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Peroxidase-Catalyzed Oxidation of 4-Thiouridine-Metabolites in Radish Seedlings*

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ダイコン幼植物体における 4-チオウリジン代謝生成物の
ペルオキシダーゼによる酸化

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Peroxidase(1.11.1.7) purified from Japanese-radish (*Raphanus sativus*) seedlings catalyzed the oxidation of 4-thiouridine, a sulfur-containing nucleoside, to 4-thiouridine disulfide in the presence of H_2O_2. 4-Thiouridine-metabolites such as 4-thiouracil, 4-thio-UMP, 4-thio-UDP, 4-thio-UTP and 4-thio-UDP glucose also were the substrate of the peroxidase reaction. Acidic peroxidase isoenzymes were mainly responsible for the 4-thiouridine oxidation. An increased 4-thiouridine-oxidase activity in the seedlings germinated and grown with the nucleoside was observed. These result suggest that the oxidation of 4-thiouridine and of its metabolites by peroxidase is not beyond the bounds of possibility in 4-thiouridine-cultured radish seedlings.

INTRODUCTION

Sulfur-containing nucleoside, 4-thiouridine, is one of minor constituents of bacterial tRNAs, but has not yet been found in plant kingdom. The 4-thiouridine absorbed during germination of Japanese-radish (*Raphanus sativus*) seeds has an inhibitory effect on chloroplast development.[1)2)] Recent our results using radish seedlings showed the metabolism of 4-thiouridine to 4-thiouracil and to the corresponding nucleotides such as 4-thio-UMP, 4-thio-UDP, 4-thio-UTP and 4-thio-UDP glucose,[3)] and the incorporation of 4-thiouridine moiety into RNA.[4)19)]

Plant peroxidase (EC 1. 11. 1. 7) catalyzes the oxidation of many redogenic compounds, regardless of their chemical structures, including some thiol-containing compounds such as cysteine and glutathione.[5)] We reportedly showed that the commercially available horseradish peroxidase oxidizes 4-thiouridine to 4-thiouridine disulfide in the presence of H_2O_2.[6)] Because both horseradish and Japanese-radish belong to the Cruciferae, we focused our attention to the oxidation of 4-thiouridine-metabolites by peroxidase purified from Japanese-radish seedlings.

MATERIALS AND METHODS

Plant growth conditions The conditions for germination and growth of radish seeds (*Raphanus sativus*, Wase-Yonjunichi) with or without 4-thiouridine were described in detail elsewhere.[1)]

* This is paper X in the series 'Chloroplast development in 4-thiouridine cultured radish seedlings'.

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Purification of Japanese-radish peroxidase Three kg of prechilled 7-day-old greened radish seedlings germinated and grown without 4-thiouridine were homogenized with 4.5ℓ of 0.2 M K-Pi, pH 7.8, in the presence of 30 g of insoluble polyvinylpolypyrrolidone, and the homogenate was made 95% saturation with $(NH_4)_2SO_4$. The precipitate was dissolved in, and dialyzed against, 0.1 M K-Pi, pH 7.8. Insoluble materials were removed by centrifugation and the supernatant was fractionated with prechilled (−20°C) acetone (35-75%, vol/vol of the supernatant). Peroxidase was extracted from the acetone powder with 0.05 M K-Pi, and the supernatant obtained by centrifugation at 20,000 x*g* for 20 min was fractionated with $(NH_4)_2SO_4$ (40 to 85%-cut). Tht precipitate was dissolved in, and dialyzed against, 30 mM Tris-HCl, pH 7.8, after which peroxidase fraction was applied to a column of DEAE-cellulose, equilibrated with the Tris buffer. The fraction passed freely from the column was collected and concentrated by adding solid $(NH_4)_2SO_4$ to 100% saturation. The precipitate was dissolved in a minimum vol of 10 mM Tris-HCl, pH 7.8, then it was applied to a column of Sephadex G-75, equilibrated with 10 mM Tris buffer. The fraction which showed guaiacol-oxidase activity was collected. The A_{403}/A_{280} ratio of the thus purified peroxidase was 0.35.

