Changes in substrate specificity of native and recombinant horseradish peroxidase under ionizing radiation

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The homogeneous recombinant horseradish peroxidase preparation from *E. coli* inclusion bodies exhibits higher specific activity towards ammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) than the native one. The differences in substrate specificity can be assigned to the native enzyme inactivation in the course of metabolic reactions in living plant cells, while the recombinant enzyme reconstructed *in vitro* completely realizes the original catalytic abilities. Application of the method of radiation-induced inactivation demonstrates the existence of different binding sites for the iodide anion, ABTS, phenol, and guaiacol and allows one to assume a common character of the binding sites of phenol and *o*-phenylenediamine.

Key words: recombinant horseradish peroxidase; inclusion bodies; guaiacol; ammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS); *o*-phenylenediamine; radiation-induced inactivation.

Horseradish peroxidase (HRP) is widely used in immunoenzymatic analysis and is one of the most studied plant peroxidases. This enzyme belongs to heme-containing peroxidases (EC 1.11.1.7) and catalyzes oxidation of many electron donors with hydrogen peroxide; the latter undergoes heterolytic cleavage in the active site with formation of so-called "Peroxidase compound I" (the oxyferryl-enzyme with the π-radical cation at the porphyrin ring). The transfer of the oxidation equivalent to the substrate proceeds in two one-electron stages (iodide and hydrosulfite ions being two-electron donors are exceptions). The literature data do not allow one to give an unequivocal answer on the role of the definite amino acid residues in the electron transfer from the substrate to heme, while some hypotheses about differences in localization of the substrate-binding sites of the iodide ion, guaiacol, and thioanisol were claimed.[1] Advances[2,3] in the HRP gene expression in *E. coli* allow one to prepare the recombinant non-glycosylated enzyme and open new possibilities in studies on the structure—property relationships using the methods of protein engineering. The first step of these studies is a comparative analysis of the properties of native and recombinant horseradish peroxidases (NHRP and RHRP, respectively), in particular, their substrate specificity, in order to estimate the degree of damages resulting from inactivation of the native enzyme under the action of radicals of the oxidized substrate in the process of its physiological functions *in vivo*.

The aim of our work was to characterize the catalytic properties of both enzymes with respect to a broad range of electron donors and to study the sensitivity of the substrate-binding sites to the damaging action of radiation. To some extent, radiation is a model of the radical character of peroxidase inactivation in the course of the reaction.

Results and Discussion

The procedure previously developed by us[3] and improved in the present work allows us to prepare from *E. coli* inclusion bodies *ca.* 25 mg of RHRP of *ca.* 4000 E* specific activity per 1 mg of protein in 1 L of culture. The characteristics of this process are presented in Table 1.

The homogeneity of the obtained preparation was confirmed by electrophoresis in the presence of sodium dodecylsulfate (SDS). RHRP appeared to be more active than NHRP; this phenomenon may be assumed on the basis of literature data on inactivation of NHRP in the course of the reaction. A study of the oxidation of ammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) by the methods of stationary kinetics in a wide range of concentrations of both substrates indicated that the elimination of the oxidized substrate

* One unit of enzymatic activity (E) is equal to the amount of enzyme (in mg) which catalyzes transformation of 1 μmol of substrate per min under the given conditions (see Ref. 4).

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Table 1. Preparation and purification of RHRP

Steps of purification*	Total protein, *m*/mg	Total activity/E	Specific activity/E mg^{-1}	Yield on activity (%)	Reactivation of protein (%)
1	20	0	0	0	—
2	20	20000	1000	100	—
3	20	24000	1200	120	—
4	5	20000	4000	100	25

* 1: Solubilization of apo-RHRP in 6 *M* urea; 2: reactivation; 3: precipitation with ammonium sulfate; 4: gel filtration.

Table 2. Apparent rate constant of oxidation of ABTS with hydrogen peroxide catalyzed by NHRP and RHRP

Rate constant	RHRP		NHRP
	Our data	See Ref. 4	
$k_1/\mu M^{-1}$ s^{-1}	4.8	5.9	3.5
$k_3/\mu M^{-1}$ s^{-1}	3.0	3.7	3.5
k_4/s^{-1}	4600	850	—

Note. 25 °C, 0.1 *M* sodium acetate buffer, pH 5.0, relative error ≤5 %.

became the rate-limiting step for the recombinant enzyme (Table 2). This fact confirms the data previously obtained.[5] Thus, the structures of the active site of the recombinant and native enzymes may differ to some extent. The kinetic data presented in double reciprocal coordinates (concentration of the substrate—rate) have the appearance of a family of parallel straight lines described by the equation:[5]

$$2[E]_0/v = 1/k_1[H_2O_2] + 1/k_3[ABTS] + 1/k_4,$$

where k_1 and k_3 are the apparent rate constants of H_2O_2 cleavage and oxidation of ABTS, and k_4 in the case of RHRP characterizes the rate-determining stage, which may be the dissociation of the oxidation product,[5] v is the rate of the enzymatic reaction. In the case of RHRP, k_1 is higher than that in the case of NHRP, and k_3 are quite equal.

