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INACTIVATION AND CLEAVAGE OF RADISH PEROXIDASE BY VARIOUS REDUCING AGENTS

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Key Word Index—*Raphanus sativus* L.; Cruciferae; radish; inactivation and cleavage; peroxidase; reducing agents.

Abstract—The inactivation and cleavage of Korean radish (*Raphanus sativus* L.) peroxidase (EC. 1.11.1.7) by three reducing agents, dithiothreitol (DTT), ascorbate and 2-mercaptoethanol (2ME) was examined. Upon incubation of the enzyme with DTT, inactivation followed by degradation and cleavage of the anionic isoperoxidase A_1 (M_r: 43 k) was observed, whereas, inactivation and degradation took place almost simultaneously in the presence of ascorbate. Nevertheless, the cleaved products generated from the inactivated enzyme showed similar M_r values of about 30 k, 23 k and 18 k in both cases. When 2ME was used, the formation of larger M_r aggregates of 66 k and 69 k was observed without cleavage of smaller peptides. When the enzyme was inactivated by DTT and ascorbate, dimethyl sulfoxide and ethanol protected the enzyme inactivation almost completely in both cases, but these scavengers did not reverse the inactivation significantly when 2ME was used as a reducing agent. The singlet oxygen scavenger histidine prevented the enzyme from inactivation by ascorbate and 2ME to some degree, but it failed to inhibit enzyme inactivation by DTT. These results suggest that there might be different inactivation and cleavage mechanisms of radish peroxidases, depending upon the reducing agents used.

INTRODUCTION

Recently it has been shown that several enzymes are inactivated and degraded by the oxidative modification system comprised of a reducing agent, oxygen and iron (or copper) [1]. The proteins cleaved by the oxidative modification system include *Saccharomyces cerevisiae* glutamine synthetase [2], *Escherichia coli* glutamine synthetase [3], erythrocyte superoxide dismutase [4] and low density lipoprotein [5]. This specific inactivation event is thought to be the result of the generation and reaction of active oxygen species at specific iron or copper binding site on these proteins [2]. However, Davison *et al.* observed that active oxygen species were not directly involved in the ascorbate mediated catalase inactivation with copper [6]. In this case, semidehydroascorbate formed from the oxidation of ascorbate was found to be the most likely damaging agent. Therefore, there might be the possibility of different inactivation and degradation mechanisms depending upon the proteins and reducing agents used.

The present study was undertaken to investigate the mechanism of inactivation and degradation of Korean radish peroxidase by reducing agents such as DTT, ascorbate and 2ME. The characteristics of inactivation and cleavage patterns of isoperoxidase A_1 by these reducing agents were compared in detail.

RESULTS AND DISCUSSION

Inactivation of Korean radish peroxidase by reducing agents

In the study presented here, we found that Korean radish isoperoxidase A_1 was inactivated in the presence of DTT, ascorbate and 2ME in a concentration and time-dependent manner. Figure 1(a) shows the effect of various concentrations of these reducing agents on the activity of isoperoxidase A_1 (final concentration 0.1 μM), suggesting that DTT and 2ME inhibited the enzyme with similar effectiveness, and that they were much more potent than ascorbate in inhibiting peroxidase activity.

Figure 1(b) shows time-dependent inactivation of isoperoxidase A_1 by DTT in the presence of EDTA and $FeCl_3$. Isoperoxidase A_1 (final concentration

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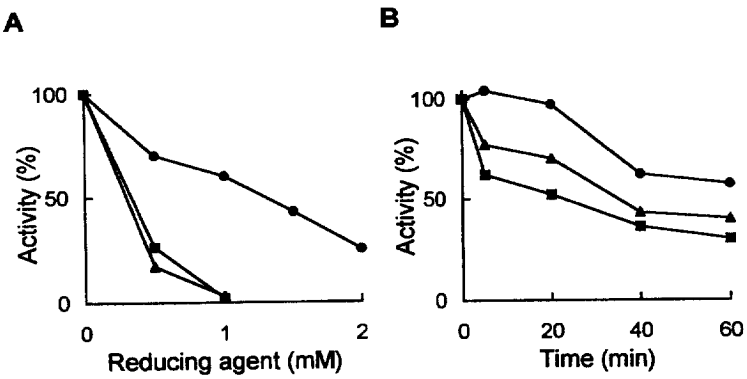

