Rapid Procedure for the Isolation of Cytochrome c Peroxidase

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Abstract

A simple, rapid method is described for the preparation of cytochrome c peroxidase from baker's yeast. The procedure involves lysis of the yeast in the presence of ethyl acetate, extraction of the peroxidase in 0.05 M sodium acetate buffer, pH 5.0, and the concentration of the crude extract on a DEAE-agarose column. The DEAE eluate is further concentrated by ultrafiltration, and gel filtration of the concentrate results in a highly purified form of the enzyme. Consistent yields with 80% recovery are easily obtained. Protein isolated by this method in the presence or absence of the protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), contains purely high-spin Fe(III) heme as monitored by its resonance Raman spectrum.

Introduction

Yeast cytochrome c peroxidase (ferrocytochrome c:hydrogen-peroxide oxidoreductase, E.C. 1.11.1.5) catalyzes the oxidation of ferrocytochrome c to ferricytochrome c in the presence of hydrogen peroxide [1]. It was first purified in 1940 from baker's yeast by Altschul *et al.* [2] following a lengthy procedure which did not yield consistent results [3]. Yonetani and Ray developed a chromatographic procedure [4] which was recently modified by Nelson *et al.* [5]. However, in this procedure approximately half the enzyme in the initial extract is lost due to irreversible binding to DEAE-cellulose. In our hands, Nelson's procedure yielded recovery rates of only 25% or less. Requiring large quantities of cytochrome c peroxidase for modification studies, we were able to significantly improve on the published procedures by a few simple modifications: (i) substituting cross-linked DEAE-agarose for DEAE-cellulose, (ii) extracting the enzyme in 0.05 M sodium acetate which allows the extract to be loaded directly on to the DEAE column, and (iii) concentrating the enzyme prior to gel filtration using ultrafiltration instead of a second DEAE column. These modifications permit us to recover ~80% of the peroxidase from the crude extract, and also lead to a much reduced processing time. In addition, the resonance Raman spectrum of the freshly purified protein indicates that it contains only high spin Fe(III) heme whether or not PMSF is added to the initial extract [6].

Materials and Methods

Activity Assay

Enzyme activity during preparations was assayed at room temperature by the method of Yonetani and Ray [4]. Cytochrome c (Sigma, type III) was reduced with a slight excess of $Na_2S_2O_4$ and added to the assay solution. The absorbance decrease of cytochrome c at 550 nm per 10 s per 10 μl of enzyme solution was used as an arbitrary unit of activity, and 1.0 units correspond to a 20 μM enzyme solution [4].

Purity Index

The ratio of the absorbance at 408 and 280 nm was used to check the purity of the enzyme solution at each step in the isolation procedure. The purified enzyme has a purity index of ~1.5 after recrystallization [5].

Isolation Procedure

Preparation of extract

Approximately 5 kg of fresh commercial baker's yeast (Fleishman) were dried following the procedure in ref. 5. Cold (4 °C) ethyl acetate (900 ml, reagent or technical grade) was added, and the mixture was kneaded in a fumehood until a sticky paste was obtained (**caution**: ethyl acetate is volatile and toxic and should be handled in a fumehood with the use of gloves). The mixture was covered, placed in an ice bath and allowed to lyse overnight in the fumehood. The icebath was allowed to warm up overnight and the temperature of the lysate was