Assays The assay methods used for peroxidase activities were those described as follows : guaiacol,[7] indoleacetic acid (IAA),[8] pyrogallol[9] and *p*-phenylenediamine[10] oxidases. Under the assay conditions used, one unit of guaiacol-oxidase and of 4-thiouridine-oxidase were defined as the activity catalyzing the formation of 1 μmol of tetraguaiacol per min, and that of 1 nmol of 4-thiouridine disulfide per 10 min, respectively.

Others 4-Thiouridine and its derivatives were synthesized and purified by the published methods.[3] Carrier ampholine, Pharmalyte, pH 3-10 was purchased from Pharmacia Fine Chemicals.

RESULTS AND DISCUSSION

The spectral changes upon addition of H_2O_2 to the reaction mixture consisting acetate buffer, pH 5.0, the purified peroxidase and 4-thiouridine are shown in Fig. 1. Under the conditions without H_2O_2, no such spectral change was detected over time period up to one hour. The A at 331 nm decreased with an apparent rate constant, 0.10 per min (inset of Fig. 1), but, after 20 min of the reaction the values (log A_{331}) deviated from the line. An isosbestic point at 304 nm and a slight increase in A at 260 nm were also observed. At the end of the reaction the initial absorption spectrum of 4-thiouridine was replaced by the one with two maxima of lower intensity at 310 and 260 nm, which resembles to that of authentic 4-thiouridine disulfide prepared by the oxidation of 4-thiouridine in KI-I_2 solution.[11] After treatment of the reaction products with 2-mercaptoethanol, which reduces 4-thiouridine disulfide to 4-thiouridine,[12] the characteristic absorption spectrum of 4-thiouridine was recovered, with a yield of 70-75% of the initial A_{331}.

These results on the spectral changes, the spectrum of the reaction product(s) and on the reduction product with 2-mercaptoethanol were the same as those obtained in the oxidation of 4-thiouridine by commercially avaiable horseradish peroxidase and H_2O_2 system,[6] in which 4-thiouridine was enzymatically oxidized to 4-thiouridine disulfide, accompanying by non-enzymatic chemical oxidation of 4-thiouridine to uridine by H_2O_2 in the reaction mixture. It is well established that 4-thiouridine in RNAs reacts with H_2O_2 to give uridine.[4][13][14] In the non-enzymatic oxidation of 4-thiouridine with H_2O_2 *i. e.* in the absence of peroxidase, a significant decrease in A at 331 nm was accompanied by a small increase in A at 260 nm,

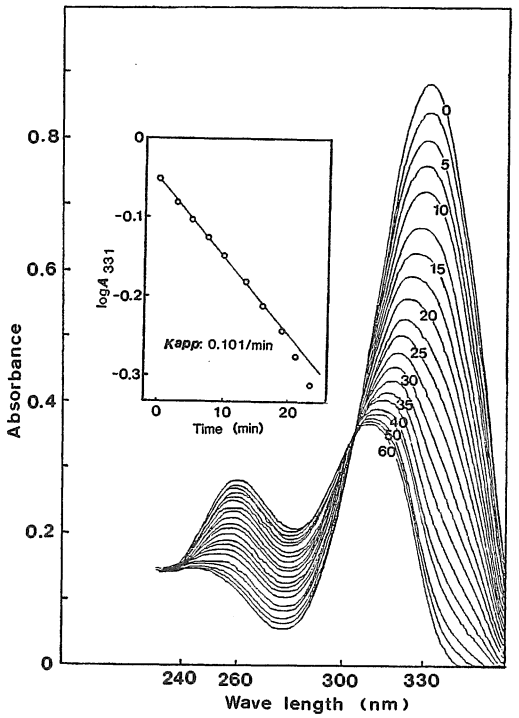

Fig. 1 Spectral shift of 4-thiouridine by peroxidase of radish seedlings. The reaction mixture contained, in a total volume of 3 ml, 0.05 M acetate buffer, pH 5.0, 40 μM 4-thiouridine, 1.51 mg of purified peroxidase and 0.4 mM H_2O_2, which was added last to initiate the reaction. The numbers in the figure indicate reaction times in minute.

