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A Purification Procedure for the Soluble Cytochrome Oxidase and some other Respiratory Proteins from *Pseudomonas aeruginosa*

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The production of the soluble cytochrome oxidase/nitrite reductase in the bacterium *Pseudomonas aeruginosa* is favoured by anaerobic conditions and the presence of KNO_3 (20g/l) in the culture medium. Of three methods commonly used for the disruption of bacterial suspensions (ultrasonication, liquid-shear homogenization and glass-bead grinding), sonication proved the most efficient in releasing the *Pseudomonas* cytochrome oxidase. A polarographic assay of *Pseudomonas* cytochrome oxidase activity with sodium ascorbate as substrate and *NNN'N'*-tetramethyl-*p*-phenylenediamine dihydrochloride as electron mediator is described. A purification procedure was developed which can be used on the small scale (40-litre cultures) or the large scale (400-litre cultures) and provides high yields of three respiratory-chain proteins, *Pseudomonas* cytochrome oxidase, cytochrome c_{551} and azurin, in a pure state. A typical preparation of 250g of *Ps. aeruginosa* cell paste yielded 180mg of *Pseudomonas* cytochrome oxidase, 81mg of *Pseudomonas* cytochrome c_{551} and 275mg of *Pseudomonas* azurin.

The water-soluble cytochrome oxidase that functions in terminal electron transfer (*Pseudomonas* ferrocytochrome c_{551}–O_2 oxidoreductase, EC 1.9.3.2) was first isolated and purified from cells of *Pseudomonas aeruginosa* grown anaerobically in the presence of nitrate by Horio *et al.* (1961). It was a dihaem protein containing haem *c* and haem *d* prosthetic groups, the latter being the autoxidizable component, and contained no metals other than iron (Horio *et al.*, 1961). Horio *et al.* (1961) also isolated two other redox components of *Ps. aeruginosa*, namely the small haem protein cytochrome c_{551} and the blue copper protein azurin, both of which could act as donors in electron transfer to the oxidase. By modifying the procedure of Horio *et al.* (1961), Yamanaka *et al.* (1962) obtained *Pseudomonas* cytochrome oxidase in a crystalline state. However, owing to the apparent difficulty in achieving consistently reproducible results with the Japanese procedure, Kuronen & Ellfolk (1972) and Gudat *et al.* (1973) developed different purification procedures, both of which produce good yields of pure enzyme.

In this paper we present details of the growth conditions and the purification procedures that we have developed independently for use both in small-scale laboratory work and on the large scale to isolate *Pseudomonas* cytochrome oxidase in a pure state, along with the other respiratory-chain proteins *Pseudomonas* cytochrome c_{551} and azurin. Some of the properties of the *Pseudomonas* cytochrome oxidase as isolated are given in Barber *et al.* (1976).

Materials and Methods

All inorganic chemicals were of analytical reagent grade obtained from Fison's (Loughborough, Leics., U.K.), unless otherwise stated. Trizma base, ascorbic acid (disodium salt) and bovine serum albumin were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., and *NNN'N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) from BDH Chemicals, Poole, Dorset, U.K. Sephadex G-25 (coarse grade) and Sephadex G-75 were obtained from Pharmacia (G.B.) Ltd., London W.5, U.K. Dialysis was carried out in Visking dialysis tubing from the Scientific Instrument Co., London W.C.1, U.K.

DEAE-cellulose (DE-52) and CM-cellulose (CM-23) were obtained from Whatman Products, H. Reeve Angel, London E.C.4, U.K., and prepared for use according to the maker's directions. Considerable time can be saved when equilibrating celluloses on the large scale by using a spin dryer. Approx. 3kg of cellulose slurry was poured into a cotton bag and spun for 4min; little cellulose was lost during the

process. In addition, batch elutions of enzyme have been carried out by this method.

Protein measurements on whole cells were carried out by the hot biuret method of Gornall *et al.* (1949), with bovine serum albumin as standard. For extract samples, either the biuret method or the method of Lowry *et al.* (1951) was used, again with bovine serum albumin as standard. Cell growth was followed by measuring the absorption of the culture in an Eel colorimeter.

