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# *Pseudomonas* Cytochrome *c* Peroxidase

## I. Purification Procedure

NILS ELLFOLK and RITVA SOININEN

*Department of Biochemistry, University of Helsinki, Helsinki 17, Finland*

A procedure is described for the purification of cytochrome *c* peroxidase from extracts of acetone-treated cells of *Pseudomonas fluorescens* cultivated in a medium equilibrated with a gas mixture containing 5 % oxygen and 95 % nitrogen. After deoxyribonuclease hydrolysis of the nucleic acids in the extracts, the purification procedure involves precipitation at pH 4.7 and chromatography on Sephadex G-100, DEAE-cellulose, and CM-cellulose. The final preparation was found to be a homogeneous, cytochrome *c*-free protein according to ultracentrifugal and disc electrophoretic criteria and showed no cytochrome *c* oxidase or catalase activity. The iron content of the preparation was 0.259 % and the ratio $A_{407}/A_{280}$ equal to 4.2. The technique of isoelectric focusing showed the presence of two peroxidatically active components. The isoelectric point of the main component was 6.7 and that of the minor one 6.5. The absorption spectrum of *P. fluorescens* cytochrome *c* peroxidase deviates from the spectrum of cytochrome *c* peroxidase from baker's yeast. The results are discussed.

Lenhoff and Kaplan[1–3] first showed that cytochrome *c* peroxidase (cytochrome *c*:$H_2O_2$ oxidoreductase, EC 1.11.1.5) exists in *Pseudomonas fluorescens*. Confirmative observations on the presence of cytochrome *c* peroxidase in *P. fluorescens* have been published recently.[4,5] Lenhoff and Kaplan described a method for partial purification of the enzyme. By ammonium sulphate fractionation and chromatography on kaolin, they obtained a preparation with an over-all purification of 12.5-fold, which, however, still contained catalase and cytochrome *c*.

This publication describes a procedure for the purification of cytochrome *c* peroxidase (CCP) of *P. fluorescens* that yields a homogeneous preparation as revealed by ultracentrifugation and disc electrophoresis.

## MATERIALS AND METHODS

*Pseudomonas fluorescens*, strain No. 8, was provided by Professor N. Kaplan. This strain had been used by Lenhoff and Kaplan in their studies on cytochrome *c* peroxidase.[1–3]

*Cultivation of P. fluorescens.* The cells were grown in a medium containing 5 g of sodium citrate, 5 g of sodium nitrate, 1 g of $KH_2PO_4$, 0.5 g of $MgSO_4 \cdot 7\ H_2O$, 4 g of powdered yeast extract (Difco), and 3 mg of $FeCl_3$ per liter that had been adjusted to a pH close to 7.0 with 1 N NaOH. When the cells were cultivated for the CCP studies, the culture was aerated with a mixture of 5 % oxygen and 95 % nitrogen by thorough mixing. To avoid foaming, an antifoam agent (Silicon Antifoam, Midland Silicones Ltd.) was added. When the cells were cultivated to isolate cytochrome *c*, the culture was not aerated. The cultivation was started by inoculating 25 ml of the medium and incubating the culture at 30°C for 3—5 h, after which the culture was transferred to 500 ml of the same medium, cultivated for 8—9 h, transferred to 8 l of the medium and incubated for 15—18 h. This culture was then transferred to a 400-l pilot plant fermentor (Getingeverken, Getinge, Sweden), which contained 300 l of the medium. The mixture was incubated at 30°C for about 20 h. The cells were harvested in a DeLaval separator and stored as a frozen paste at −16°C. The progress of growth was followed by reading the turbidity of the cell culture in a Klett colorimeter with filter 66. Cell densities were calculated from a standard curve relating optical density to dry weight. The speed of aeration was adjusted so that the culture remained saturated with the 5 % oxygen —95 % nitrogen mixture. The oxygen concentrations were measured with an Oxygraph (Gilson Medical Electronics, Middleton, Wisconsin, USA).

*Preparation of acetone-dried cell powder.* The frozen cell mass was thawed at 4°C and added in small quantities to 10 volumes of acetone that had been cooled to −2°C. The mixture, which was kept at 0 to−2°C, was stirred until all lumps had broken down (about 30 min), and then filtered on a Büchner funnel. The residue was washed with one volume of cold acetone and one volume of cold diethyl ether. The cell powder was dried to constant weight in a continuously evacuated desiccator at room temperature and stored at 4°C.

