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Cytochrome *c* Peroxidase

1. Preparation of the Crystalline Enzyme from Baker's Yeast

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A procedure for the purification of cytochrome *c* peroxidase from baker's yeast has been described. This method was found to be reproducible and has consistently given a homogeneous crystalline preparation, the iron content of which has been equal to 0.15 % per unit of dry weight corresponding to a minimal molecular weight of 37 000. The enzyme was found to be free from carbohydrates and to behave as a single homogeneous component in the ultracentrifuge and in free electrophoresis. The isoelectric point of crystalline cytochrome *c* peroxidase was found to be equal to 4.9 at an ionic strength of 0.1 at + 2°C. The enzyme was stable between pH values of 3.5 and 9.0 at room temperature.

Cytochrome *c* peroxidase, CcP, (Cytochrome *c*:H_2O_2 oxidoreductase, EC 1.11.1.5) is a hemoprotein of special interest by oxidizing mainly reduced cytochrome *c*. It was first found in baker's yeast by Altschul *et al.*[1] who also assigned a preliminary procedure for its purification. This was improved by Abrams *et al.*[2] By using chromatography on cellulose ion exchange column as a step, the purification procedure was greatly simplified.[3,4] In this publication the isolation of CcP in a crystalline state from baker's yeast is described. A preliminary report of this work has already been published.[3,5]

MATERIALS AND METHODS

Baker's yeast (Sacharomyces cerevisiae) obtained from the State Alcohol Monopoly (Alko) was used as the starting material. The yeast was stored at −16°C before use.

DEAE cellulose was prepared according to Peterson and Sober,[6] the last alcohol drying step, however, being omitted.

Column chromatography. The glass columns were filled with the same buffer as that used for the equilibration. A slurry of the ion exchange material was poured into the column and the resin was allowed to settle by gravity and the buffer was run through the column over night for final equilibration. The effluent was collected in fractions of equal volume in a fraction collector at + 4°C. The absorbance at 280 nm and 407 nm of the different fractions were determined in a Beckman DU spectrophotometer.

Ferrocytochrome c. A lyophilized preparation of horse heart cytochrome *c*, Type III (Sigma) was used. Ferrocytochrome was prepared by anaerobic gel filtration of a dithionite reduced cytochrome *c* as described by Yonetani.[7] The cytochrome was reduced to at least 95 %. *H_2O_2* Merck's Perhydrol was diluted to give a H_2O_2 solution of about 10 mM. The concentration was determined spectrophotometrically at 230 nm where E_{mM} is equal to 0.0724.

Hemin-iron. The hemin content of the CcP preparation was determined by the pyridine hemochrome method according to Paul *et al.*[8]

CcP assays were performed at 23°C in 0.02 M phosphate buffer pH 7.2 containing 12—15 μM ferrocytochrome *c*, 40 μM H_2O_2 in a final volume of 2.5 ml. The reaction was started by addition of 5 μl of enzyme. The initial absorbance decrease recorded in a Beckman DK-1 recording spectrophotometer at 550 nm per cm per 10 sec per 10 μl of the enzyme solution was used as an arbitrary unit of the enzyme activity.[5]

Electrophoresis. Free boundary electrophoresis at + 2°C in a Tiselius electrophoresis apparatus (Strübin, Focal F) was used. Before electrophoresis the protein was dialyzed for 24 h against 2 liters of buffer at + 4°C. The pH values were measured at 23°C and not extrapolated to + 2°C. Conductances were those of the equilibrated buffers at + 2°C.

Sedimentation analysis was performed in a Spinco analytical ultracentrifuge model E. Prior to ultracentrifugation each preparation was dialyzed for 24 h at + 4°C against a 0.283 μ buffer of pH 7.0 that contained 0.05 M sodium phosphate and 0.171 M sodium chloride.

Carbohydrate analysis. The total carbohydrate content was estimated by the orcinol-sulphuric acid method of Francois *et al.*[9]

Protein concentration was determined according to Lowry *et al.*[10] The absorption values were standardized against bovine serum albumin (Kabi).

RESULTS

Preparation of enzyme extract. The original method of Altschul *et al.*[1] for preparing enzyme extracts from dried baker's yeast consisted of a rather prolonged autolysis of dried yeast cells. A more time saving procedure was used by Yonetani,[4] who used ethyl acetate to autolyze the yeast cells. A comparison of these two methods in respect to the amount CcP activity extracted showed no difference. Because of this the extracts have been prepared as follows: 10 kg of thawed baker's yeast were dried on filter paper until a loss of weight to about 25—30 % was obtained after which the yeast was mixed with 1 litre of ethyl acetate and left over night in the cold (+ 4°C). Distilled water was added to give 12 litres of a suspension which was stirred for 3 h at room temperature. 5—6 litres of a yellowish brown solution was obtained after centrifugation.