Culture methods

A culture of *Ps. aeruginosa* strain N.C.T.C. 6750 was obtained from the National Collection of Type Cultures, Colindale, London NW9 5HT, U.K., and was grown in a medium containing per litre: 7g of Oxoid yeast extract: 10g of Oxoid peptone; 20g of KNO_3; 6.4g of KH_2PO_4; 3.6g of Na_2HPO_4 (anhydrous); 2.5g of NaCl; 0.01g of $CuSO_4$; the initial pH was adjusted to 6.5 with NaOH.

On the small scale, 40-litre cultures were grown in a stainless-steel keg modified to take a New Brunswick (type MF14) fermentor head. A 150ml shaken culture inoculum was grown at 37°C for 24h and used to seed the bulk medium, which was stirred at 100rev./min and grown under submerged culture conditions at 37°C for 21h, at which point the cells were harvested by using a Sharples continuous centrifuge (13200*g*) at a flow rate of 250ml/min.

On the large scale, 400-litre cultures were grown in the fermentor described by Melling & Scott (1972) and seeded with a 20-litre inoculum. Cultures were maintained at 30°C, stirred at 250rev./min, and O_2-free N_2 was passed into the vessel at 2litres/min. When growth had ceased, as indicated by no increase in absorbance over a 1h period, cultures were harvested and about 2.5kg of wet cell paste was normally obtained. This was stored in the frozen state at −20°C until required. Disruption of bacterial cell suspensions by ultrasonication was carried out by using either a Rapidis manual-tuning 150W machine (Ultrasonics Ltd., Shipley, Yorks., U.K.) or a self-tuning Dawe Soniprobe ultrasonicator (Dawe Instruments Ltd., London W.3, U.K.) fitted with a microtip probe. It should be noted that higher operating outputs (up to 100W), and hence shorter sonication times, could be achieved with the self-tuning instrument compared with the maximum outputs attainable by using the manually tuned machine.

Enzymic assay

Pseudomonas cytochrome oxidase is not only able to oxidize *Pseudomonas* cytochrome c_{551} and azurin, but can also utilize sodium ascorbate as an electron donor (Horio *et al.*, 1961). Since this is more readily available than the natural protein substrates, a polarographic assay with sodium ascorbate as substrate was developed to determine the activity of *Pseudomonas* cytochrome oxidase fractions.

Although sodium ascorbate alone in the presence of *Pseudomonas* cytochrome oxidase produced a measurable rate of O_2 utilization, the rate was enhanced 1.5 times by the addition of a molar excess of *Pseudomonas* cytochrome c_{551}. A similar enhancement of rate could be achieved by using *NNN'N'*-tetramethyl-*p*-phenylenediamine dihydrochloride as an electron mediator (Jacobs, 1960) between ascorbate and *Pseudomonas* cytochrome oxidase. Assays were performed therefore by placing the following reagents in the reaction compartment of a Rank Brothers oxygen electrode maintained at 30°C: 3.0ml of air-saturated 0.04M-potassium phosphate buffer, pH6.5; 20μl of 30mM-*NNN'N'*-tetramethyl-*p*-phenylenediamine dihydrochloride in 0.04M-potassium phosphate buffer, pH6.5; 40μl of 1M-sodium ascorbate containing 10mM-EDTA in 0.04M-potassium phosphate buffer, pH6.5.

After allowing sufficient time to determine any blank rate due to the autoxidation of ascorbate, a measured volume of enzyme solution was injected into the reaction mixture and the initial rate of O_2 depletion measured. The activity of the enzyme sample (units/ml) was quoted in μmol of O_2 used/min per ml of enzyme solution. The specific activity was calculated after the determination of protein concentrations and was quoted in μmol of O_2 used/min per mg of total protein. Sodium ascorbate and *NNN'N'*-tetramethyl-*p*-phenylenediamine dihydrochloride solutions were either prepared and used immediately, or stored at −20°C until required (Smith & Camerino, 1963).

Results and Discussion

Selection of culture conditions

Fig. 1 shows the effect of the addition of nitrate to the medium on the cellular cytochrome concentrations. A portion (150ml) of medium either without KNO_3 or in the presence of 20g of KNO_3/l was inoculated with 1ml of a cellular suspension of *Ps. aeruginosa* and grown at 37°C for 21h in an incubator shaker. The cells were harvested, washed and resuspended in 0.02M-potassium phosphate buffer, pH7.4, to give a constant cellular concentration (w/v). The reduced-minus-oxidized difference spectra in the visible region of these whole-cell suspensions recorded in an Aminco–Chance split-beam spectrophotometer (no. 4-8460) show a significant enhancement in the cytochrome concentrations in the presence of nitrate, although both in the presence and absence of nitrate, the wet weight of cells produced was identical.