*DEAE-and CM-cellulose.* DEAE-cellulose was a commercial preparation (Whatman DE 11) and CM-cellulose was prepared according to Peterson and Sober,[6] the last alcohol drying step, however, being omitted.

*Sephadex G-100* was a commercial preparation from AB Pharmacia, Uppsala, Sweden. The preparation was handled according to the instructions of the manufacturer.

*Column chromatography.* The ion exchange columns were prepared as described previously [7] and the Sephadex columns according to the directions of Flodin.[8] The absorbances of the different fractions at 280 and 407 nm were measured with a Beckman DU-2 spectrophotometer.

*Deoxyribonuclease* was a commercial preparation from Sigma Co. (DN-100).

*Cytochrome c(−551) of P. fluorescens* was prepared from acetone-dried cells by the method of Ambler.[9]

*Ferrocytochrome c of P. fluorescens* was prepared by a method previously used for the reduction of horse heart cytochrome c involving anaerobic gel filtration of cytochrome *c* reduced with sodium dithionite.[10]

*Hydrogen peroxide.* Merck's Perhydrol was diluted to give an about 10 mM $H_2O_2$ solution. The concentration was determined spectrophotometrically at 230 nm, where $\varepsilon_{mM}$ is equal to 0.0724.

*Peroxidase assays* were performed at 23°C in a 0.02 M sodium phosphate buffer of pH 7.2. The reaction mixture contained usually 38.1—38.9 μM of $H_2O_2$ and 8.33—11.67 μM of reduced *P. fluorescens* cytochrome *c*. The reaction was initiated by adding 2—20 μl of enzyme solution to 2.6 ml of the reaction mixture. The absorbance of the mixture at 551 nm was recorded on a Beckman DK—1A recording spectrophotometer. The initial decrease in absorbance per cm per 10 sec per 10 μl of enzyme solution was used as an arbitrary unit.

*Catalase* was assayed semiquantitatively at 23°C in a 0.02 M sodium phosphate buffer of pH 7.2, 4 mM in hydrogen peroxide. The reaction was started by adding 10 μl of enzyme solution to 2.6 ml of the reaction mixture. The decomposition of $H_2O_2$ was followed spectrophotometrically at 230 nm.

*Cytochrome c oxidase* was assayed at 23°C in a 0.05 M sodium acetate buffer of pH 5.3, 8.33—11.67 μM in reduced *P. fluorescens* cytochrome *c*. The reaction was initiated by adding 2—20 μl of enzyme solution to 2.6 ml of the reaction mixture. The initial decrease in absorbance at 551 nm per cm per 10 sec per 10 μl of enzyme solution as

recorded with a Beckman DK—1A recording spectrophotometer was used as an arbitrary unit.

*Iron analyses* were carried out by the modified semimicro sulfosalicylic acid method of Lorber.[11,12] The final volume of 2.5 ml was used in the determination. Before analysis, the protein samples were extensively dialyzed against deionized water.

*Pyridine hemochrome* of CCP was prepared according to Paul *et al.*[13]

*Spectrophotometry.* Absorption spectra were measured with a Cary 15 recording spectrophotometer.

*Disc electrophoresis* in polyacrylamide gel was carried out in the 2,6-lutidine-glycine KOH-glycine buffer system of Ornstein and Davis [14] as slightly modified by Carlström.[15] The runs were performed using a current of 3 mA per gel at room temperature and were terminated after 30—60 min. The zones of CCP could be located visually. Permanent protein staining was effected with 1 % Amidoschwartz in 7 % acetic acid according to Ornstein and Davis.[14]

*Isoelectric focusing* was carried out according to Svensson and Vesterberg [16,17] in an electrofocusing column of type LKB 8101 having a capacity of 110 ml. The instructions of the manufacturer were followed. The density gradient was produced with sucrose (0—50 % w/v) and the pH gradient in the range 5—8 with carrier ampholytes (LKB-Produkter AB, Stockholm) in a concentration of 1 % (w/v). The CCP sample was dialyzed against 1 % glycine and mixed with one of the middle fractions of the gradient. Electrophoresis was initially performed for 4 h at 350—700 V and subsequently for 22 h at 700 V at 9°C. When the proteins had been focused, the column was drained and 2 ml fractions were collected. The pH's of the fractions were measured with a Radiometer PHM 4c pH meter and a combination glass-calomel electrode at 9°C. The pH meter was calibrated with a Beckman standard buffer of pH 7 (No. 3501) at 9°C.

