Isolation and Purification of Rat Mammary Tumor Peroxidase

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Abstract

7,12-Dimethylbenz(a)anthracene-induced rat mammary tumors often contain high levels of the enzyme peroxidase, a putative marker of estrogen dependence. This enzyme can be effectively extracted with 0.5 M CaCl₂, giving rise to a soluble peroxidase with a molecular weight of about 50,000 as determined by gel filtration. This is the same size as the estrogen-induced peroxidase of rat uterus but smaller than other mammalian peroxidases. Further purification of the rat mammary tumor peroxidase by concanavalin A-Sepharose chromatography and hydrophobic interaction chromatography on phenyl Sepharose provides a 640-fold purification of the enzyme.

Introduction

Knowledge of the estrogen receptor content of human breast cancer is of considerable value in the management of breast cancer patients. It is generally found that the majority of the patients whose metastatic breast lesions contain significant amounts of estrogen receptor, called estrophilin, obtain objective remissions to endocrine therapy, while few patients whose cancers lack notable amounts of estrophilin obtain such benefit (18, 22). Although the success of prediction of response of breast cancer patients to endocrine therapy based on the tumor estrophilin content can be 80 to 90% (18), only about 60% of the patients with estrophilin-rich tumors respond to such therapy. Thus, cytosol estrogen receptor appears to be a necessary, but not sufficient, condition for breast cancer response. Efforts to improve the predictability of response in patients with estrophilin-rich lesions have involved assay of other parameters such as nuclear estrogen receptor (10) and progesterone receptor (23) in addition to cytosol estrophilin. On the basis of studies in the DMBA-induced rat mammary tumor system, we proposed that the enzyme peroxidase (EC 1.11.1.7) could be a marker of hormone dependence in breast cancer (2, 6). A preliminary report (9) indicates that most of the estrogen receptor-positive breast cancers had measurable peroxidase activity while few receptor-negative lesions had peroxidase. One of the problems associated with the enzymatic assay for peroxidase activity in tumors is that the presence of inhibitors, such as catalase which can compete for the substrate, H₂O₂, or mercaptans or ascorbic acid, can lead to erroneous peroxidase assay results. Such problems could be alleviated by assaying for enzyme protein by immunochemical methods. Preparation of antibody to breast cancer peroxidase could also be useful to determine whether the enzyme protein was present in the circulation of patients with peroxidase-containing metastatic breast lesions. As a prototype for an isolation of the enzyme from human breast cancer tissue and to provide a tool to elucidate the nature of the hormonal regulation of peroxidase in the animal model system, we have partially purified the peroxidase from DMBA-induced rat mammary tumors.

Materials and Methods

Animal Tumors. DMBA tumors were induced in 50-day-old female Sprague-Dawley rats by a single i.g. feeding of 20 mg DMBA in 2 ml of sesame oil as previously reported (7). MTW9A tumors were grown in female inbred Wistar-Furth rats. For transplantation of these tumors, a fine mince of the tumor was suspended in 0.9% NaCl solution (500 mg/ml), and 0.2 ml of the suspension was injected s.c. at each site.

Extraction of Peroxidase Activity. Tumor-bearing rats were killed by decapitation, and tumors were quickly removed, cleaned of any extraneous tissue, weighed, and minced with scissors. The mince was homogenized at a concentration of 50 mg/ml with 10 mM Tris-HCl buffer, pH 7.2, with a Polytron PT 10ST homogenizer at a setting of 6 and ice-bath cooling. After a sample was removed for DNA analysis, the homogenate was centrifuged at 40,000 x g for 40 min at 2° with a SM-24 rotor in a Sorvall RC-2B centrifuge. The resulting supernatant contained very little peroxidase activity and was discarded. The pellet was rehomogenized in Tris buffer, pH 7.2, containing 0.5 mM CaCl₂, the homogenate was centrifuged as before, and the resulting supernatant was assayed for peroxidase activity. Small pieces of human uterus, obtained at hysterectomy, were treated and extracted in the same way.

For extraction of peroxidase activity of human breast cancer specimens, the 250,000 x g pellets obtained from the preparation of a particulate-free cytosol fraction for estrogen receptor assay (18) were used. Since the original homogenization buffer contained 0.5 mM dithiothreitol, an inhibitor of peroxidase, a modified extraction procedure was used. For this purpose the pellets were washed by rehomogenization in 10 mM Tris-HCl buffer, pH 7.2, containing 2 mM NEM, followed by centrifugation. The NEM-washed pellets were then extracted and assayed as described for the rat tumors except that 2 mM NEM was included in all buffers.

Peroxidase Assay. Peroxidase activity was assayed by measuring the increase in absorbance at 470 nm due to the oxidation of guaiacol. The assay mixture contained 0.3 mM H₂O₂ and 13 mM guaiacol in a total volume of 3.0 ml of the extraction buffer (10 mM Tris-HCl, pH 7.2, containing 0.5 mM...
CaCl₂). The assays were performed at 25° and were started by the addition of the enzyme. Initial rates were measured over the first minute of the reaction. A unit of peroxidase activity was defined as the amount giving an initial rate of 1 absorbance unit/min under the assay conditions described here.