Inactivation of the peroxidase in the course of the reaction is caused by modification of the protein component and heme with radicals of the oxidized substrate. Application of low-dose radiolysis of the peroxidase solutions may give, to some extent, a model of the radical inactivation process; however, in this case the radicals are generated inside the protein globule, not in the active site. The radiation-induced inactivation of the RHRP solution (Fig. 1) clearly demonstrates the differences in binding sites of the iodide ion, guaiacol, ABTS, and phenol. Here, the differences in binding sites are related to both their spatial localization and the complexity of the mechanism of the electron transfer from the substrate to the enzyme, primarily, the length of the electron-transfer chain, *i.e.*, the amount of the amino acid residues involved in electron transfer. From this point of view, the character of interaction of the reaction site is the most complex for oxidation of ABTS and simplifies for guaiacol, phenol, and the iodide anion. The latter is two-electron donor, which is oxidized in one stage and directly interacts with the δ-meso-ring of heme.[1]

If radiation-induced inactivation is actually a model of the process *in vivo*, the ratios of activities with respect to guaiacol, phenol, and the iodide anion to that with respect to ABTS must be higher for the native enzyme than for the recombinant one. In fact, this phenomenon was observed in the experiment (Table 3). Moreover, for NHRP, the ratios of activities with respect to phenol and *o*-phenylenediamine to that of ABTS increases in proportion to each other, which may

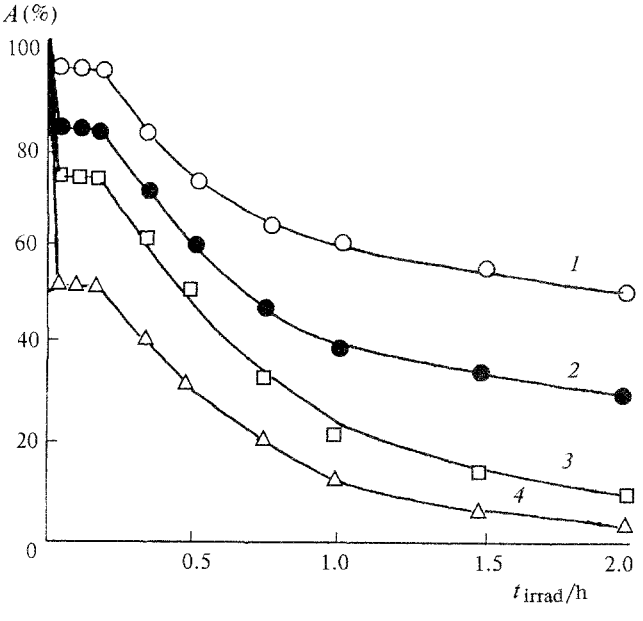

Fig. 1. Inactivation of recombinant horseradish peroxidase (10^{-7} *M* in 0.1 *M* Tris-HCl buffer, pH 8.0) under ionizing irradiation. The activity was measured with respect to the following substrates: potassium iodide (*1*); phenol/antipyrine (*2*); guaiacol (*3*); ABTS (*4*); P_γ = 0.05 Gy s^{-1}.

Table 3. Catalytic properties of native and recombinant horseradish peroxidases

HRP	Mol. wt./kDa	Specific activity with respect to ABTS/E mg^{-1}	Activity with respect to the other substrates in % of activity with respect to ABTS				
			ABTS	Guaiacol	Phenol	*o*-Phenylenediamine	Iodide
NHRP	44	2000	100	43	59	73	20
RHRP	34	4000	100	20	20	28	9

attest to the similarity of the mechanisms of oxidation of the substrates. The study of radiation-induced inactivation of the native peroxidase (Fig. 2) confirms this assumption, because the activities in both cases decrease in the same manner.

The activity of NHRP with respect to guaiacol and ABTS (see Fig. 2) is more resistant to radiation than that of RHRP. There are probably several reasons for the observed differences in catalytic properties of the native enzyme and the recombinant enzyme. If the resistance of the NHRP reaction sites of ABTS and guaiacol is related to their lower accessibility than that of the reaction site of phenol, and for the recombinant non-glycosylated enzyme the reciprocal dependence is true, one can assume that glycosylation of the native enzyme strongly distorts the structure of the enzyme around the active site, shielding the binding sites of guaiacol and ABTS and defolding the binding site of phenol. It is also necessary to take into account that reactivation by the described method may result in corrections in the conformation of the RHRP active site.