*Ultracentrifugal analyses* were run on a Beckman-Spinco model E ultracentrifuge. Prior to analysis, the preparations were dialyzed overnight at 4°C against a buffer of pH 7.0 ($\mu$=0.283), 0.05 M in sodium phosphate and 0.171 M in sodium chloride.

*Protein concentrations* were determined in the first steps of purification according to Lowry *et al.*,[18] using serum albumin (Finnish Red Cross) as standard, and in the later steps spectrophotometrically according to Kalckar.[19]

*The chemicals* were of analytical grade except those used in the cultivation medium for the bacteria, which were of technical grade.

## RESULTS

*Preliminary studies.* Different procedures were tested to find a satisfactory method to extract cytochrome *c* peroxidase from cells of *P. fluorescens*. When a cell preparation was disintegrated in an X-Press,[20] three passages were found to give a satisfactory disintegration of the bacterial cells. The disintegrated cells were suspended in a 0.1 M sodium phosphate buffer of pH 7.5 at 4°C and centrifuged for 30 min at 25 000 *g*. The enzyme activity in the supernatant was only 49 % of the total CCP activity present in the cells. When the cells were treated with alumina according to Lenhoff and Kaplan,[2] the percentage was only 41. Some improvement was observed after grinding the cells with glass beads according to Phillips *et al.*,[21] 69 % of the enzyme was extracted into solution. The same proportion was extracted after treatment of the cells with butanol according to Morton.[22] When acetone-dried cells were extracted with a 0.05 M sodium phosphate buffer of pH 7.5 for 1 h at 4°C, 93 % of CCP present in the cells was extracted into solution. An identical efficiency of extraction was obtained with distilled water. The gelatinous extract obtained was treated with deoxyribonuclease (DNAase) to hydrolyze the nucleic acids and so make the extract suitable for further purification. The enzyme in the DNAase-treated crude extract was precipitated

with ammonium sulphate between 30 and 70 % saturation but the recovery of the enzyme was only about 67 %. Fractionation of the dialyzed crude extract by adding 30 – 60 % acetone also gave a low recovery, 45 %, of the active enzyme. When the enzyme was precipitated at pH 4.7, 87 % of the enzyme was recovered. On the basis of these preliminary studies, a purification procedure was developed which comprised precipitation at pH 4.7 and chromatography on Sephadex G-100, DEAE-cellulose, and CM-cellulose as described in the following.

*Preparation of the enzyme extract.* The acetone-treated cells of *P. fluorescens* were extracted twice with distilled water to obtain as high a yield of enzyme as possible. The first extraction was performed with 7.5 volumes of distilled water at 4°C for 1 h with stirring. The temperature of the suspension was then raised to 20°C and 1 mg of DNAase per 25 g of acetone-dried powder was added and the resulting mixture stirred for 10 min, cooled and centrifuged at 25 000 *g* for 30 min at 4°C. The cell debris was suspended in 2.5 volumes of distilled water and extracted for 1 h at 4°C. The temperature of the extract was raised to 20°C and 0.5 mg of DNAase was added. The mixture was incubated and centrifuged as above. The two extracts were pooled and dialyzed before precipitation of the enzyme at a low pH.

*Precipitation at pH 4.7.* The crude extract was dialyzed against distilled water at 4°C overnight. The extract was cooled to 0 – 2°C and its pH was lowered to 4.7 with 0.5 N acetic acid. The extract was held at 0°C for 15 min. The precipitate formed was collected by centrifugation at 15 000 *g* and 4°C for 15 min and redissolved by careful addition of 2 N sodium hydroxide so that the pH did not rise above 7.

*Chromatography on Sephadex G-100.* The run was performed at 4°C on a 3.6 × 77 cm Sephadex G-100 column that had been equilibrated with a 0.1 M sodium phosphate buffer of pH 6.5. The centrifuged preparation from the preceding purification step was added to the column and eluted with the equilibration buffer under hydrostatic pressure. The rate of elution was initially about 50 ml/h, but decreased to about 13 ml/h during the run. Fractions, one every 20 min, were collected in a fraction collector. Their volumes were measured after the run. The absorbances of each fraction at 280 and 407 nm as well as the CCP activities were measured. Data from a typical run on Sephadex G-100 are presented in Fig. 1. The fractions containing CCP were pooled.