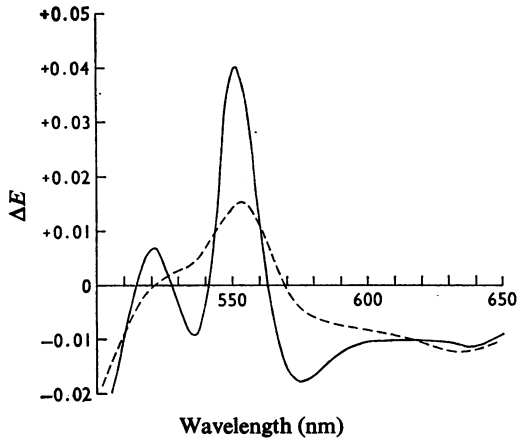

Fig. 1. *Reduced-minus-oxidized difference spectra of whole-cell suspensions of Ps. aeruginosa grown in the presence and absence of nitrate*

Cell paste (2.3 g) was suspended in 34ml of 0.02 M-potassium phosphate buffer, pH 7.4. Spectra were recorded at 20°C. Cells in the 'sample' cuvette were reduced by the addition of a few grains of $Na_2S_2O_4$ and cells in the 'reference' cuvette oxidized by the addition of 5 μl of 0.5 M-$K_3Fe(CN)_6$. ——, Cells grown in the presence of nitrate (20g/l); ----, cells grown in the absence of nitrate.

The effect of adding glucose to the culture medium as a possible carbon source was also investigated by assaying for *Pseudomonas* cytochrome oxidase activity in crude extracts of cells grown under normal culture conditions but with various amounts of added glucose. Crude extracts were prepared from a constant wet weight of cells by sonication of a cellular suspension at 0°C followed by centrifugation to remove cell debris. The effect of adding 0.5, 1, 2 and 4% (w/v) glucose, in all cases, caused a total loss of oxidase activity in the crude extracts, whereas a control with no glucose added, grown under the same conditions, gave an activity of 0.34 unit/ml of crude extract.

To investigate the production of *Pseudomonas* cytochrome oxidase at two growth temperatures, 30° and 37°C, under both aerobic and anaerobic conditions, cultures were grown in a 3-litre glass vessel similar to that described by Elsworth *et al.* (1958). Sterile air (3l/min) was passed through the aerobic cultures and anaerobiosis was maintained by gassing the cultures with 3 litres of O_2-free N_2/min. Ease of enzyme release, represented as the percentage of the total enzymic activity, which is recovered in the crude extract after removal of cell debris by centrifugation, was examined by sonication, and the results obtained are summarized in Table 1. The assay procedure described above measures the overall oxidase activity of disrupted cell suspensions, which comprises contributions from both soluble and membrane-bound cytochrome oxidases. The proportions of each oxidase under specified growth conditions can be estimated by measuring the percentage of enzymic activity that remains in the crude extract supernatant after removal of cellular debris by centrifugation. It is apparent that anaerobic conditions not only give maximum enzyme yields and specific activities, but also a significantly greater soluble enzyme release compared with aerobic conditions. This suggests that anaerobiosis favours the production of soluble *Pseudomonas* cytochrome oxidase, whereas the membrane-bound enzyme is produced to a greater extent in the presence of air. Attempts to solubilize, isolate and identify the membrane-bound component have so far proved fruitless. Maximum enzyme activity occurred in cultures grown at 30°C; thus anaerobic growth at 30°C was selected for use on the 400-litre scale.

Growth data from a typical large-scale culture are given in Table 2. Percentage enzyme release from the final culture sample is high and comparable with that achieved on the small scale. However, there ap-

Table 1. *Effect of growth temperature and aeration on the production and ease of release of the cytochrome oxidase from Ps. aeruginosa (N.C.T.C. 6750)*

Cells were disrupted by sonication of a bacterial suspension at 100W for 2min at 4°C. *Pseudomonas* cytochrome oxidase activity was assayed as described above.

