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Comparative studies of stability of native and recombinant horseradish peroxidase inactivated by radiation and other factors

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Comparative studies of the inactivation of native and recombinant horseradish peroxidase in the course of an enzymatic reaction, at elevated temperatures and in a wide range of radiation doses, have been performed. The protective effect of the carbohydrate component of the native peroxidase providing for stabilization of the enzyme against various inactivating factors was demonstrated. It was proposed that radioactive inactivation is related to dysfunction in heme interaction with the protein component and to an increase in the conformational mobility around the active site of the enzyme.

Key words: recombinant horseradish peroxidase; carbohydrate component; radiation-induced inactivation; thermal inactivation.

The preparation and reactivation of horseradish peroxidase expressed in a non-glycosylated form in *E. coli* open the possibility of studying the effect of the carbohydrate fragment of the enzyme on its catalytic properties, in particular, on inactivation in the course of an enzymatic reaction, at elevated temperatures and under ionizing irradiation. Native horseradish peroxidase (NHRP) is a protein with mol. wt. 44 kDa, containing 8 oligosaccharide chains, 4 disulfide bonds, 2 Ca^{2+} ions, and the non-covalently bound heme molecule.[1] The recombinant horseradish peroxidase (RHRP) is expressed in *E. coli* as a catalytically inactive apoprotein with mol. wt. 34 kDa forming inclusion bodies, which are then solubilized in unfolded form and undergo refolding.[2] The procedure of reactivation and purification of RHRP developed by us[3] allows a homogeneous preparation of *ca.* 3500—4000 E mg^{-1} specific activity to be obtained.* The procedure practically doubles the specific activity in comparison with the best preparations of NHRP. This fact can be tentatively explained by the presence of the enzyme inactivated in the course of the metabolic processes.

The aim of our work was a comparative study of NHRP and RHRP inactivation in the course of the reaction, at elevated temperatures and under irradiation.

* 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 given conditions (see Ref. 4).

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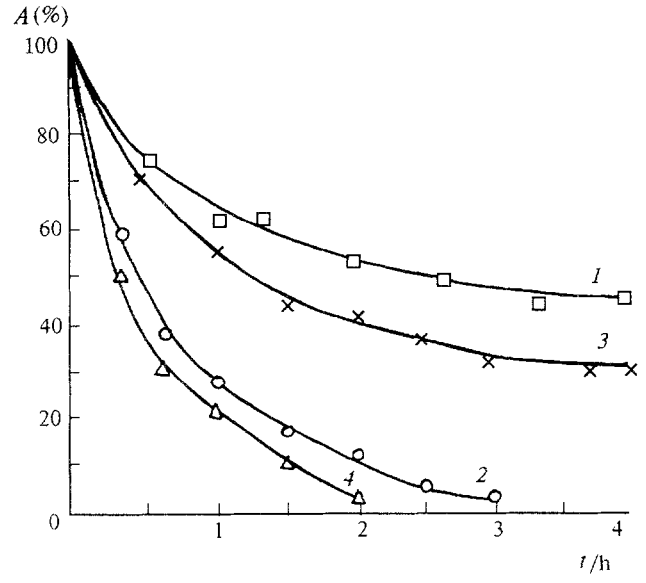

Fig. 1. Thermal inactivation of native and recombinant horseradish peroxidases (10^{-7} *M*) in phosphate buffer, pH 8.0: native (*1*, *2*) and recombinant (*3*, *4*) horseradish peroxidases at 50 (*1*, *3*) and 80 °C (*2*, *4*).

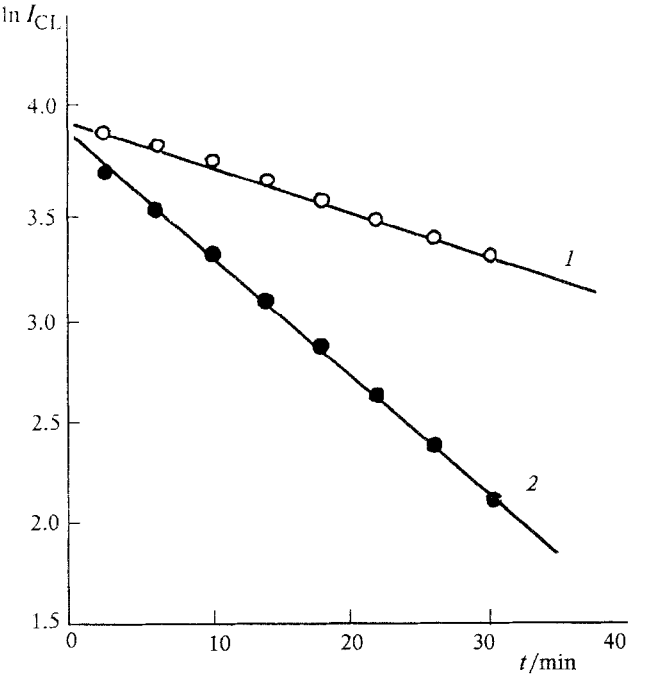

Fig. 2. Signal diminution in reaction of the enhanced chemiluminescence (see Experimental) plotted in semilogarithmic coordinates for native (*1*) and recombinant (*2*) horseradish peroxidases.