*Chromatography on DEAE-cellulose.* The pooled CCP-containing fractions obtained in the preceding purification step were concentrated with Carbowax 6000 (Shell) to about one tenth of their original volume and then dialyzed against a 0.02 M sodium phosphate buffer of pH 6.5 overnight at 4°C. The clear concentrate (centrifuged when necessary) was put on a DEAE-cellulose column (3 × 15 cm) equilibrated with a 0.02 M sodium phosphate buffer of pH 6.5 which also was used for elution of the column at a flow rate of 30 ml/h. The fractions, one every 10 min, were collected in a fraction collector and their absorbances at 280 and 407 nm as well as the CCP activities were measured. Fig. 2 shows data from a typical run on a DEAE-cellulose column.

*Chromatography on CM-cellulose.* The pooled fractions containing CCP obtained from the DEAE-cellulose column were dialyzed against distilled

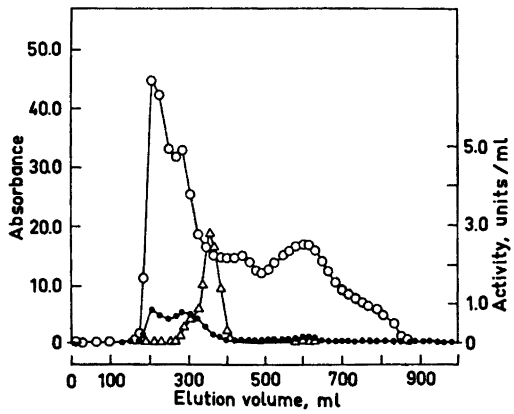


*Fig. 1.* Elution pattern of cytochrome *c* peroxidase from a Sephadex G-100 column. The column (3.6×77 cm) was equilibrated with a 0.1 M sodium phosphate buffer of pH 6.5. 38 ml of CCP preparation containing 2620 mg of protein precipitated at pH 4.7 dissolved with 2 N NaOH was applied to the top of the column. Elution was carried out at 4°C with the same buffer at a flow rate of 13 ml/h and fractions about 4.3 ml in volume were collected. ○ protein concentration expressed as absorbance at 280 nm; ● absorbance at 407 nm; △ units of CCP per ml.

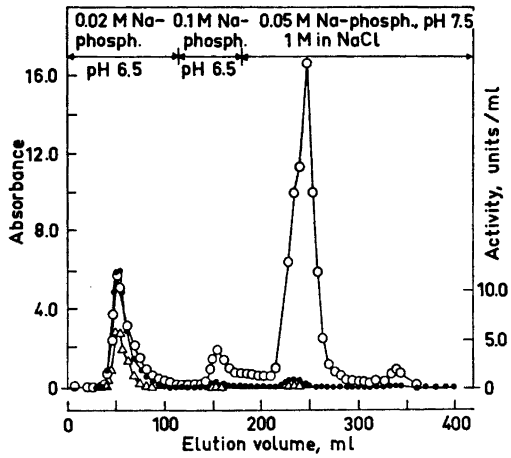


*Fig. 2.* Elution pattern of cytochrome *c* peroxidase from a DEAE-cellulose column. The column (3×15 cm) was equilibrated with a 0.02 M sodium phosphate buffer of pH 6.5. 15 ml of CCP preparation dialyzed against the same buffer and containing 353 mg of protein was applied to the top of the column and the elution was carried out at 4°C first with the same buffer, with a 0.1 M sodium phosphate buffer of pH 6.5 and with a 0.05 M sodium phosphate buffer of pH 7.5 containing 1 M sodium chloride as indicated in the figure. The flow rate was 30 ml/h and 5-ml fractions were collected. ○ protein concentration expressed as absorbance at 280 nm; ● absorbance at 407 nm, △ units of CCP per ml.

water overnight at 4°C and put on a CM-cellulose column (2×7 cm) equilibrated with a 0.01 M sodium phosphate buffer of pH 6.9. The column was eluted with the equilibration buffer at a rate of 17 ml/h. CCP was eluted with the equilibration buffer, whereas cytochrome *c* oxidase was quantitatively retained on the top of the column. By increasing the ionic strength of the sodium phosphate buffer to 0.05 M, oxidase was eluted from the column. Fractions were collected in a fraction collector at a rate of one every 10 min. Their absorbances were read at 280 and 407 nm and their CCP and cytochrome *c* oxidase activities were measured. Fig. 3 shows data from a typical run on a CM-cellulose column.