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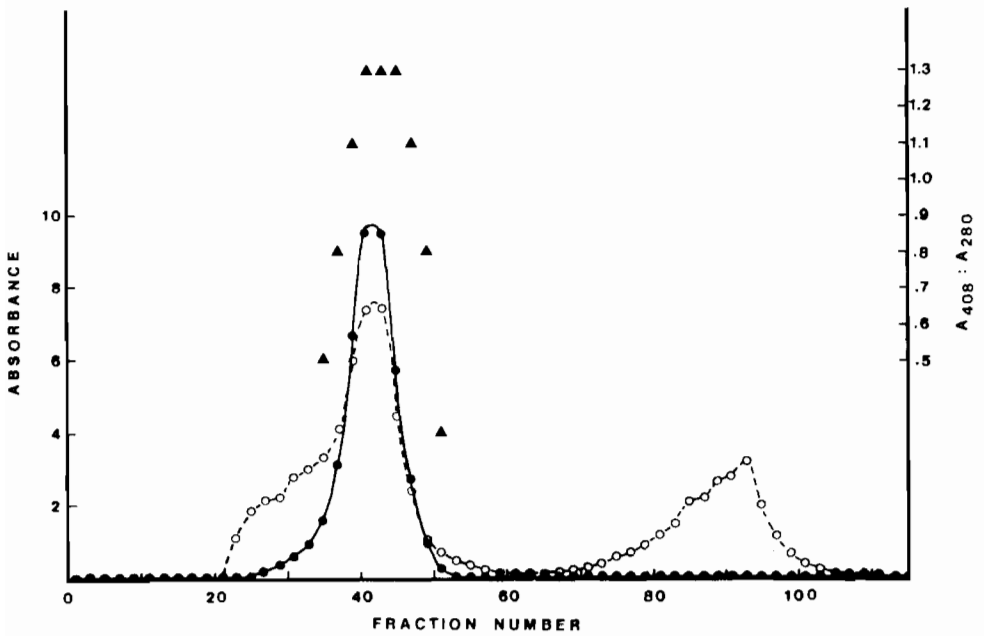

Fig. 1. Gel filtration of the cytochrome c peroxidase ultrafiltration concentrate. The eluate from the DEAE-agarose column was concentrated to 30.3 ml (A_{408}:A_{280} = 0.56; 0.24 mM) and applied to a 58 × 4 cm ϕ column of Sephadex G-75 in 0.1 M sodium acetate buffer, pH 5.0. The peroxidase was eluted with the same buffer at a rate of 60 ml/h and 7.5 ml fractions were collected. Absorbances were read at 408 (open circles, ○) and 280 nm (closed circles, ●); those fractions with A_{408}:A_{280} (triangles, ▲) ⩾ 1 were pooled (see text). The rise in the 280 nm absorbance at fraction #80 is due to the flavoprotein impurity which followed the peroxidase down the column.

~15–20 °C the following morning. The lysate was again placed in an icebath and 5 l of cold sodium acetate buffer (pH 5.0, 0.05 M, containing 1.25 mM $Na_2S_2O_5$ and 1.25 mM EDTA [5]) were slowly added with stirring. The resultant creamy extract (~9 l) was centrifuged at 3000 rpm for 20 min at 4 °C in 4 l batches in an IEC Model PR-6 centrifuge. The brownish supernatant was filtered twice in a large funnel through filter pads prepared by blending Whatman #1 filter paper with the extraction buffer.

DEAE-agarose chromatography

All chromatographic procedures were carried out in a cold room at 4 °C. The filtrate (~5 l) was loaded on to a 10 × 7.5 cm ϕ column of DEAE-agarose (DEAE-sepharose CL-6B, Pharmacia) in the extraction buffer. Initially a flow rate of 3.5 l/h was obtained but this decreased to 2 l/h towards the end of the loading process. During the time it took to load the DEAE column (~2 h) the filtrate became turbid and a substantial amount of material was deposited on top of the resin bed. The enzyme adsorbed on the top third of the resin as a dark brown band. After washing the column with 500 ml of the same buffer, the buffer head was reduced to 1 cm above the resin and the peroxidase was eluted with 0.5 M sodium acetate buffer, pH 5.0, at a flow rate of 500 ml/h. The fractions with the highest activity were pooled (~200 ml) as were those possessing marginal activity (~230 ml), and concentrated separately in a stirred ultrafiltration cell (Amicon) using a YM 10 (10000 molecular weight cut-off) filter.

Gel filtration chromatography

The combined ultrafiltration concentrates (~30 ml; 0.24 mM) were applied to a 58 × 4 cm ϕ column of Sephadex G-75 (Pharmacia) equilibrated with 0.1 M sodium acetate buffer, pH 5.0. The enzyme was eluted with the same buffer at a rate of 60 ml/h. Fractions with a purity index of ~1 were pooled (~80 ml; 0.076 mM) and frozen for storage, or crystallized by repeated dialysis against distilled water [4]; Marginal fractions with indices ⩾0.1 were added to those of previous isolations and rechromatographed. Figure 1 shows a typical elution diagram; the absorbance at 280 nm following the peroxidase elution is due to a yellow flavin band which has excitation and emission maxima at 428 and 525 nm, respectively.