Fig. 1. (a) Inactivation of isoperoxidase A_1 with several reducing agents. The enzyme (0.1 μM) was incubated at 25° for 1 h with various concentrations of dithiothreitol (▲—▲), ascorbate (●—●) or 2-mercaptoethanol (■—■). (b) Effect of EDTA and $FeCl_3$ on the inactivation rate of isoperoxidase A_1. The enzyme (0.5 μM) was preincubated with 5 mM EDTA (●—●) or 50 μM $FeCl_3$ (■—■) in 10 mM sodium phosphate buffer (pH 6) for 15 min, then treated with 1 mM dithiothreitol. Remaining enzyme activity was compared with the enzyme treated with dithiothreitol (▲—▲) at various time intervals.

0.5 μM) lost about 30% of its activity when the enzyme was incubated with 1 mM DTT for 20 min. The addition of 5 mM EDTA almost completely protected the enzyme activity under the same experimental condition, while the enzyme inactivation was much more stimulated by 50 μM $FeCl_3$. However, the protective effect of EDTA in chelating the adventitiousiron was not as effective when the incubation time was over 20 min at pH 6. The protective effects of EDTA were reported in glutamine synthetase, alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase [7] in the presence of exogenous iron and reducing agents although they are not iron-containing metalloenzymes.

SDS-PAGE analysis of the cleaved peroxidases by reducing agents

When isoperoxidase A_1 (final concentration 27.6 μM; 36 μg) was treated with 20 mM DTT for 50 h, we could not detect cleaved products at all, although the enzyme lost about 90% of its activity (lane 4 of Fig. 2). On the other hand, the enzyme began to be degraded and cleaved into several discrete smaller peptides of 30 k, 23 k and 18 k as soon as the enzyme activity disappeared completely at 0.1 M DTT (lanes 5, 6, 7 of Fig. 2). Therefore, it is likely that the cleavage event does not occur until the enzyme loses its activity completely.

On the other hand, 0.1 M ascorbate treatment for 50 h did not induce enzyme inactivation and cleavage as shown in lane 3 of Fig. 3. The cleaved peptide fragments were observed when the enzyme lost only 10% of its activity at 0.2 M ascorbate (lane 4 of Fig. 3). The measured M_rs of cleaved peptides were about 30 k, 23 k and 18 k, which were similar to the M_r values of the fragments generated by the DTT inactivation system. These results suggest that enzyme inactivation and degradation took place almost simultaneously, and that these two steps might not be strictly separate phenomena when ascorbate was used [8]. When the concentration of ascorbate was over 0.3 M, the enzyme was degraded thoroughly and the 43 k main band disappeared completely (lanes 5 and 6 of Fig. 3).

In Fig. 4, the effect of 2ME concentration on the inactivation and cleavage is shown. At 50 mM 2ME, the enzyme lost all of its activity. In this case, a notable observation was the formation of higher M_r aggregates of 66 k and 69 k which could not be found in DTT and ascorbate mediated cleavage system (lanes 3 and 4 of Fig. 4). Furthermore, we could not observe any smaller cleaved products derived from the enzyme by 2ME, although there remains the possibility that thoroughly degraded very small peptide fragments could not be detected as bands on the gel by Coomassie brilliant blue R-250. This suggestion is well supported by the observation that the more 2ME concentration was increased, the thinner the intact band of 43 k became, without the increase of cross-linked larger peptide fragments. The generation of cross-linked higher M_r adducts through radical mediated inactivation of yeast glutamine synthetase by DTT was previously reported [2, 9]. In our experimental condition, however, only 2ME caused peroxidase to make higher M_r aggregates.