Precipitation with basic lead acetate. The correct amount of basic lead acetate was determined by tests with small samples. If too much was added some of the CcP activity was lost. With our material, around 40 ml of a 19 % solution of basic lead acetate ($2PbAc_2Pb(OH)_2$) was usually required per litre of the crude enzyme extract. The precipitate formed was centrifuged off and discarded. This procedure increased the stability of the crude enyzme extract by eliminating proteolytic activity. The pH of the solution was usually around 4.3. The extract was concentrated by rotation *in vacuo* to half of its original volume. A slight precipitation was regularly formed, which was removed by centrifugation and discarded.

Acetone fractionation. The concentrated extract was cooled to 0°C and peroxide free acetone at —10°C was slowly added with stirring to give a final

solution containing 30 % acetone (v/v), the temperature being lowered progressively to −5°C. The inactive precipitate consisting mainly of lead salts was centrifuged off at −5°C and discarded. Acetone at −10°C was added to the supernatant liquid to 50 % volume of acetone per volume of the original extract. The suspension was centrifuged at −5°C. The precipitate was dissolved in cold water (0°C) and centrifuged. The enzyme was reprecipitated at an acetone concentration of 35 % (v/v). After centrifugation at −5°C the precipitate was dissolved in the least possible amount of 0.02 M acetic acid buffer of pH 5.2 and centrifuged.

Column chromatography on DEAE-cellulose. The solution obtained in the previous step was placed on a DEAE-cellulose column (1.5 × 25 cm) that had been equilibrated with 0.02 M acetate buffer, pH 5.2 at + 4°C. The column was first washed with the same buffer, the enzyme activity was all adsorbed on the column when large amounts of the protein present in the extract were not bound at this pH and ionic strength. The enzyme activity was eluted by increasing the concentration of the acetate buffer from 0.02 to 0.3 M. The gradient was produced by adding 0.3 M acetate buffer, pH 5.2 to a mixing chamber which contained 200 ml of the starting buffer. A typical elution pattern is shown in Fig. 1. Two peaks were obtained with strong absorption at 407 nm, the first of which was found to consist mainly of reduced cytochrome *c* and the second one of cytochrome *c* peroxidase. Fractions containing the

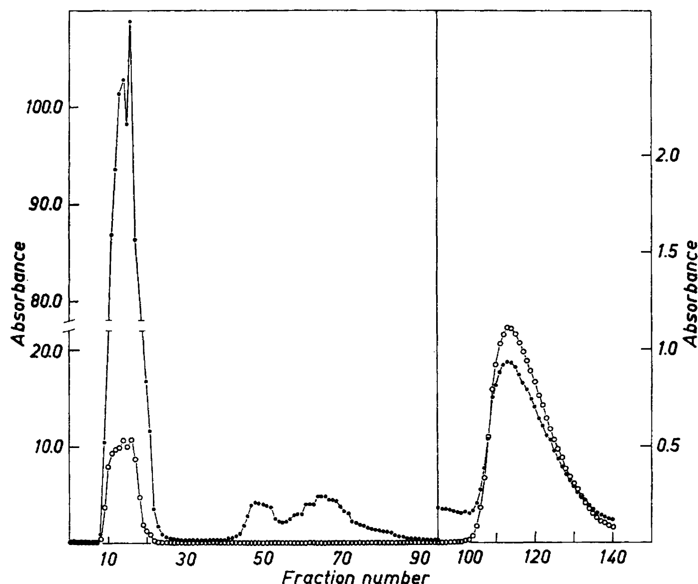

Fig. 1. Elution diagram of partially purified cytochrome *c* peroxidase on DEAE-cellulose column (1.5 × 25 cm). Experimental conditions are given in the text. The buffer reservoir initially contained 0.02 M acetate buffer of pH 5.2; at fraction 40 the reservoir was changed to 0.3 M acetate buffer of pH 5.2 the mixing chamber containing 200 ml of 0.02 M acetate buffer of pH 5.2. 5 ml fractions of the effluent were collected and the flow rate was about 30 ml/h. Absorbance at 280 nm is indicated by ●, and at 407 nm by ○.

