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Note

Application of autofocusing in the isolation of peroxidase

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Previously we have drawn attention to autofocusing as a method for isoelectric focusing without carrier ampholyte, working with the compound to be separated simply dissolved in distilled water. By autofocusing two pure enzymes have been prepared so far in our laboratory: uricase (E.C. 1.7.3.3) and α-amylase (E.C. 3.2.1.1)[1,2]. Here we report on the purification of horseradish peroxidase (E.C. 1.11.1.7), the first enzyme to be isolated by this method from plant material, present mainly in horseradish, sunflower and "giant" turnip-cabbage[3–8] from which it can be purified in three separation steps.

The necessary experimental backround and interpretation of results in enzyme autofocusing have been reported elsewhere[9–11].

EXPERIMENTAL

Horseradish was selected as a source of the enzyme peroxidase. After homogenization of 500 g of horseradish root in 2000 ml of 100 m*M* phosphate buffer (pH 7.0)[7] (3 × 5 min) and centrifugation at 1000 *g* for 15 min, the supernatant was dialysed against distilled water at 4°C for 24 h. The conductivity of raw peroxidase was adjusted to 360 μS cm^{-1} by addition of distilled water.

This solution was divided into two parts and each part was purified separately. The first part was subjected to ion-exchange chromatography on a CM-cellulose column (35 cm × 2 cm I.D.). The starting solution was a 10 m*M* sodium acetate buffer made 100 m*M* with respect to sodium chloride at pH 4.4. The second eluent was 100 m*M* sodium acetate with 1 *M* sodium chloride at pH 5.4. A linear gradient was applied at a flow-rate of 15 ml h^{-1}; 6-ml fractions were collected in which the peroxidase activity was determined[12]. The active fractions were pooled and subjected to ultrafiltration with disposable Centriflo membrane cones rated at MW 50 000 (for filtrate) and at MW 25 000 (for residue).

The second portion was subjected to autofocusing; 1 l of crude peroxidase solution with a conductivity of 360 μS cm^{-1} was focused in an autofocuser (Realizing Centre of Slovak Academy of Sciences, Košice, Czechoslovakia) at 4°C for 32 h in an

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electric field of strength varying from 250 to 1000 V d.c. until the current decreased to its minimum value. The autofocused medium was then divided into twenty equal fractions in which the pH, peroxidase activity and protein concentration were determined. The fractions containing peroxidase activity were pooled and loaded on to a 62 × 3 cm I.D. Spheron P-40 column equilibrated with 0.05 *M* phosphate buffer (pH 7.0)[8]. The column was operated at 4°C at a flow-rate of 100 ml h^{-1} and 15-ml fractions were collected by an automatic fraction collector (FCC 60, Laboratorní přístroje, Prague, Czechoslovakia). All fractions were tested for their protein content and peroxidase activity and the active fractions were pooled for final evaluation.

For peroxidase activity detection 9 m*M* pyrogallol and 4 m*M* hydrogen peroxide solution was freshly prepared in 4 ml of the peroxidase solution and incubated at 30°C for 5 min. The reaction was then stopped by adding 0.2 ml of 100 m*M* potassium cyanide to the reaction mixture. The yellow-brown colour at 380 nm was measured against a blank sample[12]. The protein concentration in individual fractions was determined by the Lowry method[13].

RESULTS AND DISCUSSION

Fig. 1 shows the purification of peroxidase by ion-exchange chromatography on CM-cellulose. The course of the purification process using ion-exchange chromatography is summarized in Table I. The active peroxidase fraction after ion-exchange chromatography contained as many as six surrounding protein fractions by polyacrylamide gel electrophoresis (PAGE) (data not shown) and still three fractions after the ultrafiltration step (for the effect of ultrafiltration in peroxidase purification see Table II).

Fig. 2 shows the results of autofocusing. The bulk of proteins focused within the pH range 2.4–3.1 while the fractions containing peroxidase activity occurred between

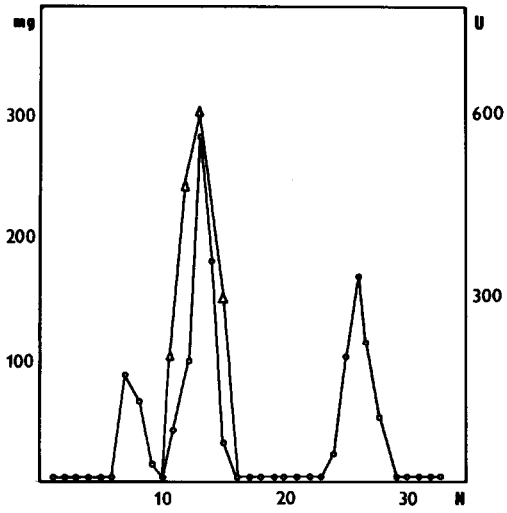

Fig. 1. Purification of peroxidase by ion-exchange chromatography. *N* = fraction number. ○, Protein concentration in mg per fraction; △, activity of peroxidase in U.