TABLE I. Isolation of Cytochrome c Peroxidase from 5 kg of Baker's Yeast

Fraction	Volume (ml)	Activity[a]	Specific[b] activity	Purity[c] index	% Recovery[d]	mg CcP
Crude extract (filtered)	4950	0.09	0.04	0.059	100	300
DEAE eluate (total)	430				93	279
Main band	200	1.48	0.42	0.120	67	201
Marginal fractions	230	0.50	0.03	0.060	26	78
Ultrafiltration concentrate	30.3	12.4	0.54	0.562	85	256
G-75 eluate (total)	172				82	246
Main band	82.5	3.75	0.53	1.27	70	210
Marginal fractions	90.0	0.59	0.53	0.448	12	36

[a]Arbitrary units (see text). [b]Activity/A_{408}. [c]A_{408}:A_{280}. [d]% Recovery of activity in the crude extract.

Results and Discussion

The results obtained are summarized in Table I. Recovery rates were generally 80% or better, and the purity index of 1.27 obtained for the main G-75 fraction compares favorably with the values obtained previously [4, 5].

Contrary to the observations of Nelson *et al.* [5], the enzyme was found to be quite stable in dilute acetate buffer at pH 5.0; the lysate supernatant showed little or no decrease in activity on standing at 4 °C in 0.05 M acetate over a 2–3 day period. Consequently, 0.05 M buffer was used to extract the enzyme rather than the 0.5 M buffer used previously [5]. By keeping the buffer salt concentration low, dilution of the extract was avoided, and the volume to be loaded on the DEAE was reduced from 22 to 5 l. This volume reduction considerably decreased the time the enzyme spent in contact with the DEAE resin which is highly desirable since enzyme recovery varied inversely with contact time.

Substitution of cross-linked DEAE-agarose for DEAE-cellulose further decreased the contact time. This is due to the higher flow rates obtainable with the agarose and the lack of shrinkage of the resin bed on increasing the ionic strength. Ultrafiltration allowed us to eliminate the second DEAE column as well as increasing the purity index from 0.1 to 0.5, presumably due to the excretion of low molecular weight material in the filtrate.

During the course of this work we observed some variation in the conditions necessary to lyse the yeast with ethyl acetate. In a number of preparations carried out in the spring, we obtained activities in the range 0.12–0.16 following 4 °C lysis. However, during the summer months the activity dropped to 0.03 under the same lysing conditions. Various commercial brands of yeast were tested and similar results were obtained in each case. Examination of the ethyl acetate past under a microscope indicated that most of the yeast cells had not been lysed. Therefore, we let the paste sit overnight at 20 °C which resulted in activities of 0.10–0.18. The lysing procedure given in the experimental section is now routinely used and the average activity of the lysate is ~0.15. It should also be mentioned that irrespective of the lysing conditions, the extract showed maximum activity once the lysate was homogeneously mixed with buffer. Thus, stirring the extract for 4 h as done previously [4, 5] does not increase the activity of the extract.

Recently, a report by Ho *et al.* [7] noted that the EPR spectrum of the Fe(III) enzyme isolated in the presence of PMSF [8] did not show any evidence of low spin material. Hence, they suggested a possible correlation between damage by proteolytic attack and the presence of low spin forms of the peroxidase. In our hands the above procedure resulted in purely high spin forms of the freshly isolated protein as determined by resonance Raman spectroscopy [6], irrespective of whether or not PMSF was added to the initial extract. However, we did observe a low spin component in a sample which had been left in contact with the DEAE-agarose resin overnight instead of the usual 2–3 h [6]. Also, a number of aged samples (*i.e.*, samples stored at −10 to −30 °C for several months) contained a fraction of low spin material [6]. These results suggest that the varying amounts of low spin material observed in different preparations [1] may be due to variation in the contact time with the ion-exchange resin and/or to aging-induced changes in the protein.

Acknowledgements

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