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Purification of Rat Uterine Peroxidase

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Using a combination of gel filtration, affinity chromatography on immobilized concanavalin A and hydrophobic adsorption chromatography, a peroxidase has been isolated from the uteri of oestrogen-primed rats. The enzyme was purified some 306-fold with respect to the uterine extract and to >95% homogeneity. The final product had an apparent molecular weight of 48 000, an absorption maximum at 412 nm ($A_{412}/A_{280}=0.47$) and a specific activity very similar to those of several other pure haemoprotein peroxidases.

Oestrogen-induced peroxidase is a potential biochemical marker for tissues, both normal and malignant, which are dependent on this hormone for growth.[1-7] It is thus of great interest to purify and characterize this enzyme activity. McNabb and Jellinck [8] reported a purification method of the uterine peroxidase giving a 40-fold increase in specific activity and a molecular weight of about 40 000. We have previously shown that at least two separable peroxidase activities are present in the 0.5 M $CaCl_2$ uterine extract.[9] These activities have apparent molecular weights of about 92 000 and 40 000 and we have reported a partial purification of the latter activity.[9] We have now purified this enzyme some 306-fold to >95% homogeneity.

EXPERIMENTAL

Preparation of uterine extract. Twenty female Wistar rats, 160–180 g (Möllegaard, Ejby, Denmark) were given daily injections of 5 μg of oestradiol-17β in 0.2 ml sesame oil for three days. Twenty-four h after the last injection the animals were sacrificed by decapitation under ether anaesthesia. After the uteri had been removed and adhering fat dissected away, the uteri were washed well in 0.15 M NaCl, weighed and minced into small pieces. This mince was homogenized in 0.1 M potassium phosphate buffer, pH 7.4 (3% w/v tissue in buffer) using a Dounce tissue grinder. The crude homogenate was centrifuged at 37 000 *g* for 30 min at 2 °C and the resultant supernatant discarded. This washing procedure was repeated twice on the pellet and after the last centrifugation the pellet was homogenized in 0.5 M $CaCl_2$. The homogenate was centrifuged for 30 min at 37 000 *g* and 2 °C. The $CaCl_2$ extraction procedure was repeated on the pellet and the combined supernatants were dialyzed overnight at room temperature against a solution containing 0.5 M $CaCl_2$, 25% (w/v) glycerol, 0.05% (w/v) cetyltrimethylammonium bromide and 0.1 M sodium acetate buffer (pH 5.6). This solution is henceforth referred to as buffer A. Due to the high viscosity of buffer A, all subsequent purification steps were carried out at room temperature (22–24 °C).

Gel filtration. Two different columns were used: one (2.8 × 84 cm) of Ultrogel AcA34 (LKB) using a flow rate of 18 ml/h and later in the purification another column (1.6 × 74 cm) of Ultrogel AcA54 (LKB) at a flow rate of 12 ml/h. Buffer A was used as eluant and fractions of 4.8 ml were collected from the former column and 2.6 ml from the latter. When necessary, enzyme solutions were concentrated using Diaflo Ultrafiltration equipment (Amicon Corporation) with a PM-10 membrane.

Affinity chromatography. A small column (1.5 × 1.5 cm) of concanavalin A-Sepharose (Pharmacia) was first washed with ten bed volumes of buffer A without glycerol but containing 1 mM $MgCl_2$ and 1 mM $MnCl_2$. The enzyme solution was equilibrated against this solution by dialysis (2 h) and then applied to the column. The column was then washed with two bed volumes of this buffer before elution with the same buffer but containing 0.2 M 1-*O*-methyl-α-D-glycopyranoside. Fractions (3 ml) were collected. The eluted enzyme activity was then dialyzed overnight against buffer A.

Hydrophobic adsorption chromatography. The enzyme solution was applied to a column (1.5 × 2.5 cm) of phenyl-Sepharose (Pharmacia) which had been equilibrated with buffer A. After sample application,

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the column was washed with two bed volumes of buffer A and then eluted with buffer A containing 70 % (w/v) glycerol.

Enzyme assay. The peroxidase assay used was based on the method of Himmelhoch *et al.*[10] The reaction mixture (3 ml) contained guaiacol (2-methoxyphenol) (13 mM), H_2O_2 (0.33 mM) and was buffered at pH 8.0 with Tris-HCl (0.04 M) containing 0.15 M $CaCl_2$. $CaCl_2$ was omitted from the reaction mixture when samples in phosphate buffer were assayed. One unit of peroxidase activity was defined as giving an initial rate of 1 absorbance unit/min in the above system in 1 cm path length cuvettes at 23 – 24 °C. Protein was determined by the method of Schaffner and Weissmann[11] with bovine serum albumin as standard.

