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Received May 8, 1972.

## *Pseudomonas* Cytochrome *c* Peroxidase. VI. Large Scale Purification Procedure

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A method for the purification of cytochrome *c* peroxidase (PsCCP) from 25 g of acetone-dried cells of *Pseudomonas* has been previously reported.[1] The need for larger amounts of enzyme for further studies of its structural and enzymatic properties led to the development of a large-scale preparation method, described in this communication, starting with 125 g cells. The sequence of operations is basically as before, except that the final steps have been modified.

*Experimental. Pseudomonas aeruginosa* (previously reported as *P. fluorescens*, see Ref. 2) was cultivated and the acetone-dried cells were prepared as previously described.[1] CM-cellulose, DEAE-cellulose (Whatman DE-11), and Sephadex G-100 (Pharmacia) were used as previously described.[1] Peroxidase assays with *Pseudomonas* cytochrome c-551 (prepared from the same organism by the method of Ambler [3]) as substrate were performed as earlier.[1] Protein concentrations were determined according to Lowry *et al.*[4] with serum albumin (Finnish Red Cross) as standard. After the last step the protein concentration was determined spectrophotometrically using $A_{280}$ (1 %, 1 cm) = 12.1 calculated on the basis of dry weight determinations of the purified preparation.[2] Disc electrophoresis in polyacrylamide gel was carried out according to Maurer [5] (pH 8.6, 7 % gel) and the protein bands were stained according to Weber and Osborne.[6] About 50 $\mu$g of protein was applied per gel.

*Results.* All the steps of the procedure were performed at 4°C unless otherwise stated. 125 g of acetone-dried cells were extracted with distilled water, treated with DNAase, and precipitated at pH 4.7 as previously described.[1] The precipitate was dissolved and the solution was left overnight before being centrifuged at 15 000 *g* for 15 min to remove a small amount of insoluble material.

The preparation was chromatographed on Sephadex G-100 (column 8.9 × 80 cm, eluent 0.1 M sodium phosphate buffer pH 6.5, hydrostatic pressure 17 cm, flow rate about 80 ml/h, 13 ml fractions). The elution pattern was similar to that of the small column.[1] The fractions containing PsCCP activity were pooled, those fractions forming the first part of the PsCCP peak but containing opalescent impurities being omitted, however. The pooled fractions were dialyzed against 0.02 M sodium phosphate buffer pH 6.5, overnight. The concentration step of the original method was omitted.

The dialyzed preparation was passed through a DEAE-cellulose column (6 × 40 cm, eluent 0.02 M sodium phosphate buffer pH 6.5, flow rate 60 ml/h, 10 ml fractions). All coloured fractions were pooled; the absorbance ratio $A_{280}/A_{260}$ at this stage should be more than 1.2. The pooled fractions were dialyzed against distilled water overnight.

The pH of the preparation was adjusted to 6.5 with 0.005 M $NaH_2PO_4$ before it was fed onto a column of CM-cellulose (2.5 × 20 cm, equilibration buffer 0.005 M sodium phosphate pH 6.5, flow rates: sample 80 ml/h, eluent 23 ml/h, 5.8 ml fractions). The coloured proteins were adsorbed at the top of the column, forming a narrow band. The column was washed with 300 ml of the equilibration buffer. The band moved slightly down the column. Elution was performed with 0.01 M sodium phosphate buffer pH 6.8, and a red zone containing PsCCP was collected. Fractions with a spectral purity ($A_{407}/A_{280}$) greater than 3.1 were pooled. A green-red zone containing cytochrome oxidase moved down the column but remained separate from the PsCCP zone; cytochrome oxidase can be eluted with 0.05 M sodium phosphate buffer pH 6.8.

*Table 1.* Purification of *Pseudomonas* cytochrome *c* peroxidase from 125 g of acetone-dried cells (fresh weight about 500 g).

| Purification procedure | Vol., ml | Activity, units/ml | Activity, total units | Prot. concn., mg/ml | Specific activity, units/mg | Enrichment | Recovery % | $A_{407}/A_{280}$ |
|---|---|---|---|---|---|---|---|---|
| 1. Pooled extracts from acetone-dried cells | 975 | 5.3 | 5160 | 20.3 | 0.26 | 1 | 100 | — |
| 2. Precipitation at pH 4.7 | 174 | 25.0 | 4350 | 79.8 | 0.31 | 1.2 | 84 | — |
| 3. Pooled fractions from Sephadex G-100 column | 1110 | 3.6 | 4000 | 8.5 | 0.42 | 1.6 | 77 | — |
| 4. Pooled fractions from DEAE-cellulose column | 1460 | 2.6 | 3800 | 0.61 | 4.3 | 16.5 | 74 | 0.66 |
| 5. Pooled fractions from CM-cellulose column | 35 | 61 | 2140 | 1.13 | 54 | 208 | 41 | 3.30 |
| 6. Pooled fractions from Sephadex G-100 Superfine column | 14.5 | 127 | 1840 | 1.76 | 72 | 277 | 36 | 4.51 |