Temperature (°C)	Gas (3 litres/min)	Specific activity of cells (units/g of protein)	% release* (soluble enzyme)	Enzyme activity† (units /ml of culture)
30	Air	51.5	50	0.091
30	N_2	135.0	94	0.119
37	Air	12.8	77	0.039
37	N_2	89.4	95	0.112

* $\frac{\text{Enzyme activity in solution after removal of cell debris}}{\text{Enzyme activity in whole disrupted suspension}} \times 100.$

† Enzyme activity in whole disrupted suspension.

Table 2. *Data obtained during a 400-litre batch culture of Ps. aeruginosa*

Growth was carried out at 30°C. The culture was stirred at 250rev./min and 2 litres of O_2-free N_2/min were passed into the vessel.

Time (h)	pH	Laboratory dry wt. (g/l)	Enzyme activity (unit/ml)	% release (soluble enzyme)
4	6.55	0.588	0.106	73.5
10	6.95	0.892	0.114	91.2
14	7.3	—	0.171	67.8
16	7.5	1.617	0.308	94.4
18	7.6	—	0.281	90.7
19	7.6	1.596	0.266	92.1

Table 3. *Comparison of different methods of bacterial disruption on the release of cytochrome oxidase from Ps. aeruginosa*

A 25% (w/v) suspension of cell paste was used in each case. The Manton–Gaulin operating pressure was 54 MPa (8000lb/in^2) and the output of the ultrasonicator was approx. 50W.

Manton–Gaulin		Dynomill		Sonication	
No. of passes	Activity (unit/ml of supernatant)	Residence time (min)	Activity (unit/ml of superantant)	Time (min)	Activity (unit/ml of supernatant)
1	0.273	0.5	0.105	1	0.28
2	0.42	1.0	0.091	2	0.448
3	0.483	2.0	0.21	3	0.602
		4.0	0.196	4	0.728
		10.0	0,191	5	0.728
				6	0.784

peared to be some loss of enzyme activity on storage of the cell paste at −20°C.

Release of Pseudomonas cytochrome oxidase

In small-scale enzyme purifications the technique of sonication was used for the disruption of the whole-cell suspension. Although sonication was not possible on the large scale, it was compared with two methods commonly used for the disruption of large quantities of cell paste, namely the Manton–Gaulin (liquid-shear) homogenizer and the Dynomill (glass-beed grinding mill) (Melling & Phillips, 1975). The results in Table 3 indicate that, of the two methods suitable for large-scale work, maximum enzyme release was obtained by using liquid-shear homogenization. However, sonication is clearly the most efficient technique for the release of *Pseudomonas* cytochrome oxidase.

Small-scale purification procedure for Pseudomonas cytochrome oxidase

Step 1, *extraction of cellular protein.* Approx. 250g wet wt. of *Ps. aeruginosa* cell paste was thawed and uniformly suspended in 1 litre of 0.02M-potassium phosphate buffer, pH 7.0, at 4°C. The cells were ruptured by ultrasonication of approx. 300ml batches of the cell suspension. Each batch was sonicated for 1½min at 100W at 0°C. The sonicated suspension was stirred vigorously and then centrifuged at 10000*g* for 20min to remove cell debris. The dark green-brown crude extract supernatant was decanted from the precipitated debris, which was resuspended in buffer, stirred vigorously, and centrifuged as before. Again, the supernatant was decanted from the precipitate and combined with the first extract.

Step 2, $(NH_4)_2SO_4$ *fractionation.* The crude extract was brought to 45% saturation by slowly adding solid $(NH_4)_2SO_4$ (277g/l) over a period of about 15min. This solution was centrifuged at 20000*g* for 25min, and the precipitate of unwanted proteins discarded. The clear golden-yellow supernatant was taken to 95% saturation by a further addition of solid $(NH_4)_2SO_4$ (372g/l, see Table 4). This solution was centrifuged at 23000*g* for 45min and the pale-yellow supernatant, which contained only a small amount of oxidase activity, was discarded. The light-brown precipitate containing the cytochrome oxidase, cytochrome c_{551} and azurin was resuspended in a minimum volume of 0.02M-potassium phosphate buffer, pH 7.0, and dialysed at 4°C for 20h againt 15 litres of this same buffer. The