Polyacrylamide gel electrophoresis. Sodium dodecylsulfate polyacrylamide gel electrophoresis studies were carried out according to Weber and Osborn[12] using 10 % gels. Before application, the samples were boiled for 15 min in the presence of 2-mercaptoethanol. The molecular weight was estimated by calibrating the gel with standard proteins (Electrophoresis calibration kit, low molecular weight proteins, Pharmacia). The gels were stained according to Holbrook and Leaver[13] and also inspected using a Gilford 2400-S spectrophotometer with gel scanning attachment. Analytical polyacrylamide gel electrophoresis was carried out as described previously.[9]

Purification of enzyme. The peroxidase activity was extracted from the uteri of 20 rats using 0.5 M $CaCl_2$ and the solubilized activity then dialyzed against buffer A and concentrated by ultrafiltration. The peroxidase solution was then chromatographed on Ultrogel AcA34. The elution profile (not shown)

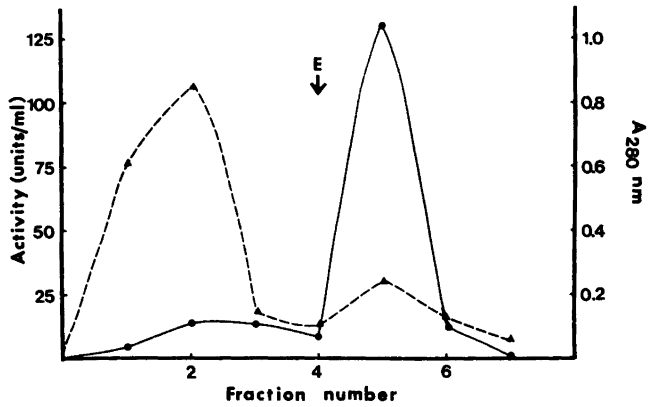

Fig. 1. Affinity chromatography of uterine peroxidase on concanavalin A – Sepharose. E indicates start of elution. Fractions were tested for absorption at 280 nm (– – –) and peroxidase activity (——).

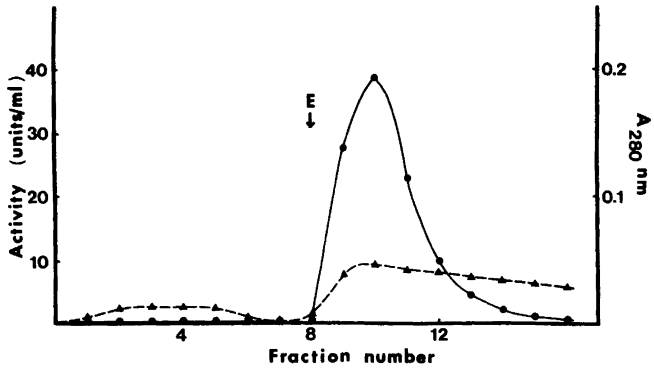

Fig. 2. Hydrophobic adsorption chromatography of uterine peroxidase on ohenyl – Sepharose. E indicates start of elution. Fractions were tested for absorption at 280 nm (– – –) and for peroxidase activity (——).

Table 1. Purification of rat uterine peroxidase. The uteri of 20 oestradiol-treated rats were used.

Step	Total volume/ml	Total activity/units	Total protein/mg	Specific activity/units mg^{-1}	Recovery/%
Homogenate	230	184	448.5	0.41	–
0.5 M $CaCl_2$ extract	165	2146	127.9	16.8	100
Gel filtration (AcA34)	70	683	13.0	52.5	31.8
Concanavalin A – Sepharose	5.5	382	0.84	455	17.8
Phenyl – Sepharose	7.5	317	0.2	1585	14.8
Gel filtration (AcA54)	2.0	180	0.035	5143	8.4

resembled the one we have published previously for the uterine extract.[9] Fractions corresponding to the low molecular weight were pooled and concentrated to 5 ml and further purified on immobilized concanavalin A (Fig. 1). During loading and washing, some leakage of peroxidase activity from this column was noted. This effect may be due to leakage of the ligand itself from the column.[14,15] The enzyme was further purified by hydrophobic adsorption chromatography (Fig. 2). As a final step, gel filtration on Ultrogel AcA54 was used to separate the enzyme from a small amount of high molecular weight material (not shown). Fractions containing enzyme activity were pooled and concentrated and stored at −20 °C.