Results and Discussion

The results of a comparative study of the thermal inactivation of NHRP and RHRP in Fig. 1 demonstrate that the recombinant enzyme is less stable than the native one. The inactivation curves are described by an exponential dependence. The rate constant of RHRP inactivation at 50 °C is practically twice as high as that of NHRP ($4.5 \cdot 10^{-3}$ and $2.4 \cdot 10^{-3}$ min^{-1}, respectively). Upon increasing the temperature to 80 °C, the differences in the behavior of the recombinant and native enzymes become less appreciable (the rate constants of inactivation are $1.9 \cdot 10^{-2}$ and $1.5 \cdot 10^{-2}$ min^{-1}, respectively).

Using the fact that the decline of the curve of enhanced chemiluminescence (see Experimental) catalyzed with peroxidase results from inactivation of the enzyme in the course of the reaction,[5] we performed a comparative study of both forms of the enzymes in the reaction. The optimum conditions of the reaction are equal for the native and recombinant enzymes, but under these conditions RHRP is inactivated 2—3 times faster than NHRP (Fig. 2). The rate constant of RHRP inactivation in the course of the reaction is $5.74 \cdot 10^{-2}$ min^{-1}, and that of NHRP is $2.14 \cdot 10^{-2}$ min^{-1}.

Thus, the recombinant enzyme appears significantly more labile than the native one; this, in particular, may be a result of the absence of oligosaccharide fragments protecting the native enzyme from inactivation. To specify the role of glycosylation in stabilization of the peroxidase, the method of radiation-induced inactivation in a wide range of radiation doses was applied.

Previously, we have demonstrated the existence of an induction period in dose curves of inactivation for angiotensin-converting enzyme (ACE),[6] the appearance

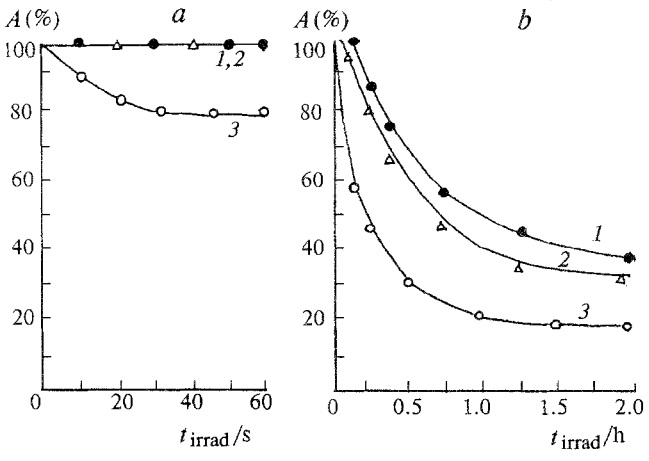

Fig. 3. Changes in enzymatic activity of NHRP from irradiation dose and concentration of the enzyme (pH 6.0, 0.1 *M* K-phosphate buffer): 10^{-6} (*1*); 10^{-7} (*2*); 10^{-8} (*3*) *M*. $P_\gamma = 0.05$ Gy s^{-1}: $D \leq 3$ Gy (*a*); $D > 3$ Gy (*b*).