CcP activity and having an E_{407}/E_{280} ratio equal to 1.0 or larger were pooled and dialyzed for 6 h against distilled water and collected on a 2 cm DEAE-cellulose column equilibrated to pH 5.2 by 0.02 M acetate buffer and eluted with 0.3 M buffer of pH 5.2 giving a strong solution in some ml of solution.

Crystallization. The concentrated CcP solution obtained in the previous step was dialyzed against cold distilled water (+ 4°C). Usually after 1—2 days large brown needle-like crystals appeared. The crystals were dissolved by careful addition of 0.1 N NaOH solution and recrystallization carried out by repeating the dialysis procedure against cold distilled water. By this technique a final ratio of E_{407}/E_{280} equal to 1.28 was obtained. No improvement of this ratio was observed by additional crystallization up to 5 times. On the contrary, a decrease could be observed evidently due to a degradation of the hemin ring, because the original ratio of 1.28 was restored after chromatography on a short DEAE-cellulose column. Fig. 2 represents a microphotograph of twice

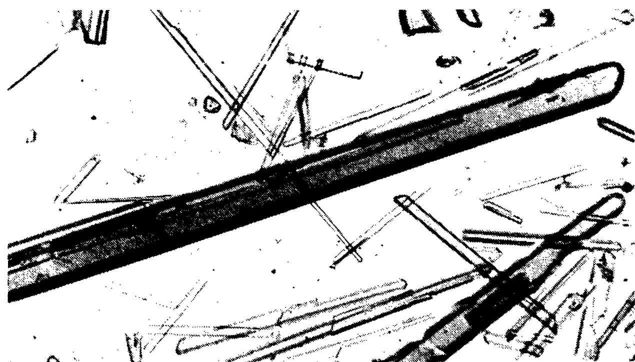

Fig. 2. Twice crystallized cytochrome *c* peroxidase, 140 ×.

crystallized cytochrome *c* peroxidase with a ratio of E_{407}/E_{280} equal to 1.28. The yield of the enzyme and the specific activity at each step of a typical purification is summarized in Table 1. The over all purification of the initial extract was 865 times with a recovery of 9 % of the activity as crystalline enzyme. Crystallization is evidently an important step in the purification procedure. Two crystallizations increased the ratio E_{407}/E_{280} from 1.171 to 1.285. In separate experiments it was found that a starting material with even lower ratios of E_{407}/E_{280} could be successfully purified only by crystallization; *e.g.*, a preparation with the ratio of 0.85 gave in the first crystallization a ratio of 1.15, in the second a ratio of 1.23 and in the third one of 1.28. Microscopically no difference was observed among the different crystalline preparations.

Properties of CcP. Free electrophoresis analysis of the final crystalline preparation showed only one protein component with an I. P. of about 4.9, which can be seen in the mobility curve of the protein (Fig. 3). Confirmatory results were obtained by sedimentation analysis (Fig. 4). The crystalline preparation was found to be free of carbohydrates as far as could be decerned

Table 1. Details of a representative preparation of crystalline cytochrome *c* peroxidase. Values shown are for 10 kg of baker's yeast.

Purification procedure	Volume ml	Protein conc. mg/ml	Absorbance $E_{407}^{1\,cm}$	Absorbance $E_{280}^{1\,cm}$	E_{407}/E_{280}	Activity units/ml	Activity Total units	Specific activity units/mg prot.	Enrichment —fold	Recovery %
1) *Ethyl acetate treated yeast extracted with water.* Supernatant	4670	18	1.560	42.600	0.037	25	116 800	1.39	—	100
2) *Lead acetate treatment* Supernatant	4630	14	1.560	[96.000]*	[0.016]*	23.5	108 800	1.68	1.2	93
Conc. *in vacuo*	2700	20	1.200	[77.000]*	[0.016]*	38.0	102 600	1.90	1.4	88
3) *Acetone fractionation* a) Precipitation with 30 % acetone. Supernatant	3560	13	1.680	60.000	0.028	25.5	90 800	1.96	1.4	78
b) Precipitation of 3 a with 50 % acetone. Precipitate dissolved in dist. water, centrifuged.	750	16	1.260	27.420	0.046	114.2	85 650	7.14	5.1	73
c) Precipitation of 3 b with 35 % acetone. Precipitate dissolved in 0.02 M acetate buffer and centrifuged	126	31	18.900	12.900	0.146	470.0	59 690	15.16	10.9	51
4) *Column chromatography on DEAE-cellulose* Fractions 109—127 (Fig. 1)	93	0.38	0.820	0.700	1.171	274.	25 482	721.05	518.7	22
5) *Crystallization* Crystals dissolved in 2.2 ml dist. water + 35 μl 0.1 N NaOH	2.2	7.0	9.816	7.872	1.245	5695	12 053	813.57	585.3	10
1st Recrystallization	1.67	8.8	7.032	5.472	1.285	6367	10 633	1202.8	865.3	9

* The high absorbance depends on the large amounts of lead acetate still present in the solutions.