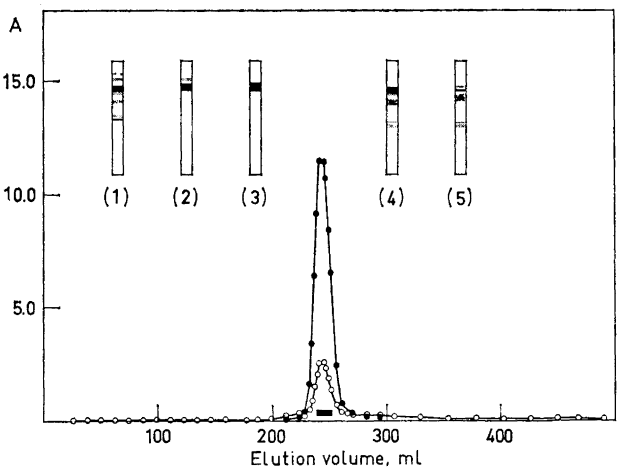


*Fig. 1.* Chromatography of PsCCP on a Sephadex G-100 Superfine column (experimental conditions are described in the text) and disc electrophoretic patterns of the sample (concentrated preparation from CM-cellulose column) and eluted fractions containing PsCCP. (1) sample, $A_{407}/A_{280}=3.30$, (2) fraction eluted at 233 ml, $A_{407}/A_{280}=3.47$, (3) fractions eluted at 237–249 ml, indicated by the black bar, $A_{407}/A_{280}=4.51$, (4) fraction eluted at 257 ml, $A_{407}/A_{280}=3.49$, and (5) fraction eluted at 261 ml, $A_{407}/A_{280}=2.15$. ○ $A_{280}$. ● $A_{407}$. The PsCCP activity parallels the absorbance at 407 nm.

The pooled PsCCP fractions were concentrated by ultrafiltration in a Diaflow ultrafiltration apparatus (Model 202, Aminco) and chromatographed on Sephadex G-100 Superfine (2.5 × 102 cm column, eluent 0.01 M sodium phosphate buffer pH 6.8, hydrostatic pressure 55 cm, flow rate 9.6 ml/h, 2.4 ml fractions). A single red band containing PsCCP was eluted (Fig. 1). Those fractions giving one protein band in disc electrophoresis (Fig. 1) were pooled.

The purification procedure is summarized in Table 1.

*Discussion.* The last steps of the original procedure have been modified. The conditions for the CM-cellulose chromatography gave a quantitative retention of PsCCP during the passage of a large volume of solution through the column. This was achieved by lowering the ionic strength and pH of both equilibration buffer and preparation. Chromatography on Sephadex G-100 Superfine eliminated the need for rechromatography on CM-cellulose, which was found to be necessary sometimes with the earlier method.[1] Chromatography on Sephadex G-100 Superfine removed some minor impurities, as can be seen from Fig. 1. This large scale procedure gives a better recovery of PsCCP than does the original method; further, the degree of purification achieved is higher.

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Received June 5, 1972.

# General Acid Catalysis in the Hydrolysis of a Furan Derivative

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Although reactions of furan and its derivatives have been thoroughly studied, only a few papers deal with the hydrolytic cleavage of the ring in these compounds. Stamhuis *et al.*[1] proposed that the rate-determining stage in the acid-catalyzed hydrolysis is proton transfer to the α-position of the ring. In the light of recent kinetic data on the hydrogen exchange of furans,[2,3] this mechanism seems to be excluded because the rate of α-hydrogen exchange was found to be higher than that of the subsequent cleavage of the ring. The α-hydrogen exchange was studied using different isotopes of hydrogen and the kinetic data were found to be in accordance with a two-step $A-S_E2$ mechanism.[3] As no β-hydrogen exchange was observed *prior* to further reactions, two alternatives remain. The first is that hydrogen exchange at the β-position of the ring takes place at a lower rate than the protolytic cleavage of the ring. Unfortunately, no verification is possible in aqueous solutions. Despite this, Unverferth and Schwetlick[4] recently concluded that β-hydrogen exchange is slower than the cleavage in the hydrolysis of furan derivatives. Consequently the hydrolytic decomposition proceeds by an α-protonation pre-equilibrium and a subsequent rate-determining cleavage of the ring. The second alternative is that the hydrolysis proceeds *via* a rate-determining β-protonation of the ring. Indeed, all the known facts seem to be in agreement with this alternative.[3] It was therefore of considerable interest to study the possibility of general acid catalysis in the hydrolysis of furan derivatives. Unfortunately, the susceptibility of most furans to hydrolytic decomposition is so low that the rates in buffer solutions are too low to be experimentally studied. Therefore we investigated whether the hydrolysis of 2-methoxyfuran (I) is subject to general acid catalysis. The alkoxy substituent in this compound enhances the susceptibility