RESULTS AND DISCUSSION

Table 1 summarizes the results of a typical purification. The overall recovery of activity relative to the total measured peroxidase activity in the uterine extract is only 8.4%. It should, however, be remembered that the uterine extract contains more than one peroxidase activity so that the recovery of the present peroxidase enzyme will be greater than the overall recovery of peroxidase activity. Another point of interest is that the total measured peroxidase activity in the initial uterine homogenate is only some 10% of that in the subsequent extract of this homogenate. Similar results have been reported with mammary tumour peroxidase.[16]

The present purification results in a 306-fold increase in specific activity relative to that of the uterine extract, a value considerably higher than those reported previously.[8,9] Upon concentration of the enzyme obtained after the final gel filtration, a slightly brown-coloured solution resulted. The sample had an absorption maximum at 412 nm (Fig. 3) and the A_{412}/A_{280} was calculated to be 0.47. These results are consistent with previous indications that the uterine peroxidase is a haemoprotein.[9]

The final product appeared to be very nearly homogeneous in sodium dodecylsulfate polyacrylamide gel electrophoresis (Fig. 4). One strongly staining band was observed, but two other very faint bands were just discernable. Photodensitometric scanning of the gel indicated >95% homogeneity. The apparent molecular weight of the major band in this system was 48 000. In analytical gel electrophoresis a single protein-stanining band which coincided with the single peroxidase activity band was also found (not shown). Analytical disc gel electrophoresis may not, however, be a reliable guide to the purity of this enzyme. In our previous partial purification, the final product which had a specific

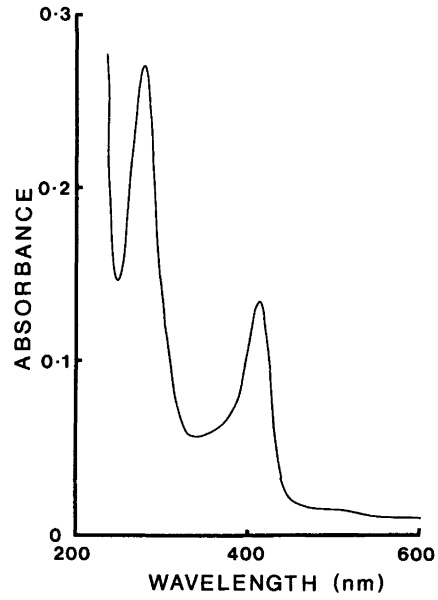

Fig. 3. Absorption spectrum of purified uterine peroxidase. Enzyme (0.137 mg/ml) was dissolved in buffer A.

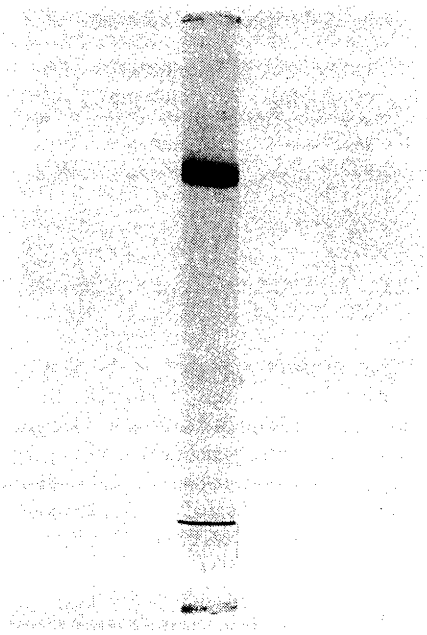

Fig. 4. Gel electrophoretic pattern of the final product after reduction and denaturation run in the presence of sodium dodecylsulfate. Protein (8 μg) was applied to the gel. The metal string indicates the position of the marker.

activity of only 1.6 % of the present purified enzyme appeared almost homogeneous in analytical gel electrophoresis; the mobilities of the present and the previous enzyme preparations were also identical in this system.

Using the extinction coefficient for the oxidation product of guaiacol published by Alexander,[17] the specific activity of the final product from the present purification scheme becomes approximately 44 000 μmol/min (μmol enzyme). This is very similar to the specific activities shown for guaiacol by highly purified samples of thyroid peroxidase, lactoperoxidase and horseradish peroxidase (specific activities 50 000, 34 000 and 32 000 μmol/min(μmol enzyme), respectively).[17] This is further evidence for the proposed classical haemoprotein nature of this peroxidase.[9]

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