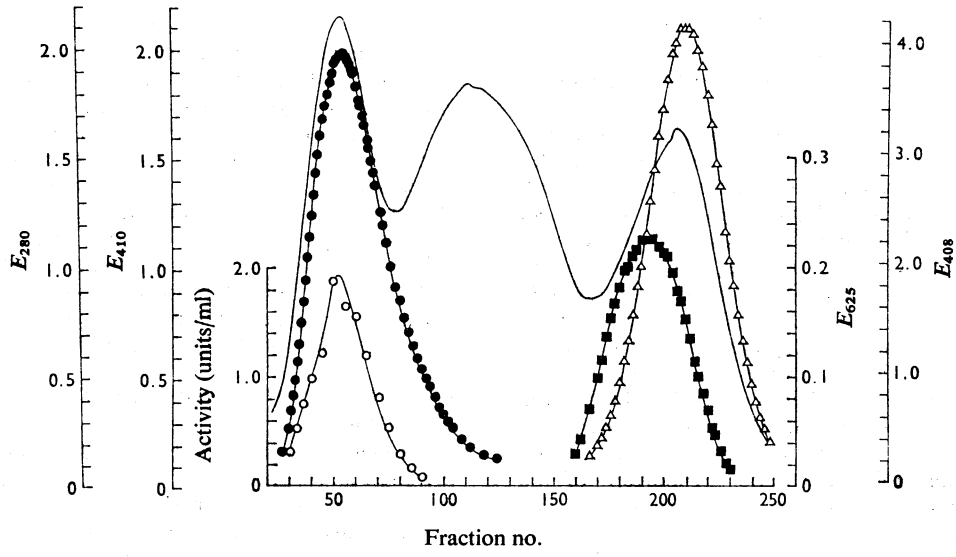

Fig. 2. *Elution profile of the respiratory proteins of Ps. aeruginosa from a Sephadex G-75 column (step 4)*

The column (9cm × 50cm) was equilibrated with 0.01M-Tris/HCl buffer, pH 7.5, and 7ml fractions were collected. ——, Protein concentration expressed as E_{280}; ●, E_{410} (*Pseudomonas* cytochrome oxidase); ○, enzymic activity; ■, E_{625} (azurin); △, E_{408} *Pseudomonas* cytochrome c_{551}).

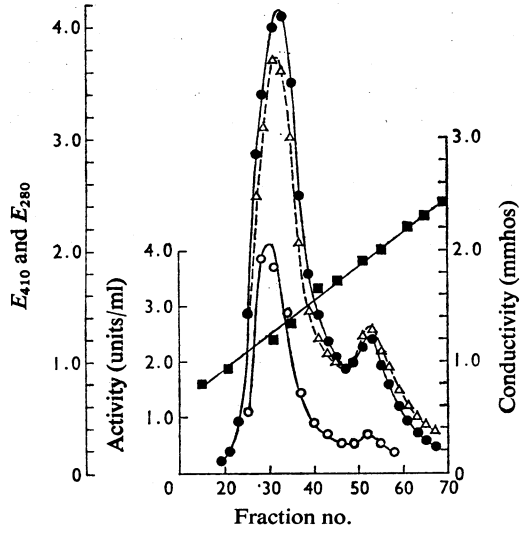

Fig. 3. *Elution diagram of Ps. cytochrome oxidase from a DEAE-cellulose (DE-52) column (step 5)*

The column (3cm × 10cm) was equilibrated in 0.01M-Tris/HCl buffer, pH 7.5, and eluted by a gradient made from 230ml of 0.01M- and 230ml of 0.1M-Tris/HCl buffer, pH 7.5; 6ml fractions were collected. △, E_{280}; ●, E_{410}; ○, enzymic activity; ■, conductivity.

dialysis medium was changed once during the period of dialysis.

Assays of cytochrome oxidase activity and protein determinations were made of the 45 and 95% (w/v) $(NH_4)_2SO_4$ supernatants after dialysing a small measured volume overnight against 0.02M-potassium phosphate buffer, pH7.0.