Effect of radical scavengers on the isoperoxidase A_1 inactivation

Various radical scavengers were used to examine whether free radicals might be involved in the inactivation of peroxidase by reducing agents. In Table 1, the effect of several radical scavengers on the inactivation of isoperoxidase A_1 by reducing agents was shown. Upon incubation of 0.1 μM isoperoxidase A_1 with three reducing agents (DTT, ascorbate and 2ME), different protective effects were obtained

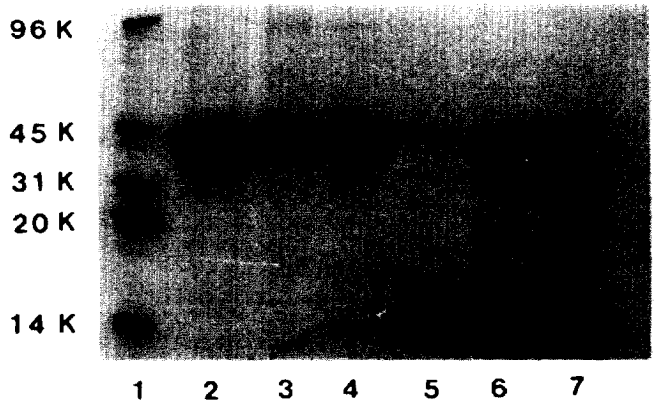

Fig. 2. Effect of dithiothreitol concentration on the inactivation and cleavage of iosperoxidase A_1. The enzyme (27.6 μM; 36 μg) containing various concentrations of dithiothreitol was incubated at 25° for 50 h. Lane 1, M_r markers (96 k, 45 k, 31 k, 20 k and 14 k); lane 2, no DTT; lane 3, 10 mM DTT; lane 4, 20 mM DTT; lane 5, 100 mM DTT; lane 6, 200 mM DTT; lane 7, 300 mM DTT.

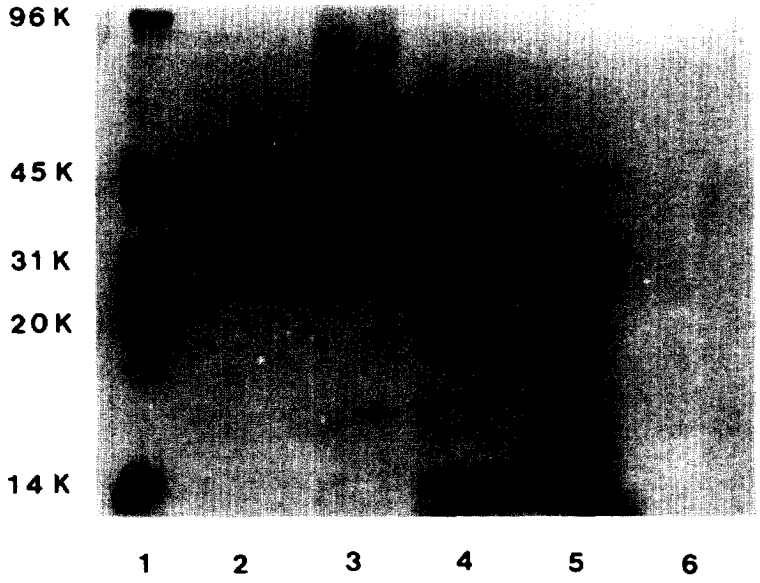

Fig. 3. Effect of ascorbate concentration on the inactivation and cleavage of iosperoxidase A_1. The enzyme (27.6 μM; 36 μg) containing various concentrations of ascorbate was incubated at 25° for 50 h. Lane 1, M_r markers (96 k, 45 k, 31 k, 20 k and 14 k; lane 2, no ascorbate; lane 3, 0.1 M ascorbate; lane 4, 0.2 M ascorbate; lane 5, 0.3 M ascorbate; lane 6, 0.5 M ascorbate.