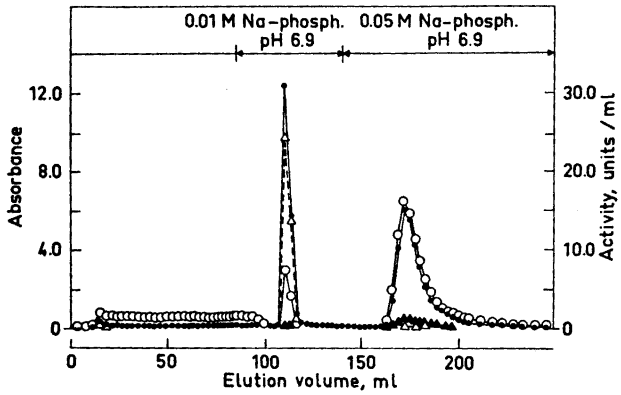


*Fig. 3.* Elution pattern of cytochrome *c* peroxidase from a CM-cellulose column. The column (2×7 cm) was equilibrated with a 0.01 M sodium phosphate buffer of pH 6.9; 64 ml of CCP preparation dialyzed against distilled water and containing 199 mg of protein was applied to the top of the column and elution was carried out at 4°C first with the equilibration buffer and then with a 0.05 M sodium phosphate buffer of pH 6.9 at a flow rate of 17 ml/h. Fractions 2.8 ml in volume were collected. ○ protein concentration expressed as absorbance at 280 nm, ●absorbance at 407 nm; △ units of CCP per ml; ▲ units of cytochrome *c* oxidase per ml.

The yields of the enzyme and the specific activities in each step during a typical purification of CCP from *P. fluorescens* are summarized in Table 1. The over-all purification of the initial extract (starting with 25 g of acetone-dried cells) was 189-fold with a recovery of 18 %.

*Properties of CCP.* Disc electrophoresis of the final preparation yielded one coloured zone. When the polyacrylamide gels were stained with Amidoschwartz, the zone was the only one visible as can be seen in the photograph in Fig. 4. The photograph shows that the preparation contained no other proteins than the hemin protein. The ultracentrifugal analysis showed also that only one homogeneous protein was present in the preparation (Fig. 5). On isoelectric focusing of the preparation, two components were obtained both of which exhibited CCP activity (Fig. 6). The isoelectric point of the main component was 6.7 and that of the minor one 6.5. The minor component is evidently a subcomponent of CCP. The iron content of the preparation was

*Table 1.* Details of a representative preparation of purified cytochrome *c* peroxidase from *Pseudomonas fluorescens*. The values shown are for 25 g of acetone-dried cells (equivalent to 100 g of fresh bacteria).

| Purification procedure | Volume, ml | Activity, units/ml | Activity, total units | Protein concn., mg/ml | Specific activity, units/mg protein | Enrichment —fold | Recovery % | $A_{280}/A_{260}$ | $A_{407}/A_{280}$ | Obs. |
|---|---|---|---|---|---|---|---|---|---|---|
| 1. Pooled extracts from acetone-dried cells | 190 | 8.3 | 1580 | 24.4 | 0.34 | 1 | 100 | — | — | Oxidase +[a] Catalase + |
| 2. Precipitated at pH 4.7, dissolved with 2 N NaOH, centrifuged | 36 | 30.6 | 1100 | 71 | 0.43 | 1.3 | 70 | — | — | Oxidase + Catalase + |
| 3. Pooled fractions from Sephadex G-100 column | 134 | 7.8 | 1050 | 7.2 | 1.08 | 3.2 | 66 | 0.67 | 0.15 | Oxidase + Catalase + |
| 4. Pooled fractions from DEAE-cellulose column | 60 | 10.3 | 620 | 2.68[b] | 3.84 | 11.3 | 39 | 1.56 | 0.91 | Oxidase + Catalase — |
| 5. Pooled fractions from CM-cellulose column | 4.6 | 62.9 | 289 | 0.98[b] | 64.2 | 189 | 18 | 1.06 | 3.9[c] | Oxidase — Catalase — |

[a] + or — indicates the presence or absence of cytochrome *c* oxidase or catalase activity in the preparation.
[b] Measured spectrophotometrically according to Kalckar.[19]
[c] Highest ratio obtained was 4.2. The preparation from step 5 can be rechromatographed on CM-cellulose to achieve this purity.