Step 3, batchwise treatment with DEAE-cellulose. About 250ml of a slurry of DEAE-cellulose (DE-52) (about 100g) equilibrated in 0.02M-potassium phosphate buffer, pH7.0, was added to the dark-green dialysate and stirred for 15min. The suspension was centrifuged at 10000*g* for 15min and the green supernatant decanted. The DEAE-cellulose precipitate was resuspended in a further volume of buffer to remove any unattached protein and centrifuged as before. The two supernatant solutions were combined.

Under these conditions *Pseudomonas* cytochrome oxidase, cytochrome c_{551} and azurin do not absorb to the gel but remain in the supernatant. However, much unwanted protein is removed, particularly the yellow flavoproteins, before the column chromatographic steps.

Step 4, chromatography on Sephadex G-75. The proteins were precipitated by bringing the supernatant solution from Step 3 to 100% saturation with solid $(NH_4)_2SO_4$ (760g/l). After centrifugation, the super-

natant, which was blue in colour owing to a proportion of unprecipitated azurin, was decanted but retained for further purification (see below), and the precipitate was dissolved in a minimum volume (70ml) of 0.01 M-Tris/HCl buffer, pH7.5, and applied in batches to a column (2.5cm × 36cm) of Sephadex G-25 (coarse grade). This column served to desalt the sample and stop channeling on the subsequent Sephadex G-75 column. The coloured eluate from the Sephadex G-25 column, (approx. 150ml) was applied to a column (9cm × 50cm) of Sephadex G-75 equilibrated in 0.01 M-Tris/HCl buffer, pH7.5, and eluted with the equilibrating buffer. Fig. 2 shows a typical elution profile from such a column. The absorption and activities of the fractions of the fast-running green band, which was eluted just behind the void volume, were measured and those fractions containing the major proportion of the *Pseudomonas* cytochrome oxidase were pooled. The later fractions of the overlapping red and blue bands of *Pseudomonas* cytochrome c_{551} and azurin were pooled to be kept for further purification.

Step 5, chromatography on DEAE-cellulose. The pooled *Pseudomonas* cytochrome oxidase fractions were applied directly to a column (3cm × 10cm) of DEAE-cellulose (DE-52) equilibrated in 0.01 M-Tris/HCl buffer, pH7.5. The oxidase was adsorbed as a tight green band to the top of the column, which was washed thoroughly with the equilibrating buffer. The oxidase was then eluted from the column by linear gradient elution by using 230ml each of 0.01 M-Tris/HCl, pH7.5 and 0.1 M-Tris/HCl, pH7.5. Fig. 3 shows the elution profile from such a column. Those fractions containing oxidase activity were pooled.

Step 6, chromatography on CM-cellulose. The pH of the above pooled fractions was adjusted to 6.4 with 0.01 M-KH_2PO_4 before adsorption of the *Pseudomonas* cytochrome oxidase to a column (3cm × 12cm) of CM-cellulose (CM-23) equilibrated in 0.01 M-potassium phosphate buffer, pH6.4. After sample application, the column was washed thoroughly with the equilibrating buffer before elution of the oxidase as a concentrated band by using 0.04 M-potassium phosphate buffer, pH6.9.

A flow chart of a typical preparation of *Pseudomonas* cytochrome oxidase by the purification procedure described is shown in Table 4.

Purification of cytochrome c_{551} and azurin from Ps. aeruginosa

Pseudomonas cytochrome c_{551} and azurin could be prepared as by-products of the cytochrome oxidase preparation. The cytochrome c_{551} and a proportion of the azurin remain associated with the oxidase fractions in the initial stages (steps 1–3 above), but separate on the Sephadex G-75 column (Fig. 2).

As mentioned above, however, a large proportion of the azurin does not precipitate in 100% $(NH_4)_2SO_4$ before sample application to the Sephadex G-75 column. To recover this azurin fraction, the blue 100%-$(NH_4)_2SO_4$ supernatant was washed through old unequilibrated CM-cellulose in a Buchner funnel. It appears that the azurin is precipitated at the surface of the cellulose and hence remains as a band that can be eluted by washing the gel with distilled water (R. P. Ambler, personal communication). The blue eluate is then dialysed overnight against running tapwater, after which it is combined with the cytochrome c_{551} and azurin fractions

Table 4. *A small-scale purification of Pseudomonas cytochrome oxidase*

After step 6 the ratio $E^{ox}_{410}/E_{280} = 1.16$, corresponding to a purity of 96% (Yamanaka *et al.*, 1962). The total amount of *Pseudomonas* cytochrome oxidase isolated was 180mg [$E^{ox}_{410} = 149 \times 10^3$ litre·mol^{-1}·cm^{-1} (Horio *et al.*, 1961), mol.wt. = 120000 (Kuronen & Ellfolk, 1972)].