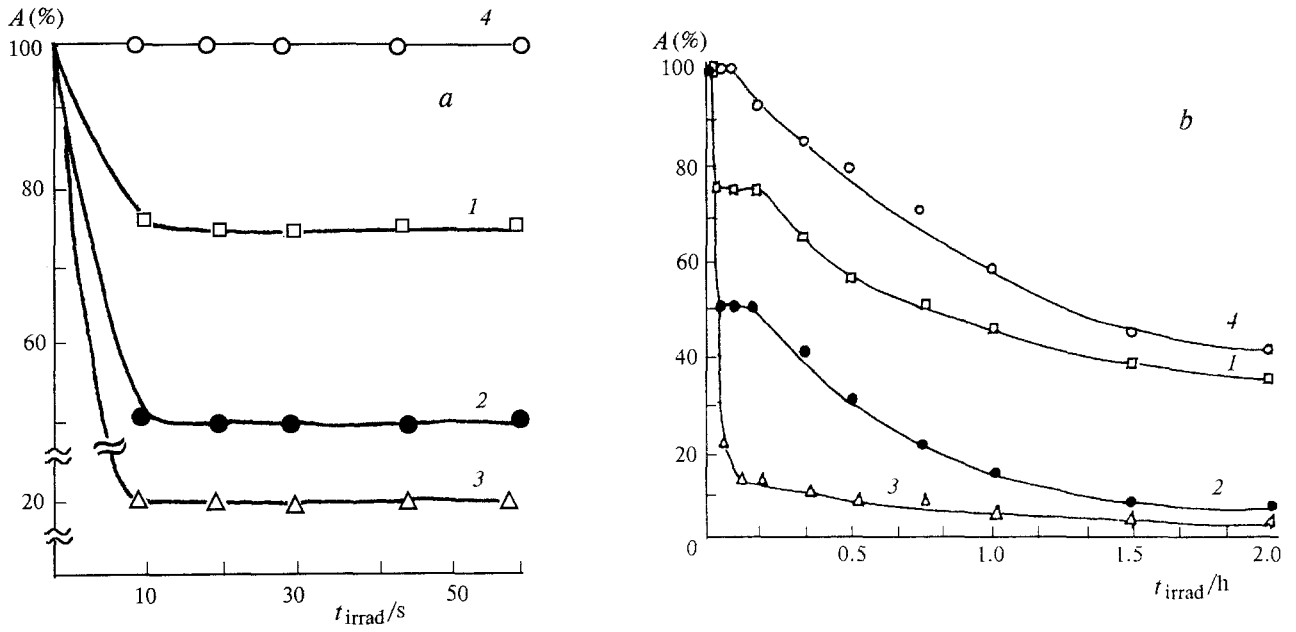

Fig. 4. Dose dependence of inactivation for RHRP samples of different concentrations: 10^{-6} (*1*); 10^{-7} (*2*); 10^{-8} (*3*) *M* in 0.01 *M* Tris-HCl buffer, pH 8.0. Dose dependence of radiation inactivation of NHRP 10^{-7} *M* (*4*) under the same conditions is presented as reference. P_γ = 0.05 Gy s^{-1}: $D \leq 3$ Gy (*a*); $D > 3$ Gy (*b*).

of which is most probably related to the protective effect of the polysaccharide chains. The contribution of the latter to the mol. wt. of the enzyme is *ca.* 30 %. However, ACE contains a large amount of aromatic amino acid residues, and some of them which are not directly connected with the catalytic activity possess a radioprotective effect. The fact that horseradish peroxidase containing a low percentage of those residues also has an induction period of radiation inactivation (Fig. 3) is a tentative confirmation of the more significant contribution to this autoprotective effect of carbohydrate residues. The assumption becomes quite evident after a study of the dose dependences of RHRP inactivation (Fig. 4), for which no induction period was observed at 10^{-6}—10^{-8} *M* concentrations of the enzyme.

It should be noted that the value of the induction period in radiolysis of NHRP (as in the case of ACE[5]) increased with an increase in the initial enzyme concentration. When the concentration was 10^{-8} *M*, the induction period was not observed even at doses less than 3 Gy.

A significant distinction of the dose dependences of RHRP inactivation (see Fig. 4) in comparison with those of NHRP is the existence of a plateau in the 0.5—3 Gy and 15—90 Gy dose ranges. The literature data[7] demonstrate that folding and inactivation of peroxidase occur *via* the formation of a rather stable intermediate, the so-called "molten globule". One cannot exclude that the ionizing irradiation causes the appearance of this state, which may be stable in the given dose ranges. It should be noted that a decrease in enzyme concentration shifts the plateau area to smaller doses; the fact attests to the dependence of the process of formation of the intermediate from the ratio of the amount of active radicals to that of active enzyme molecules.

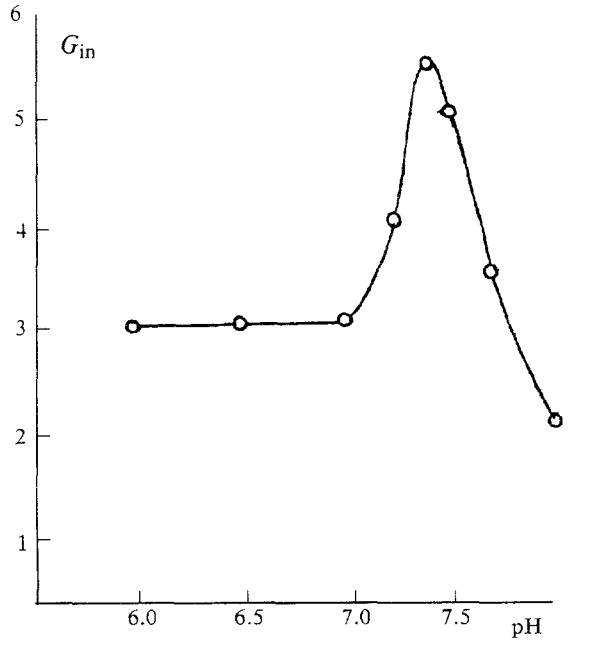

Fig. 5. Dependence of radiation-chemical yield (without regard for the induction period) of NHRP inactivation (10^{-7} *M*) on pH in 0.1 *M* phosphate buffer.