*Fig. 4.* Disc electrophoretic pattern of the purified preparation of cytochrome *c* peroxidase in the 2,6-lutidine-glycine/KOH-glycine system in a polyacrylamide gel. The preparation of CCP containing about 63 μg of protein was subjected to electrophoresis as described in the text. The gel was stained with Amidoschwartz to visualize the protein zones. The cathode was on the top of the tube.

*Fig. 5.* Schlieren diagram of the ultracentrifugal sedimentation of the purified preparation of cytochrome *c* peroxidase. The concentration of the enzyme was 3.23 mg/ml in a buffer of pH 7.0, $\mu = 0.283$, 0.05 M in sodium phosphate and 0.171 M in sodium chloride. The photographs were taken at a phase angle of 45° 8, 24, 32, 56, and 72 min after 59 780 rpm were reached at 20°C. The sedimentation is from left to right.

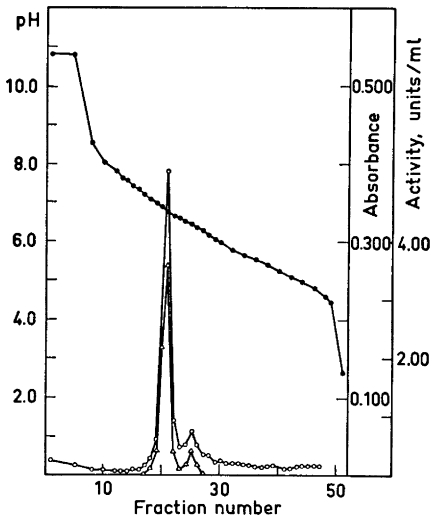


*Fig. 6.* Isoelectric focusing pattern of the purified preparation of cytochrome *c* peroxidase (the cathode at left). About 2 mg of protein was focused in the pH range 5—8 as described in the text. ○ absorbance at 407 nm; ●pH; △ units of CCP per ml.

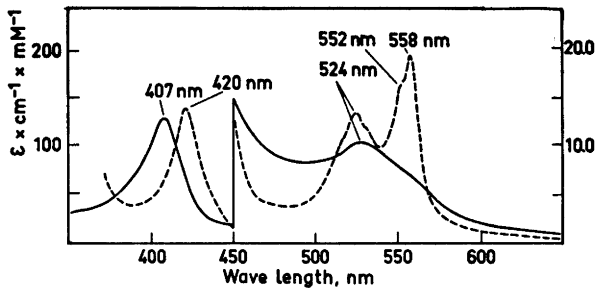


*Fig. 7.* Absorption spectra of cytochrome *c* peroxidase and reduced cytochrome *c* peroxidase in a 0.01 M sodium phosphate buffer of pH 6.9. CCP was reduced with sodium dithionite. Millimolar absorptivities were calculated on the basis of the iron content of the preparation. ———— CCP, — — — — the reduced CCP.

found to be $0.259 \pm 0.010$ %. The highest value of the ratio $A_{407}/A_{280}$ was 4.2. The spectra of CCP and its reduced form are shown in Fig. 7. The spectrum of oxidized CCP is essentially the same as that of CCP. Fig. 8 shows the spectrum of the alkaline pyridine ferrohemochrome of the enzyme. An identical spectrum was obtained for reduced denaturated CCP. The pH stability of the enzyme is given in Fig. 9. The activity of a purified preparation stored for several months at $-16$°C was equal to that of the original purified preparation.

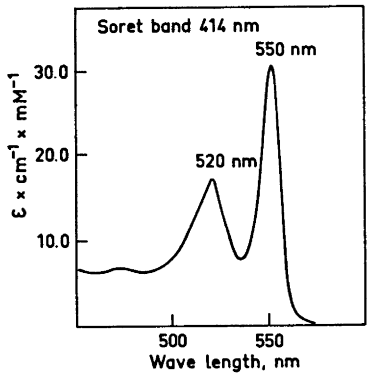


*Fig. 8.* Absorption spectrum of the alkaline pyridine ferrohemochrome of cytochrome *c* peroxidase. Millimolar absorptivities were calculated on the basis of the iron content of the preparation.