Mammary Tumor Peroxidase Purification. Ten DMBA-induced mammary tumors (about 30 g) were selected, based on the peroxidase content derived from assay of aliquot portions, from a group of 34 tumors from intact rats given 5 µg estradiol 20 hr previously. The remaining portions of these tumors were extracted with 0.5 M calcium chloride as described, except with 100 mg of tissue per ml, and the extracts were combined. A total of 365 ml of 0.5 M CaCl₂ tumor extracts (2190 units) were pumped at about 15 ml/hr onto a 46-ml bed volume of Con A-Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) which had been packed and washed at 4° with the 10 mM Tris-0.5 M CaCl₂ extraction buffer, pH 7.2. The Con A-Sepharose fractionation was conducted at 4°. The column was washed with 200 ml of the same buffer following which the elution buffer was changed to 2.5 M CaCl₂-0.5 M α-methylmannoside in 10 mM Tris, pH 7.2. The pooled eluted peroxidase activity (Fractions 221 to 231) consisted of 1320 enzyme units (60.3% of the total). Twenty-seven ml (1188 units) of the Con A-Sepharose-purified peroxidase were placed on a 34-ml bed volume column of phenyl Sepharose (Pharmacia) at a flow rate of 20 ml/hr. The phenyl Sepharose column was run at room temperature since better recoveries of enzyme were obtained at room temperature than in the cold. The column was washed with 2 column volumes of 0.5 M CaCl₂ in 10 mM Tris buffer, pH 7.2, followed by 250 ml of 35% ethylene glycol in the same buffer. The phenyl Sepharose column size was decreased from 3 to 1.4 ml) to elute the enzyme. The major peak of enzyme, Fractions 193 to 210, provided 463 units of enzyme (39% recovery), 643-fold purified relative to the initial homogenate specific activity.

Molecular Weight Determination. A Sephadex G-200 column, 2.5 x 33 cm, was equilibrated at 4° with 10 mM Tris-0.5 M CaCl₂ buffer, pH 7.2. The column was calibrated with the following proteins as standards: γ-globulin, bovine serum albumin, horseradish peroxidase, myoglobin, and cytochrome c. Nonenzymic proteins were detected by the absorbance at 280 nm, and the peroxidase activity of the tissue extracts and horseradish enzyme were detected by the guaiacol assay. Elution in all cases was with 0.5 M CaCl₂ in 10 mM Tris-HCl, pH 7.2, at a flow rate of 6 ml/hr.

Results

The range of peroxidase content in normal and neoplastic endocrine-responsive tissues of the rat and human are quite similar (Table 1). In the endocrine-deprived rat (immature or ovariectomized), we find that peroxidase levels of both the uterus and mammary tumor are near zero but that administration of estrogen for several days can induce levels of over 100 units of enzyme per g of tissue as are seen in intact animals. Assay of about 12 MTW9A mammary tumors from mature rats given 5 µg estradiol 20 hr before tumor excision showed more moderate, but still appreciable, peroxidase levels, in the range of 5 to 6 units/g. Separation of endometrium in some samples of human uterus indicated that the high peroxidase levels probably arise from the epithelial and glandular tissue elements. Although a modified extraction and assay procedure had to be used for the human breast cancer samples because the tissue was homogenized in the presence of dithiothreitol for steroid receptor assay (18), about 30% of the samples had appreciable (i.e., >1 unit/g) peroxidase content and a few had over 100 units of enzyme per g of tissue.

In order to purify the rat mammary tumor peroxidase, we first studied various methods of solubilization of the enzyme. As previously reported (6) this enzyme is associated with the endoplasmic reticulum and nuclear membrane of the cell. Like the enzyme of the uterus (20), the rat mammary tumor peroxidase was best solubilized with calcium chloride. As shown in Chart 1, a sharp increase in enzyme
yield and specific activity occurred at about 0.6 M calcium chloride. Although a little more enzyme could be extracted with 1 M CaCl₂, further increases in salt concentration only reduced the specific activity of the solubilized enzyme due to increased extraction of other proteins.

The mammary tumor peroxidase appears to have a molecular weight of about 50,000. As shown in Chart 2, the elution position of the calcium chloride-extracted mammary tumor enzyme from a calibrated Sephadex G-200 column was similar to the rat uterine peroxidase (20), but significantly smaller than other reported mammalian peroxidases such as thyroid (1) and myeloperoxidase (11).