Step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity (units/ml)	Total activity (units)	$10^2 \times$ Specific activity (μmol of O_2/min per mg)	Purification factor	Recovery (%)
1. Crude extract from 253g of cell paste	1290	14.5	18777	0.72	928	4.9	1	100
2. 45%-Satd.-$(NH_4)_2SO_4$ supernatant	1370	7.2	9873	0.47	638	6.5	1.3	69
3. Supernatant from DEAE-cellulose batchwise treatment	980	3.2	3136	0.46	454	14.5	3.0	49
4. Sephadex G-75 column eluate	350	2.3	822	1.10	385	47	9.6	41
5. Eluate from DE-52 cellulose column	208	1.6	340	1.59	330	97	19.8	36
6. Eluate from CM-cellulose column	25	7.5	187	9.72	243	130	26.5	26

from the Sephadex G-75 column and purified further by a method similar to that described by Ambler (1963).

pH adjustment. The pH of the combined fractions was adjusted to pH3.9 with acetic acid, and any resulting precipitate removed by centrifugation at 23000*g* for 20min.

Chromatography on CM-cellulose. The clear supernatant was applied to a column (2.5cm × 30cm) of CM-cellulose (CM-23) equilibrated in ammonium acetate buffer, pH3.9, made by adjusting the pH of 0.05M-acetic acid with 2M-NH_3. At this pH a dense-coloured band absorbs at the top of the column, which is washed with the equilibrating buffer until the eluate is free of protein. The cytochrome c_{551} is eluted from the column as a broad dilute band by using ammonium acetate buffer, pH4.45. When the eluate becomes colourless the pH of the eluting buffer is raised to pH4.65 and the azurin is eluted, again as a broad dilute band.

To concentrate the proteins, the respective fractions were adjusted to pH3.9 with acetic acid and each adsorbed on a fresh column (1.25cm × 15cm) of CM-cellulose equilibrated at pH3.9. The two proteins were then obtained as concentrated solutions by slow elution with ammonium acetate buffer, pH6.0. Table 5 shows the yields and purities of the samples obtained from a typical purification.

Table 5. *Pseudomonas cytochrome c_{551} and azurin isolated from a small-scale purification*

	Pseudomonas cytochrome c_{551}	Azurin
Volume (ml)	12	12.5
Concentration (μM)*	753	1370
Total amount (mg)	81	275
Purity ratio: $\frac{E^{red}_{551}-E^{red}_{570}}{E_{280}}$	1.14	—
Purity ratio: E^{ox}_{652}/E_{280}	—	0.57

* For *Pseudomonas* cytochrome c_{551} $E^{red}_{M551} = 28.3 \times 10^3$ litre·mol^{-1}·cm^{-1} (Horio *et al.*, 1960), and for azurin $E^{ox}_{M625} = 3.5 \times 10^3$ litre·mol^{-1}·cm^{-1} (Brill *et al.*, 1968).

Large-scale purification of Pseudomonas cytochrome oxidase

The large-scale purification follows the procedure described above, but with a 10-fold increase in scale and the following modifications.

In step 1 the bacterial cells were disrupted by twice passing a suspension of 2.5kg of wet cell paste through a Manton–Gaulin homogenizer operating at 50 litres/h and a pressure of 54MPa (8000lb/in^2). Since the temperature of the suspension rises approx. 10°C on passing through the homogenizer, the homogenate was collected in an ice-cooled vessel and allowed to cool to 4°C between each pass. Step 2 was performed as in the small-scale preparation. In step 3 approx. 2.5kg of DEAE-cellulose was added, and, after stirring for 30min at 4°C, the cellulose was removed by using the spin dryer.