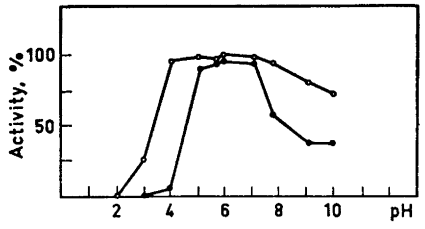


*Fig. 9.* Stability of cytochrome *c* peroxidase as a function of pH. The enzyme was incubated in glycine-HCl, phosphate, acetate, and glycine buffers ($\mu = 0.1$) for 1 min and 3 h at room temperature. Incubation time 1 min ○, 3 h ●.

## DISCUSSION

The location of CCP in the cells of *P. fluorescens* is not known. However, attempts to extract the enzyme from bacteria using different techniques to effect disintegration resulted in low yields. This indicates that the enzyme is associated with insoluble membrane material. The CCP of baker's yeast has been found to be located in the mitochondria.[23]

Treatment of the bacterial cells with acetone is essential to bring the enzyme quantitatively into aqueous solution. This technique resulted in a higher specific activity of the crude extract than any of the other procedures tested and facilitated the subsequent purification steps. The precipitation at pH 4.7 is carried out at a low ionic strength and low temperature to obtain as quantitative a precipitation of enzyme as possible. This step facilitates considerably the chromatography on Sephadex G-100, which was found to remove considerable amounts of inactive protein. The concentration of the pooled CCP containing fractions after the run on Sephadex G-100 is essential because the DEAE-cellulose column does not retain the enzyme. The main purpose of this step is to remove nucleic acids present in the preparation. As a practical rule, the ratio $A_{280}/A_{260}$ of the preparation should be 1.20 or higher before the next purification step on the CM-cellulose column. For successful final separation of CCP and cytochrome *c* oxidase, a pH of 6.9 and a buffer molarity of 0.01 are essential. CCP was eluted with this buffer, but cytochrome *c* oxidase was retained quantitatively on the top of the column. The latter was removed by increasing the ionic strength of the buffer. If the column was run at a slightly lower pH, both enzymes moved down the column and were poorly separated.

The over-all purification of CCP in this procedure is approximately 190-fold and the yield of enzyme 18 % of that in the original crude extract. This means that 0.5 % of the proteins present in the crude extract consisted of CCP and that 1000 g of fresh bacteria contained 250 mg of the enzyme. Finnish baker's yeast was found to contain only 16.4 mg CCP per 1000 g of fresh yeast.[7]

The final purified preparation did not contain measurable amounts of cytochrome *c*, catalase, or cytochrome *c* oxidase. In analytical ultracentrifugation and disc electrophoresis the preparation behaved as a homogeneous protein. The homogeneity was also indicated by the elution patterns on cellulose ion exchange chromatography. The isoelectric focusing, however, showed the presence of two components with nearly identical isoelectric points. The minor component is obviously a subcomponent of CCP. Its slightly lower isoelectric point as compared with that of the main component indicates that it is formed by the splitting of an amide group from the main component. Such subcomponents have been observed in the CCP of baker's yeast [24] as well as in several other peroxidases.[25–34]

The spectra of *Pseudomonas* CCP deviate considerably from those of CCP of baker's yeast as well as from spectra of peroxidases like horse radish peroxidase. Similar peroxidases with $\alpha$- and $\beta$-bands have been reported and classified by Morita [28] as *b*-group or by Yamazaki [35] as "low spin" peroxidases. *Pseudomonas* CCP does not form the alkaline pyridine ferrohemochrome

with an α-band at 557 nm as does protohemin. Instead, a hemochrome spectrum with an α-band at 550 nm was obtained. An identical band recorded after reduction of a denaturated preparation of CCP. This indicates that the heme in CCP is present as a hemochrome in the native enzyme.

The enzyme preparation was found to be stable between pH 4 and 7.7 for 1 min and between 5 and 7 for at least 24 h. CCP from baker's yeast is somewhat more stable at extreme pH values than the *Pseudomonas* enzyme.[7]

We wish to thank Professor N. Kaplan for his kind gift of the *Pseudomonas fluorescens* strain. The investigation was supported in part by grants from the *Finnish National Research Council of Sciences*.

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