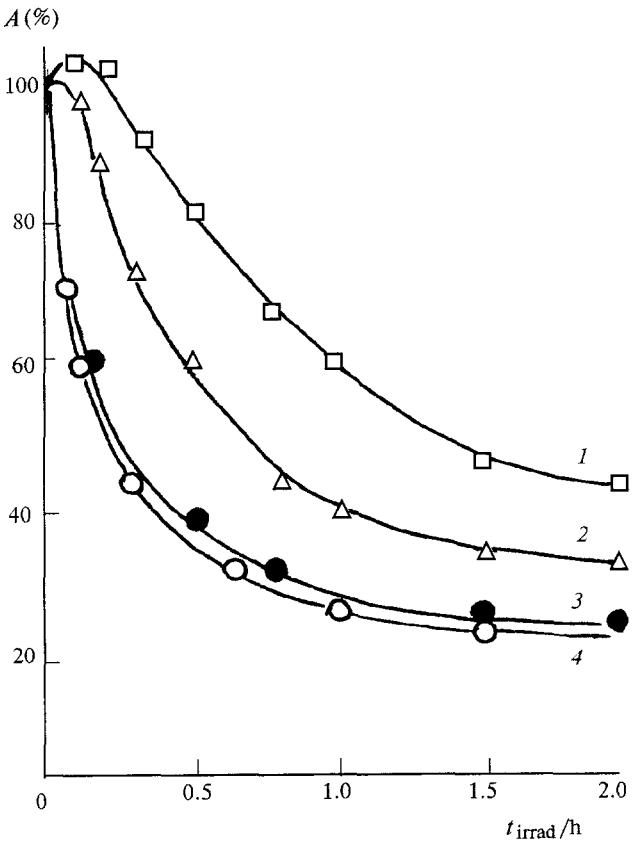

Fig. 2. Inactivation of native horseradish peroxidase (10^{-7} *M* in 0.1 *M* Tris-HCl buffer, pH 8.0) under ionizing irradiation. The activity was measured with respect to the following substrates: guaiacol (*1*); ABTS (*2*); phenol/antipyrine (*3*); *o*-phenylenediamine (4); $P_\gamma = 0.05$ Gy s^{-1}.

It is most probable that in the living process in the cell the radical modification of the reaction sites of oxidation of ABTS and guaiacol is "exhaustive" and the further radical attack in the first minutes of radiolysis does not cause additional changes in the active site. The existence of the intermediate conformation resistant to radiation in the dose range 15—45 Gy (corresponding to the plateau in Fig. 1) also confirms this assumption. Under prolonged irradiation, stochastic modification of the amino acid residues proceeds, thus resulting in changes in the conformation of the molecule, which lead to destruction of the catalytic site and, hence, the total loss of catalytic activity.

Thus, the application of the method of radiation-induced inactivation for the study of substrate specificity of native and recombinant horseradish peroxidases allows one to assume structural differences in the active sites of the enzymes and to demonstrate the existence of differences in the reaction sites of the iodide anion, guaiacol, phenol (*o*-phenylenediamine), and ABTS. In addition, a more complex mechanism of the process of electron transfer from substrate to enzyme in the case of guaiacol and ABTS in comparison with phenol and the iodide anion was observed.

Experimental

ABTS, 4-aminoantipyrine, *o*-phenylenediamine, guaiacol, isopropyl-β-D-thiogalactopyranoside (IPTG), sodium dodecylsulfate (SDS), oxidized glutathione, dithiothreite (DTT), hemin (Sigma, USA), bactotripton, and yeast extract (Difco), horseradish peroxidase (Biozyme, USA) were used in the present work. The original plasmide pSA261 with the HRP gene under control of the tac-promotor was kindly supplied by Amersham International plc, Great Britain.

The measurements of the peroxidase activities with respect to the different substrates were performed with a Shimadzu UV 120-02 spectrophotometer (Japan) at 25 °C in accordance with the following procedures.

ABTS. An ABTS solution (0.05 mL, 8 mg mL^{-1}) and an aliquot of the enzyme were added to 2 mL of 0.1 *M* Na-acetate buffer (pH 5.0), the reaction was initiated by addition of a 0.5 % solution of hydrogen peroxide (0.1 mL). The extinction coefficient at 405 nm was accepted equal to 36800 L mol^{-1} cm^{-1} (Ref. 6).