depending upon the reducing agents used. When the reaction condition was adjusted to obtain 50% enzyme activity inhibition by DTT, dimethyl sulfoxide protected the enzyme almost completely. Ethanol and mannitol showed some protective effect against DTT, whereas thiourea and histidine had no effect. Ethanol and dimethyl sulfoxide also protected the enzyme inactivation due to ascorbate, although they did not reverse the inactivation by 2ME significantly. Considering the above results, reactive oxygen species are thought to be involved in the inactivation, since radical scavengers such as ethanol and dimethyl sulfoxide prevented the enzyme inactivation. Curiously, histidine reduced peroxidase inactivation by ascorbate and 2ME to some degree, indicating presumptive evidence for a role of singlet oxygen [6]. However, the possibility that it scavenges hydroxyl radicals or serves merely to chelate metal ions must be considered, as suggested in the NADH oxidase system [7].

Effect of substrate and several compounds on the cleavage of isoperoxidase A_1 by DTT

The effects of substrate and several compounds on the inactivation and cleavage of isoperoxidase A_1 are shown in Table 2. The enzyme (final concentrations 27.6 μM, 36 μg) preincubated with 0.5 M guaiacol retained 95% of the activity, suggesting that guaiacol might prevent accessibility of DTT to the enzyme. Recent reports indicate that a site-specific oxidative

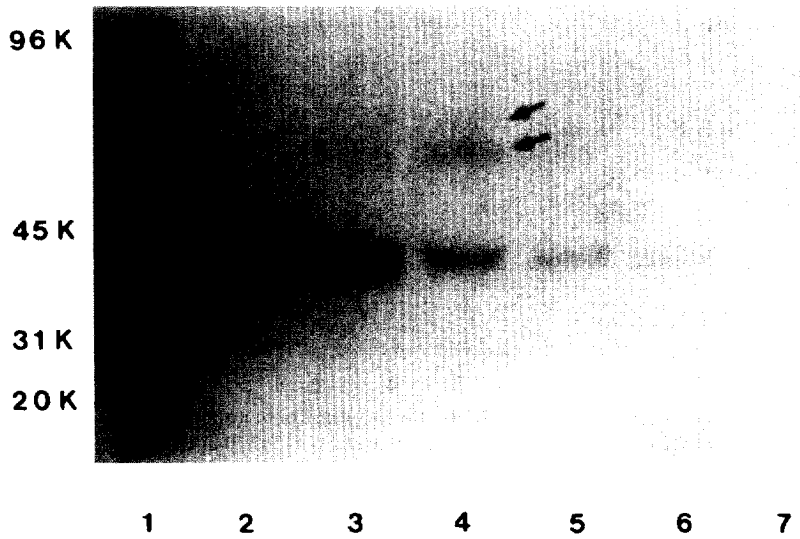

Fig. 4. Effect of 2-mercaptoethanol concentration on the inactivation and cleavage of isoperoxidase A_1. The enzyme (27.6 μM; 36 μg) containing various concentrations of 2-mercaptoethanol was incubated at 25° for 50 h. Lane 1, M_r markers (96 k, 45 k, 31 k and 20 k; lane 2, no 2-mercaptoethanol; lane 3, 50 mM 2-mercaptoethanol; lane 4, 0.1 M 2-mercaptoethanol; lane 5, 0.2 M 2-mercaptoethanol; lane 6, 0.3 M 2-mercaptoethanol; lane 7, 0.5 M 2-mercaptoethanol.

Table 1. Effect of various scavengers on the inactivation of isoperoxidase A_1* by reducing agents

	Loss of activity†		
Scavengers‡	Ascorbate (5 mM, 10 min)	Dithiothreitol (1 mM, 10 min)	2-Mercaptoethanol (1 mM, 10 min)
None	100	100	100
Ethanol	5	45	82
Dimethyl sulfoxide	13	0	73
Mannitol	95	69	118
Thiourea	138	144	94
Histidine	64	104	70

* Concentration of iosperoxidase A_1 is 0.1 μM.

