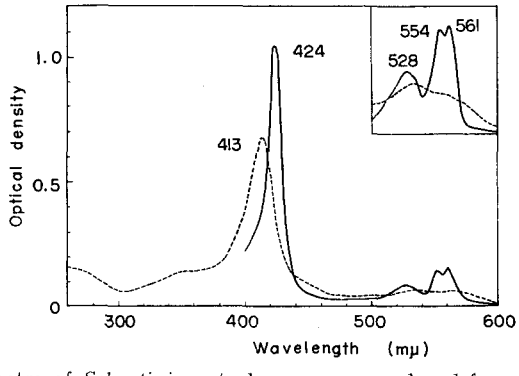

Fig. 1. Absorption spectra of *Sclerotinia* cytochrome. ——, reduced form; – – –, oxidized form. A more detailed absorption spectrum of the α- and β-absorption peaks is shown in the inserted graph.

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the procedure of adsorption and elution using the aluminium oxide column, the eluate containing the cytochrome was saturated with $(NH_4)_2SO_4$, the precipitate formed was removed by centrifugation, and the supernatant was dialysed against tap water. The cytochrome present in the solution was again adsorbed onto the aluminium oxide column and eluted with 10% saturated $(NH_4)_2SO_4$ (pH 5.6). The eluate obtained was light red in colour which turned into pink when treated with a small amount of $Na_2S_2O_4$. In Fig. 1 are reproduced the absorption spectra shown by the oxidized and reduced form of the cytochrome. While the oxidized form showed a sharp absorption peak at 413 mμ and two weaker bands at 532 mμ and 560 mμ, the $Na_2S_2O_4$-reduced form had sharp absorption peaks at 424 mμ, 528 mμ, 554 mμ and 561 mμ with a shoulder at around 534 mμ.

This feature of the absorption spectrum appeared to indicate that the solution we have obtained might have contained two cytochromes with the α-band lying at 561 mμ and 554 mμ, respectively. The solution was, therefore, subjected to a zone electrophoresis using a vertical starch column. Repeated experiments conducted under widely different conditions gave, however, no indication at all that the solution contained two different cytochromes. When the solution was shaken with a mixture of pyridine and 0.2 *N* NaOH (1:2, v/v), the water layer lost its colour, while the pyridine layer became red. When treated with $Na_2S_2O_4$, the pyridine layer showed an absorption spectrum having peaks at 419 mμ, 524 mμ and 556 mμ, which are characteristic for protohaemochromogen (Fig. 2). It may, therefore, be concluded that the cytochrome we have isolated was of the *b*-type, although its α-band differed from those of ordinary *b*-type cytochromes in showing two peaks even at room temperature (about 20°). That by our purification procedure the cytochrome had been obtained in a fairly pure form may be evidenced by the fact that the ratio of $E^{\text{reduced}}_{561\ m\mu}/E^{\text{oxidized}}_{280\ m\mu}$ was

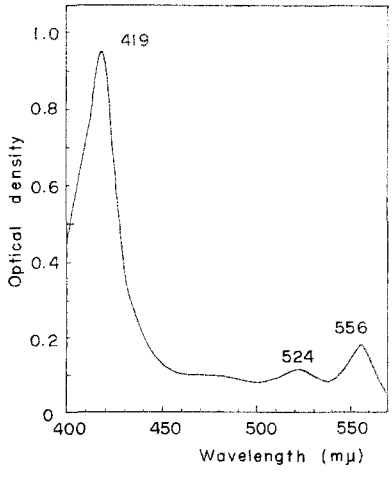

Fig. 2. Absorption spectrum of the pyridine haemochromogen of *Sclerotinia* cytochrome. The red colour of the cytochrome was easily extracted with alkaline pyridine. When $Na_2S_2O_4$ was added to the pyridine layer, the absorption spectrum of protohaemochromogen was seen.