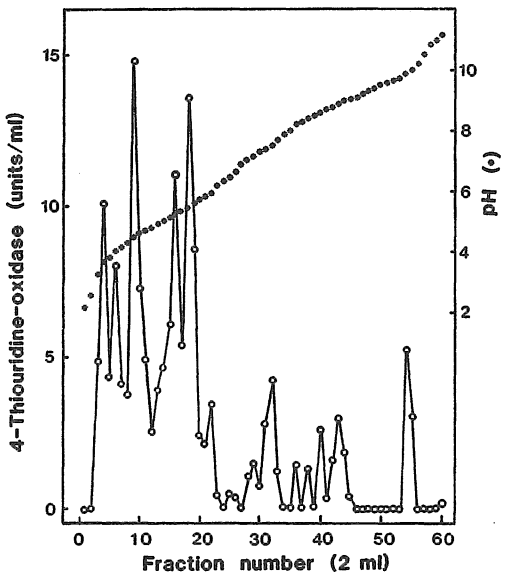

Fig. 2 Oxidation of 4-thiouridine by peroxidase isoenzymes separated by isoelectric focusing. Peroxidases in the crude peroxidase fraction were subjected to an isoelectric focusing using a wide range ampholine that gave a pH 3 to pH 10 gradient. After electrophoresis and fractionation, 4-thiouridine-oxidase activity was determined as in Fig, 1 except that purified peroxidase was replaced by one in the each separated fractions.

indicating a chemical oxidation of 4-thiouridine to uridine.

The formation of 4-thiouridine disulfide and a small amount of uridine as a by-product in the radish peroxidase-H_2O_2 system was further demonstrated by the tlc method as described in detail previously.[6] As the results under the conditions used in Fig. 1, 120 nmol of 4-thiouridine was converted enzymatically to 55.4 nmol of the disulfide, and non-enzymatically to 9.3 nmol of uridine by the chemical oxidation. The reaction mechanism in the formation of 4-thiouridine disulfide will follow that 4-thiouridine thiyl radicals formed by the peroxidase reaction may dimerize itself to produce the disulfide, as discussed previously.[6]

The numerous forms of peroxidase are pre sent in plant tissues, especially, in the extract of mature Japanese-radish roots contains eighteen isoenzymes distinguishable on polyacrylamide gel electrophoresis.[15] To investigate the isoenzyme specificity for 4-thiouridine-oxidation, we separated isoenzymes in the crude peroxidase fraction by isoelectricfocusing using a wide range ampholine that gave a pH 3 to pH 10 gradient. At least fourteen isoenzymes of 4-thiouridine-oxidase were distinguishable on the pH gradient (Fig. 2). The separated acidic isoenzymes could oxidize 4-thiouridine effectively. The neutral and basic ones were found to be less effective. Because the acidic isoenzymes are the major ones in the extract of mature radish roots,[15] the isoenzyme specificity obtained in Fig. 2 may depend on their relative abundances in

Table 1 Catalytic properties of peroxidase isoenzymes separated by isoelectric focusing.

Isoperoxidases*		Distribution of Substrate-Specific Peroxidase Activity(%)				
		4-Thio-uridine	Guaiacol	Pyroga-llol	*p*-Phenylene-diamine	IAA
pI	3.5-6.5	65.6	25.0	8.2	36.4	9.3
pI	7.0-9.1	22.1	3.6	10.3	21.4	4.8
pI	9.5-10.5	12.2	71.4	81.5	42.2	85.9

*Isoperoxidases separated by isoelectric focusing as described in Fig 2 were divided into three fractions according to their pIs. Solid $(NH_4)_2SO_4$ was added to the each fractions to 85% saturation. The resulting precipitates were collected and dissolved in, and dialyzed against 5 mM phosphate buffer pH 6.5, then peroxidase activities in the each fractions were determined using five different kinds of substrates.

the radish seedlings. This will not be the case by considering the following results. To investigate the distribution of other isoperoxidase activities, we divided the isoperoxidases separated in Fig. 2 into the following three acidic (pI 3.5-6.5), neutral (pI 7.0-9.1) and basic (pI 9.5-10.5) fractions according to the distribution of 4-thiouridine-peroxidase, after which peroxidase-activities in each three fractions were determined using the five different kinds of substrates (Table 1). More than 65% of 4-thiouridine-peroxidase activity was recovered in the acidic fraction, in accordance with the results obtained in Fig. 2. In contrast with the distribution of 4-thiouridine-peroxidase, about 70-85 % of guaiacol, pyrogallol- and IAA-oxidases were found in the basic fraction, indicating that the major isoenzymes for these kinds of substrates might be basic in the extract of radish seedlings. A considerable activity of *p*-phenylenediamine-oxidase was also present in both acidic and neutral fractions. From these results, we concluded that acidic isoperoxidases in radish seedlings are mainly responsible for 4-thiouridine-oxidation. There are reportedly many examples that individual isoperoxidases themselves differ in their ability to oxidize substrates.[16)17)18)]