Since both plant and animal peroxidases have been reported to be carbohydrate-containing proteins, several lectin affinity adsorbents were tested with a view to purification of the enzyme. Con A showed remarkable affinity for the mammary tumor peroxidase and was found to provide a highly effective purification step (Chart 3). While the majority of the protein (judged by 280 nm absorption) did not absorb to Con A-Sepharose 4B, more than 95% of the peroxidase activity was adsorbed. The enzyme could be eluted in about 60% yield with α-methylmannoside in 2.5 M CaCl₂. The specific eluant, α-methylmannoside, was required for elution of peroxidase. The 2.5 M CaCl₂ was included so that the eluted enzyme would be in an optimal ionic environment for hydrophobic interaction chromatography as the next step. Other studies had indicated that peroxidase exhibited a strong hydrophobic interaction with the agarose. It was found that this phenomenon could be used to advantage for purification. As shown in Chart 4, hydrophobic interaction chromatography on phenyl Sepharose 4B effected further purification of the Con A-Sepharose-purified enzyme. The partially purified tumor peroxidase adsorbed to phenyl Sepharose, while a major portion of extraneous protein passed through the column and was eluted by lowering the calcium concentration to 0.5 M. Additional protein was eluted when 35% ethylene glycol was added to this buffer. Elution with 50% ethylene glycol provided the highly purified peroxidase in about 39% yield.

As shown in Table 2, the reported sequence of purification steps provided a significant purification of mammary tumor peroxidase, 643-fold overall, with a good recovery. Solubilization with calcium chloride actually provided increased total enzyme over that assayable in the homogenate so that the recovery was based on the extracted enzyme. This increased enzyme activity does not appear to be due to catalase activity present in the homogenate. Most effective was the Con A-Sepharose step which gave over 20-fold purification with 60% recovery of peroxidase.
Discussion

There is now an accumulating body of evidence that the enzyme peroxidase is a meaningful marker of estrogen-induced growth response of reproductive tissues (2, 4, 5, 12, 13, 14–16, 19–21). Although absent in the immature or castrated rat, treatment with estrogen induces peroxidase in the uterus, vagina, and oviduct but not the pituitary (2, 15, 20). Uterine peroxidase induction by estrogen is dose dependent, is effected by various estrogens, and relates to the relative efficacy of the estrogens to induce uterine growth (16, 20). Similarly, the extent of the estrogen induction of uterine peroxidase in various species relates to the uterine growth sensitivity to estrogen (15). In the immature rat following a single dose of estradiol, uterine peroxidase is first detected at about 4 hr, increases to a maximum level at about 20 hr, and decreases thereafter (20). The estrogen induction of uterine peroxidase is inhibited by actinomycin D and cycloheximide (20), suggesting that new RNA and protein syntheses are required. Antiestrogens also inhibit the estrogen induction of uterine peroxidase (20, 24), and the time dependence of the antiestrogen effect suggests that this inhibition is due to depletions of the cytoplasmic content of estrogen receptor by antiestrogen (8).

Peroxidase has also been identified histochemically in the rat mammary gland (3). As shown in this paper and elsewhere (2, 6, 17), the rat mammary tumors often contain appreciable amounts of peroxidase. This tumor peroxidase, like the uterine enzyme, appears to be hormone dependent since it disappears from the tumor following ovariectomy of the host. Recent studies have indicated that significantly increased levels of peroxidase are found in the hormone-dependent GR mouse tumors compared with their independent counterparts.4 High levels of peroxidase are found in about 30% of human breast cancer samples (19), and current studies in our laboratory are attempting to correlate peroxidase, cytoplasmic estrogen receptor, and patient response to endocrine therapy. Since peroxidase activity is inhibited by azide and mercaptans, compounds in general use for tissue preparation for receptor studies, modified methods are required for assay of both steroid receptors and peroxidase in the same sample of tissue. The inhibitory effect of mercaptans can, however, be reversed by NEM. Immunochemical detection of peroxidase would eliminate these difficulties but requires pure tumor peroxidase.

For elucidation of the nature of the hormonal regulation of peroxidase in the rat mammary tumor, it will also be necessary to be able to detect enzyme protein as well as enzyme activity. The enzyme appears to be a carbohydrate-containing heme protein like other reported peroxidase enzymes. Thus translation of peroxidase mRNA in vitro would not be expected to give rise to active enzyme since such a product would lack the porphyrin necessary for enzymatic activity. This product could be detected with a specific antibody to the protein portion of the enzyme. Preparation of such an antibody, however, requires highly purified enzyme. As shown in this paper, a significant purification of mammary tumor peroxidase can be accomplished by solubilization of the enzyme with calcium chloride and by taking advantage of 2 interesting properties of the enzyme, namely, its carbohydrate content and its hydrophobic character. The procedure described, including solubilization and chromatography on Con A-Sepharose followed by phenyl Sepharose, provided over 600-fold purification of the tumor enzyme. Final purification, with the use of classical techniques such as gel filtration and electrophoresis, and antibody production are in progress.

Acknowledgments

We are grateful for the excellent technical assistance of Richard Garay and Restituto Dizon.

References


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