TABLE I

PURIFICATION OF PEROXIDASE BY ION-EXCHANGE CHROMATOGRAPHY AND ULTRAFILTRATION

Step	*Total protein (mg)*	*Peroxidase activity (U)*	*Specific activity (U/mg)*	*Purification*	*Recovery (%)*
Centrifugation	1320	1740	1.32	1.00	100
Ion-exchange chromatography	580	1500	2.59	1.96	86
Ultrafiltration (MW 50 000)	390	1176	3.02	2.29	68
Ultrafiltration (MW 20 000)	208	890	4.28	3.24	51

TABLE II

PURIFICATION OF PEROXIDASE BY ULTRAFILTRATION USING FILTERS LIMITING THE PROTEIN MOLECULES IN THE RANGE MW 25 000–50 000

Parameter	*MW 50 000*		*MW 25 000*	
	Residue	*Filtrate*	*Residue*	*Filtrate*
Total protein (mg)	75	390	208	166
Total activity (U)	–	1176	890	–

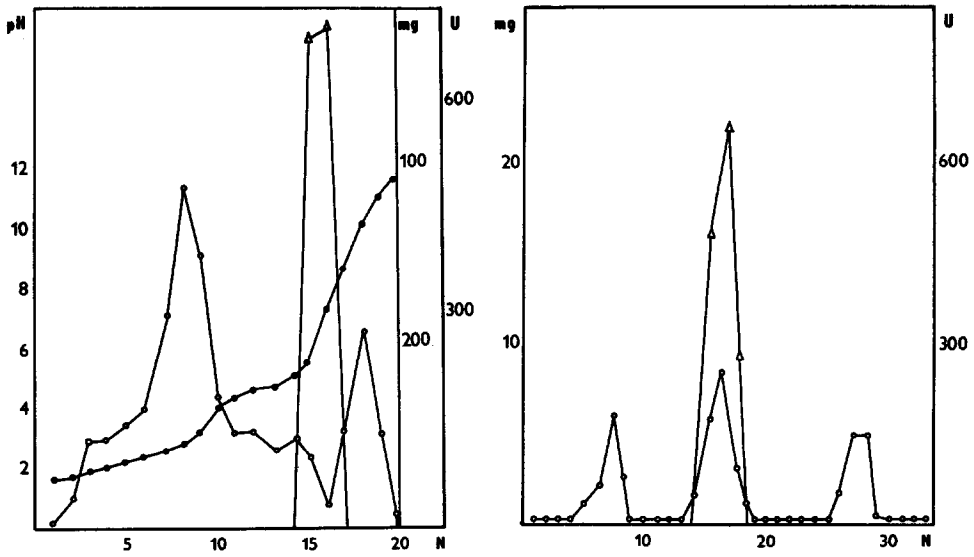

Fig. 2. Isolation of peroxidase by autofocusing. N = fraction number. ○, Protein concentration in mg per fraction; ●, pH gradient; △, activity of peroxidase in U.

Fig. 3. Purification of peroxidase after autofocusing by Spheron P 40 gel filtration. N = fraction number, ○, Protein concentration in mg per fraction; △, activity of peroxidase in U.

TABLE III
PURIFICATION OF PEROXIDASE BY AUTOFOCUSING AND GEL CHROMATOGRAPHY

Step	*Total protein (mg)*	*Peroxidase activity (U)*	*Specific activity (U/mg)*	*Purification*	*Recovery (%)*
Centrifugation	1650.00	1800	1.14	1.00	100
Autofocusing	25.50	1715	67.26	59.00	91
Spheron pool	17.20	1472	85.58	75.07	78

pH 5.45 and 7.12. Fig. 3 shows the purification of the focused peroxidase by subsequent gel chromatography. As can be seen in Table III, the specific activity of the enzyme increased 59-fold after autofocusing. At this stage four surrounding protein fractions were found by PAGE. After the gel chromatography step the specific activity increased 75-fold and the isolated enzyme was electrophoretically homogeneous (Fig. 4).

The purified enzyme was freeze-dried on addition of 25 μM glutathione. The enzyme activity of 85 U per mg protein remained unchanged for as long as 6 months.

The advantages of autofocusing over ion-exchange chromatography are obvious from the comparison of Tables I and III. While ion-exchange chromatography yields a 3.24-fold enrichment (compare also refs. 14–16), autofocusing followed by gel permeation chromatography offers a 75-fold enrichment with a very high recovery.

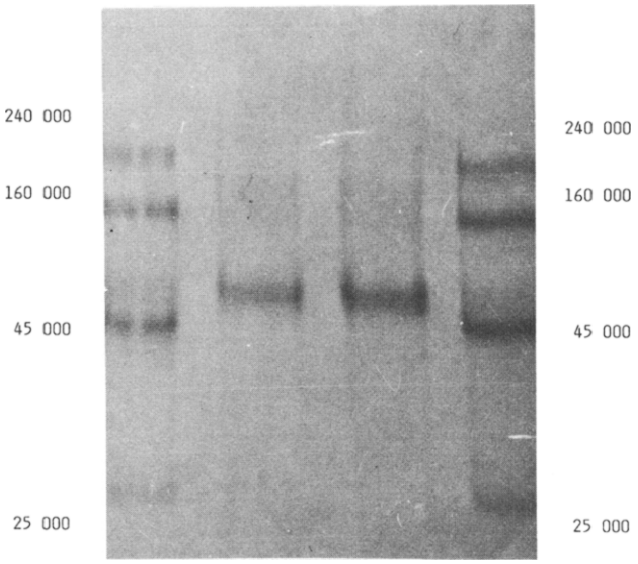

Fig. 4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of the peroxidase purified by autofocusing and gel chromatography. Lanes 1 and 4, molecular weight standards given in the margin; lanes 2 and 3, purified peroxidase.

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