† Results are expressed as a percentage of the activity loss without scavenger additions. Thus, values less than 100% indicate protection from inactivation. In the absence of scavenger additions, about 50% of the peroxidase activity was lost during the inactivation.

‡ Concentration of scavengers is 250 mM except ethanol (800 mM).

modification system generates activated oxygen derivatives that attack His or other oxidizable amino acids (e.g. Cys, Met, Trp and Tyr) in the catalytic site of the enzyme [10]. In this regard, it is noteworthy that the guaiacol-protected enzyme did not generate 23 k cleaved peptide which had been apparently observed in the DTT treated sample (Table 2). The same cleavage patterns without 23 k peptide were found in the EDTA treated enzyme having 20% of the activity, although complete protection of EDTA against glutamine synthetase cleavage were reported [2]. Dimethyl sulfoxide treated enzyme showed three discrete peptide fragments with identical M_r to DTT treatment sample, suggesting that dimethyl sulfoxide did not protect against enzyme cleavage by DTT at

Table 2. Effects of substrate (0.5 M) and various compounds (0.5 M) on the cleavage and inactivation of isoperoxidase A_1 by dithiothreitol. The enzyme (27.6 μM; 36 μg) was pre-incubated with several compounds for 3 min and subsequently reacted with dithiothreitol (0.5 M) for 3 h at 35° before SDS-PAGE

Addition	Enzyme activity (%)	Formation of 23 k peptide
Enzyme only	100	
DTT	0	Yes
DTT + guaiacol	95	No
DTT + dimethyl sulfoxide	20	Yes
DTT + EDTA	20	No

all (data not shown), although it was an effective protector against enzyme activity inhibition as shown in Table 1.

Therefore, the inactivation and degradation phenomena observed in Korean radish peroxidase seem to be ascribed to thiol/metal ion/O_2 oxidative modification system, and the existence of heme iron in the peroxidase structure might make the enzyme more susceptible to easy attack by reducing agent in the presence of O_2 [2, 17].

EXPERIMENTAL

Experimental plant and enzyme purification

The fresh Korean radish (*Raphanus sativus* L.) root was obtained from a local market in Seoul and used for the purification of isoperoxidase A_1. The enzyme purification was performed as previously reported in our laboratory [11–13].

Peroxidase assays and protein determination

The peroxidase activity with guaiacol as a substrate was assayed by a modified method of Ref. [14]. Protein concn was determined by the method of Ref. [15].

Enzyme cleavage reaction

The cleavage reaction mixture was composed of Korean radish isoperoxidase A_1 and 10 mM Na-Pi buffer (pH 6) containing various concns of reducing agents such as DTT, ascorbate and 2ME. Isoperoxidase A_1 (final concentration 27.6 μM; 36 μg) was incubated with 10 mM Na-Pi buffer (pH 6) containing 0.5 M DTT for 50 h at 25° in a total vol. of 30 μl. In order to investigate the effect of concns of various reducing agents such as DTT, ascorbate and 2ME on the inactivation and cleavage of isoperoxidases, the reducing agents were added at different final concns (10 mM–0.5 M) in 10 mM Na-Pi buffer (pH 6) at 25° for 50 h, and then the residual enzyme activity was measured.

Effect of substrate and several compounds on the cleavage of isoperoxidase A_1 by DTT

To investigate the effect of substrate and several compounds on the inactivation and cleavage process of iosperoxidase A_1 by DTT, the enzyme (27.6 μM; 36 μg) was incubated with 0.5 M DTT for 3 h at 25° in the presence of substrate or other compounds in a total vol. of 30 μl. These additions include substrate (guaiacol) of 0.5 M, 0.5 M dimethyl sulfoxide and 0.1 M EDTA.

SDS-polyacrylamide gel electrophoresis

After the cleavage reaction was performed, the reaction mixtures were analysed by SDS-PAGE according to methods of Ref. [16].

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