In Fig. 5, the dependence of the radiation-chemical yield of inactivation for 10^{-7} *M* solutions of the native peroxidase (without regard for the induction period) on pH is presented. The peak at pH 7.0—7.5 indicates the significant decrease in radiation stability of the enzyme. The pK_a values of the histidine residues in proteins are thought to be similar. Thus, for example, in cytochrome-C-peroxidase His-181 has pK_a 7.4 (see Ref. 8). In horseradish peroxidase three residues of histidine are present. Two residues coordinate heme in the active site and their pK_a are in a more acidic region, and the third one, *i.e.*, His-40 is located in the distal area in relation to heme and, hence, does not play a key role in peroxidase catalysis (on the basis of up-to-date literature data). Possibly, deprotonation of this histidine residue leads to a conformational transition through the intermediate state distinguished by elevated radiosensitivity, perhaps due to an increase in the surface area of the active site accessible for products of the radiolysis of water. In earlier works[9] on the conformational mobility of the active site of NHRP, a supposition on the existence of two conformers of peroxidase at pH > 6—6.5 was put forward, which was confirmed by our data.

Thus, a comparison of the effects of several inactivating factors on NHRP and RHRP revealed a decrease in the stability of the latter. The method of radiation-induced inactivation indicates that the phenomenon is most likely due to the stabilizing effect of the carbohydrate fragments. The radiation inactivation changes are likely related to a dysfunction in the interaction of heme with the protein component of the molecule and an increase in conformational mobility around the active site that is additionally confirmed by the preliminary data on the second derivatives of the UV spectra.

Experimental

Hemin, ABTS (ammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate)), and horseradish peroxidase type IX (Sigma, USA) were used in the present work. Other reagents, *i.e.*, salts, acids, hydrogen peroxide, were of Russian production and have "extra pure" and "pure for analysis" grades.

Recombinant horseradish peroxidase of specific activity 2000 E mg^{-1} was prepared using the known procedure.[3]

The reaction rate of oxidation of ABTS was measured with a Shimadzu UV 120-02 instrument (Japan) using the following procedure: to 0.1 *M* Na-acetate buffer, pH 5.0 (2 mL), an aqueous solution of ABTS (50 μL, 8 mg mL^{-1}), an aliquot of the enzyme, and 0.5 % aqueous H_2O_2 (0.1 mL) were added. The extinction coefficient at 405 nm was accepted equal to 36800 L mol^{-1} cm^{-1} (Ref. 10).

The irradiation of the aqueous solutions of peroxidase was performed on a γ-source with $P\gamma$ 0.05 Gy s^{-1}. Then their enzymatic activities were measured taking into account spontaneous and post-radiation inactivation.

The radiation-chemical yield of inactivation (the number of inactivated molecules per 100 eV of absorbed energy) was calculated using the slope of the tangent to the dose curve without regard for the induction period by the formula:

$$G_{in} = 0.96 \cdot 10^6 \, \Delta E/\Delta D,$$

where ΔE is the change in concentration (in *M*) of the catalytically active enzyme upon increase in irradiation dose by ΔD (kRad).

The thermal stability of peroxidase was studied using incubation of NHRP and RHRP (10^{-7} *M*) at 50 and 80 °C in 0.1 *M* phosphate buffer, pH 8.

Calculations of operation stability were performed on the basis of the total curve of the enhanced chemiluminescence obtained under optimum conditions: concentration of luminol, *p*-iodophenol, and hydrogen peroxide were 1 m*M*, 50 μ*M*, and 1.8 m*M*, respectively, in 5 m*M* Tris-HCl buffer, pH 8.6. Measurements were carried out with a Wallac 1251-002 luminometer, the sample volume was 1 mL, the reaction was initiated by simultaneous addition of the solutions of substrates. The concentration of peroxidase was 10^{-10} *M*.

The protein content was determined using the formula $183A_{230} - 75.8A_{260}$ (μg mL^{-1}); it was shown that these determinations were in good agreement with the quantitative protein determination by Lowry.[11]

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