In steps 4 and 5, because of difficulties in achieving good separations on large Sephadex G-75 columns with samples containing high protein concentrations, this step was omitted from the large-scale procedure. The filtrate from step 3 was concentrated, therefore, by ultrafiltration by using an Amicon TC/5 thin-channel system with a PM10 (10000-mol.-wt. cut-off) membrane. The concentrate was adjusted to pH7.5 with 1M-NaOH and to a conductivity of 1500μS with distilled water. This solution was then applied to a column (7cm × 50cm) of DE-32 cellulose equilibrated in 0.01M-Tris/HCl buffer, pH7.5. The *Pseudomonas* cytochrome oxidase, together with cytochrome c_{551} and azurin adsorbed at the top of the column, which was washed with 4 column volumes of the equilibrating buffer. The column was then eluted with an 8-litre ionic-strength gradient from 0.01M to 0.1M-Tris/HCl buffer, pH7.5. At this stage the pooled oxidase fractions still contained some cytochrome c_{551} and azurin.

Step 6 was performed as in the small-scale procedure, at 0.01M-potassium phosphate buffer, pH6.4.

Table 6. *A large-scale purification of Pseudomonas cytochrome oxidase*

Step	Volume (litres)	Total enzyme activity (units)	$10^2 \times$ Specific activity (units/mg)	Recovery (%)	
				Per stage	Overall
1. Crude extract from 2.8kg of cell paste	7.6	9420	4	100	100
2. 50%-Satd.-$(NH_4)_2SO_4$ supernatant	6.8	3100	5.5	33	33
3. Supernatant from DEAE-cellulose batchwise treatment	10.9	3666	8	100	39
4. Ultrafiltrate	1.4	1906	13.3	52	20
5. Eluate from DE-52 cellulose column	0.35	882	42	46	9
6. Eluate from CM-cellulose column	0.05	474	150	54	5

Under these conditions *Pseudomonas* cytochrome oxidase adsorbs at the top of the CM-cellulose column and cytochrome c_{551}, azurin and other contaminating cytochromes still remaining at this stage are eluted with the equilibrating buffer. The *Pseudomonas* cytochrome oxidase can then be eluted as before. A flow chart of a typical large-scale purification is shown in Table 6.

In summary, the bacterial-growth conditions and purification procedures described here provide good yields (approx. 25% on the small scale) of *Pseudomonas* cytochrome oxidase with a high spectral purity ratio (Table 4). These procedures compare very favourably with those previously reported (Yamanaka *et al.*, 1962; Kuronen & Ellfolk, 1972; Gudat *et al.*, 1973). As by-products of the oxidase preparation, *Pseudomonas* cytochrome c_{551} and azurin could also be obtained, again with high spectral purity ratios (Table 5) and in amounts comparable with those achieved by Ambler (1963) using direct purification procedures.

References

Ambler, R. P. (1963) *Biochem. J.* **89**, 341–349

Barber, D., Parr, S. R. & Greenwood, C. (1976) *Biochem. J.* **157**, 431–438

Brill, A. S., Bryce, G. F. & Maria, H. (1968) *Biochim. Biophys. Acta* **154**, 342–351

Elsworth, R., Capell, G. H. & Telling, R. C. (1958) *J. Appl. Bacteriol.* **21**, 80–85

Gornall, A. G., Bardawill, E. F. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766

Gudat, J. C., Singh, J. & Wharton, D. C. (1973) *Biochim. Biophys. Acta* **292**, 376–390

Horio, T., Higashi, T., Sasagawa, M., Kusai, K., Nakai, M. & Okunuki, K. (1960) *Biochem. J.* **77**, 194–201

Horio, T., Higashi, T., Yamanaka, T., Matsubara, H. & & Okunuki, K. (1961) *J. Biol. Chem.* **236**, 944–951

Jacobs, E. E. (1960) *Biochem. Biophys. Res. Commun.* **3**, 536–539

Kuronen, T. & Ellfolk, N. (1972) *Biochim. Biophys. Acta* **275**, 308–318

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Melling, J. & Phillips, B. W. (1975) in *Handbook of Enzyme Technology* (Wiseman, A., ed.), pp. 190–195, Ellis Horwood, Chichester

Melling, J. & Scott, G. K. (1972) *Biochem. J.* **130**, 55–62

Smith, L. & Camerino, P. W. (1963) *Biochemistry* **2**, 1428–1432

Yamanaka, T., Kijimoto, S., Okunuki, K. & Kusai, K. (1962) *Nature (London)* **194**, 759–760