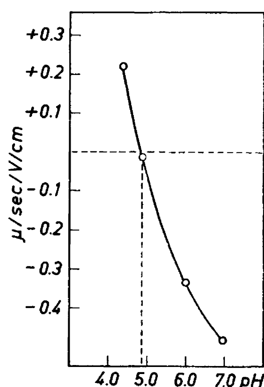

Fig. 3. Electrophoretic mobility (μ/V/sec/cm) of cytochrome *c* peroxidase as a function of pH in acetate and phosphate buffers (μ = 0.1) at + 2°C. The isoelectric point of CcP was defined as pH 4.90.

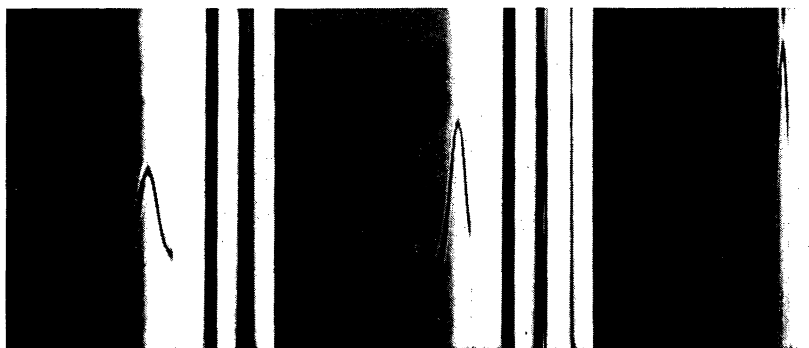

Fig. 4. Schlieren diagram of an ultracentrifuge run of cytochrome *c* peroxidase. The concentration of the enzyme was 5.38 mg/ml in phosphate buffer (pH 7.0,μ = 0.284). The photographs were taken at a phase plate angle of 45° at 4, 16, and 40 min after 59 780 rev./min were reached at 20°C. The sedimentation is from right to left.

from the orcinol-sulphuric acid test. The iron content of the preparation was found to be equal to 0.15 % per unit of dry weight corresponding to a minimal molecular weight of 37 000. The ratio E_{407}/E_{280} was found to be constant after reaching the value of 1.29. This was usually obtained after two crystallizations, naturally depending on the starting material. The enzyme was found to be stable between pH 3.5 and 9.0 as can be seen in Fig. 5.

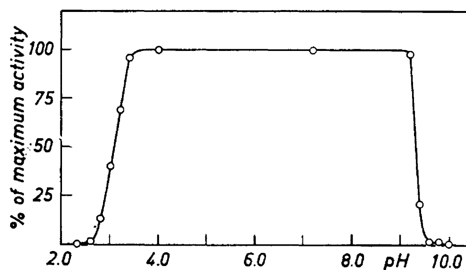

Fig. 5. Stability of cytochrome *c* peroxidase as a function of pH. The enzyme was incubated in 0.1 M acetate, phosphate and glycine buffers, respectively, for 15 min at room temperature.

DISCUSSION

The purification procedure presented in this publication involves precipitation of inactive material with basic lead acetate, acetone fractionation in the cold, column chromatography on DEAE-cellulose and finally crystallization of the enzyme at low ionic strength. Crystallinity as such was not necessarily evidence of purity of CcP, a fact which has been observed with many enzymes before. No attempts were made to obtain crystals by salting-out procedures.

The recovery of the enzyme units was about 9 % (Table 1). Thus, there must have been about 164 mg enzyme in the crude extract of 10 kg of baker's yeast comprising about 0.2 % of the total protein of the extract.

From the elution pattern (Fig. 1) it is apparent that no isoenzymes of CcP are present in baker's yeast. Crystalline CcP was found to be a single homogeneous protein as tested by sedimentation and electrophoretic procedures. However, multiple components have been demonstrated in several peroxidases. Paul [11] separated five horse radish peroxidase components by ion-exchange chromatography. Several active components have also been observed in the peroxidase preparations from sweet potato [12,13] Japanese radish,[14] turnip,[15] wheat,[16] and pea.[17]

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