The 4-thiouridine taken up by radish seeds was convertible to 4-thiouridine-metabolites such as 4-thiouracil, 4-thio-UMP, 4-thio-UDP, 4-thio-UTP and 4-thio-UDP glucose.[3)] The results described above on the 4-thiouridine oxidation by peroxidase system have prompted us a search for the possibility of the peroxidase-catalyzed oxidation of 4-thiouridine metabolites in plant cells. The objectives were to ; (a) determine if these metabolites are able to be the substrate of peroxidase, and (b) examine any change in peroxidase (especially 4-thiouridine-oxidase) activity of the seedlings germinated with 4-thiouridine.

Firstly, (a) all five kinds of the 4-thiouridine metabolites examined also were oxidized in the peroxidase-H_2O_2 system. The relative oxidation rates are shown in Table 2. 4-Thiouracil was the most effective substrate and increasing phosphorylation of the 4-thiouridine nucleotides decreased the oxidation rate. 4-Thio-UDP glucose also was the substrate. Secondly, (b) the relative peroxidase activities per mg protein basis in the crude peroxidase

Table 2 Oxidation of 4-thiouridine-metabolites by peroxidase-H_2O_2 system.

Metabolite	Relative Oxidation Rate*
4-Thiouridine	1.00
4-Thiouracil	1.28
4-Thio-UMP	0.56
4-Thio-UDP	0.40
4-Thio-UTP	0.36
4-Thio-UDP glucose	0.48

*Oxidation of the each 4-thiouridine-metabolites was measured under the conditions described in the legend for Fig. 1. The amounts due to the chemical oxidation caused by H_2O_2 in the absence of peroxidase were corrected.

Table 3 Peroxidase activities in radish seedlings germinated and grown with 4-thiouridine.

4-Thiouridine in Germination Medium*(mM)	Chlorophyll (μg/g Fresh cotyledons)	Relative Specific Activity		
		Guaiacol	IAA	4-Thiouridine
0.0	695	1.0	1.0	1.0
0.25	459	1.24-1.35	0.6-0.7	1.15-1.22
0.5	238	1.25-1.28	0.94-1.02	1.20-1.35
0.75	173	1.29-1.39	0.8-0.9	1.57-1.58
1.0	78	1.07-1.09	0.62-0.72	1.72-2.25

*Radish seeds were germinated and grown with or without 4-thiouridine in the dark for 4 days, then the seedlings were illuminated for 1 day. The contents of chlorophyll in the cotyledons and the peroxidase activities in the crude peroxidase fractions were determined. The results on the peroxidase activities obtained from three independent experiments were listed.

fractions prepared from the seedlings germinated and grown with 4-thiouridine (0-1.0 mM) in the dark for 4 days followed by under white light (2,000 lux) for 1 day, are listed in Table 3, which also shows the chlorophyll contents in the cotyledons. Although fresh weight and soluble protein content of the seedlings were not affected significantly by the 4-thiouridine cultur,[2] chlorophyll contents of the cotyledons decreased with increasing concentrations of 4-thiouridine in the culture medium, indicating an inhibition of chloroplast development. The 4-thiouridine feeding brought about a slight (25-40%) increase and a decrease (10-20%) in guaiacol- and IAA-oxidase activities, respectively. It is of interest to emphasize that the 4-thiouridine-oxidase activities increased, with increasing concentrations of 4-thiouridine in the culture medium, approximately twice the activity at 1.0 mM of 4-thiouridine as high as that of control seedlings. These results reveal that the oxidation of 4-thiouridine and of its metabolites by peroxidase is not beyond the bounds of possibility in radish seedlings. Since this type of oxidation requirs supplementation with H_2O_2 (Fig. 1), however, it will be necessary to take into account the H_2O_2-generating system and the compartmentalization of peroxidase, H_2O_2 and 4-thiouridine-metabolites in the plant cells.

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