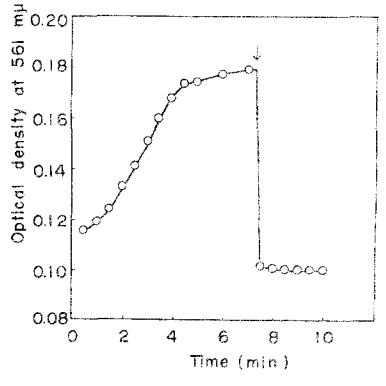

Fig. 3. Reduction of *Sclerotinia* cytochrome by lactate in the presence of baker's yeast lactic dehydrogenase under anaerobic conditions. When air was introduced as indicated by the arrow, the reduced cytochrome was rapidly oxidized. pH 6.5. Temp., 25°.

as high as 1.1 (see Fig. 1), which is comparable to the ratio of $E^{\text{reduced}}_{550\,m\mu}/E^{\text{oxidized}}_{280\,m\mu}$ obtained for a typical cytochrome *c**.

The *b*-type cytochrome was reduced by lactate in the presence of baker's yeast lactic dehydrogenase[6-9] under anaerobic conditions, but not under aerobic conditions. The enzymic reduction of the cytochrome was measured spectrophotometrically *in vacuo* in a Thunberg tube modified for spectrophotometry (Fig. 3). When air was introduced into the tube, the reduced cytochrome was immediately oxidized. The autoxidation of the cytochrome was not inhibited by 10^{-2} *M* KCN. Neither KCN nor CO caused any modification in the absorption bands as judged from the observation made with a microspectroscope. The biological function of the cytochrome, for which we propose the name "cytochrome *b*-561, 554 (*Sclerotinia libertiana*)"[10], is a matter for further investigation.

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1 J. YAMASHITA, T. HIGASHI, T. YAMANAKA, M. NOZAKI, H. MIZUSHIMA, H. MATSUBARA, T. HORIO AND K. OKUNUKI, *Nature*, 179 (1957) 959.
2 I. SEKUZU AND K. OKUNUKI, *J. Biochem.*, 43 (1956) 107.
3 G. HÜBSCHER AND M. KIESE, *Naturwissenschaften*, 39 (1952) 524.
4 G. HÜBSCHER, M. KIESE AND R. NICOLAS, *Biochem. Z.*, 325 (1954) 223, 299.
5 Y. SATOMURA, S. OI AND A. SAWADA, *Bull. Agr. Chem. Soc. Japan*, 22 (1958) 194.
6 S. J. BACH, M. DIXON AND L. G. ZERFAS, *Biochem. J.*, 40 (1946) 229.
7 C. A. APPLEBY AND R. K. MORTON, *Nature*, 173 (1954) 749.
8 T. YAMANAKA, T. HORIO AND K. OKUNUKI, *J. Biochem.*, 45 (1958) 291.
9 J. YAMASHITA, T. HORIO AND K. OKUNUKI, *J. Biochem.*, 45 (1958) 707.
10 F. EGAMI, M. ISHIMOTO, T. MORI, Y. OGURA, K. OKUNUKI AND R. SATO, *J. Biochem.*, 44 (1957) 619.

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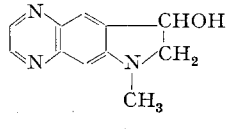
*It should be remarked that no *c*-type cytochrome has ever been extracted from this fungus.

The condensation of catechols with ethylenediamine

It has been shown by HARLEY-MASON AND LAIRD[1] that the main product of the reaction of ethylenediamine with adrenaline is dihydro-3-hydroxy-1-methylpyrrolo-(4,5-*g*)-quinoxaline (I), while the reaction with catechol yields 1,2,3,4-tetrahydro-1,4,5,8-tetraaza-anthracene (II). HARLEY-MASON (personal communication) further



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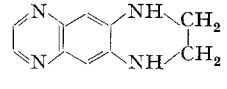

II

found that the main product formed from noradrenaline is identical with that formed