Guaiacol. A solution of guaiacol in water (0.15 mL, 1 mg mL^{-1}) and an aliquot of the enzyme were added to 0.1 *M* Na-acetate buffer (pH 5.0) (2 mL), the reaction was initiated by the addition of a 0.5 % hydrogen peroxide solution (0.1 mL). The extinction coefficient at 436 nm was accepted equal to 25500 L mol^{-1} cm^{-1} (Ref. 7).

Phenol/antipyrine assay. A solution of phenol (0.5 mL) and 4-aminoantipyrine (16.2 and 0.5 mg mL^{-1}, respectively) and an aliquot of the enzyme were added to 0.1 *M* K-phosphate buffer (pH 7.0) (1.5 mL), the reaction was initiated by addition of 0.5 % hydrogen peroxide solution (0.1 mL). The extinction coefficient at 510 nm was accepted equal to 6580 L mol^{-1} cm^{-1} (Ref. 8).

***o*-Phenylenediamine.** A solution of *o*-phenylenediamine (0.05 mL, 16 mg mL^{-1}) and an aliquot of the enzyme were added to 0.1 *M* Na-acetate buffer (pH 5.0) (2 mL), the reaction was initiated by addition of a 0.5 % hydrogen peroxide solution (0.1 mL). The extinction coefficient at 445 nm was accepted equal to 11100 L mol^{-1} cm^{-1} (Ref. 9).

The iodide anion. A solution of KI (0.05 mL, 11.6 mg mL^{-1}) and an aliquot of the enzyme were added to 0.1 *M* Na-acetate buffer (pH 5.0) (2 mL), the reaction was initiated by addition of a 0.5 % hydrogen peroxide solution (0.1 mL). The extinction coefficient at 350 nm was accepted equal to 26000 L mol^{-1} cm^{-1} (Ref. 10).

The activity was presented in E (μmol min^{-1}) per 1 mg of a protein.

The determination of the apparent rate constants for NHRP and RHRP was performed on the basis of the data on stationary kinetics of oxidation of ABTS with hydrogen peroxide at the following concentration ranges: $(0.015—0.15) \cdot 10^{-3}$ *M* of ABTS, $(0.01—0.1) \cdot 10^{-3}$ *M* of hydrogen peroxide, and $1 \cdot 10^{-9}$ *M* of the enzymes.

The samples of the aqueous solutions of the peroxidase were irradiated on a γ-source with P_γ 0.05 Gy s^{-1}. Then their enzymatic activities with respect to the different substrates were measured taking into account spontaneous and post-radiation inactivation.

The recombinant enzyme was prepared using the method described by us previously[3] with the following corrections. The transformed *E. coli* JM109/pSA261 cells were grown in 300 mL of the medium prepared from 10^{-2} *M* Tris-HCl buffer, pH 8.0, containing ampicilline (100 μg mL^{-1}) and glycerol (0.4 %) at 30 °C. The gene expression was induced by addition of $2 \cdot 10^{-4}$ *M* IPTG in the middle of logarithmic phase of cell growth. The biomass was collected and disintegrated by sonication (22 kHz, 10 min) in the presence of 2 *M* NaCl and 10 m*M* DTT. The mixture was incubated for 1.5 h at the ambient temperature and then sonication was repeated. The supernatant was removed and the residue was washed with 0.05 *M* Tris-HCl buffer, pH 8.5 with subsequent solubilization in 10 mL of 6 *M* urea containing DTT (1 m*M*). The solubilized HRP apoprotein (95 % purity, 2 mg mL^{-1}) was added dropwise to the medium (2 L) containing urea (2 *M*), oxidized glutathione (0.7 m*M*), DTT (0.1 m*M*), calcium chloride (5 m*M*), glycerol (5 %) in $5 \cdot 10^{-2}$ *M* Tris-HCl buffer, pH 9.8, and the mixture was incubated at 4 °C. Hemin (5 μ*M*) was added after overnight incubation and right after the enzymatic activities of aliquots from the medium were measured. When the growth of activity was finished, the medium was saturated with ammonium sulfate (60 %), the precipitate was centrifuged and dissolved in water (30 mL). The preparations obtained were applied portionwise (15 mL) on a column (5.2 × 80 cm) with Toyopearl HW 55F equilibrated with 0.05 *M* Tris-HCl buffer, pH 7.0, containing 0.1 *M* NaCl. Active fractions were collected and frozen.

Protein content was determined spectrophotometrically.[11] The homogeneity of the enzyme preparations was tested by electrophoresis on polyacrylamide gel in the presence of SDS.

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