Chlorophyll Degradation by Peroxidase in Parsley Leaves

Naoki Yamauchi and Takahisa Minamide
College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 591

Summary

The mechanism of chlorophyll degradation by peroxidase in parsley leaves was studied. Chlorophylls present in the ethyl alcohol (EtOH) extract of parsley leaves were degraded by a peroxidase—hydrogen peroxide system. Purified chlorophylls, however, were not affected by the system, suggesting the existence of a substance which acts together with peroxidase to degrade chlorophyll in parsley leaves.

The substance was isolated from the EtOH extract by the methods of solvent fractionation and column chromatography. After hydrolysis, it was identified as an apigenin, which is a major flavonoid contained in parsley, by measuring Rf value on thin layer chromatogram and spectral characteristics.

From the results obtained, it is concluded that in parsley leaves chlorophylls are not degraded by peroxidase directly, but that peroxidase oxidizes the apigenin and then the oxidized apigenin degrades chlorophylls.

Introduction

Leaf vegetables deteriorate rapidly after harvest as compared with other fruits and vegetables. One of the main factors causing deterioration of freshness is yellowing of leaves. Eskin summarized that the yellowing phenomenon, the degradation of chlorophylls, appears to involve one or more of the following reactions: 1) pheophytin formation from chlorophyll by organic acids, 2) chlorophyllide formation by chlorophyllase, and 3) bleaching reaction of chlorophyll by oxidative reactions (4). The formation of pheophytin is particularly found in processed foodstuffs, like brined cucumber (12) and frozen spinach (3). Although the chlorophyllase has been thought to catalyze the degradation of chlorophylls, a function for the enzyme in the synthesis of chlorophylls has also been postulated (8, 10, 24). The bleaching of chlorophyll seems to involve lipoxygenase and peroxidase (9, 11, 14, 17, 28, 30). Orthoefer and Dugan Jr. observed that chlorophyll a was bleached in a system which consisted of linoleic acid and lipoxygenase (17). Holden also noted that chlorophyll was bleached when leaves of various species of plants, which were rich in lipoxygenase, were incubated in an aqueous acetone solution at pH 6.0 in the dark (9). Matile (14) and Huff (11) reported that chlorophylls were degraded by a peroxidase—hydrogen peroxide system in the presence of phenolic compounds, such as 2, 4-dichlorophenol and resorcinol. In a previous paper we also reported that chlorophyll in parsley was degraded, not by chlorophyllase but by peroxidase, and that the degradation was retarded by L-ascorbic acid (30).

This report deals with the mechanism of chlorophyll degradation by peroxidase in parsley leaves.

Materials and Methods

Materials
Parsley (Petroselium sativum Hoffm., cv Paramount) used in this study was purchased at a local market.

**Purification of chlorophylls a and b**

Chlorophylls a and b were purified by the method of Yoshiura and Iriyama(31). The pigments were extracted with 80 ml acetone from 20 g of parsley leaves. Into the extract, 1,4-dioxane and distilled water were added and the mixture was allowed to stand until a precipitate was formed. The precipitate was collected by centrifugation at 10,000 g for 15 min., and dissolved in a small quantity of acetone. Chlorophylls a and b were then separated by thin layer chromatography (silica gel, solvent : n-pentane : t-butyl alcohol=90:5:5). Chlorophyll a and b fractions also contained chlorophylls a' and b', respectively.

**Determination of chlorophyll degradation**

The reaction mixture contained 0.2 ml of 80% ethyl alcohol (EtOH) extracts of parsley leaves (or 30 μg purified chlorophyll plus flavonoid), 0.04% Triton X-100, 0.012% hydrogen peroxide, 20 μg peroxidase (Sigma chemical, horseradish), and 80 mM phosphate buffer (pH 6.0) in a total volume of 2.5 ml. After 10 min. at 25°C, the reaction was stopped by the addition of EtOH (2.5 ml). The remaining (non-degraded) chlorophylls were extracted with hexane and were assayed by reading the absorbance at 663 nm (chlorophyll a) or 644 nm (chlorophyll b), and degraded chlorophylls were calculated subtracting this value from the blank (distilled water instead of hydrogen peroxide) one.

**Isolation of unknown substances involved in chlorophyll degradation**

The unknown substances involved in chlorophyll degradation were extracted with boiling EtOH from parsley leaves (10 g). Fractionation with different solvents was carried out as shown in Fig. 1 and each fraction was isolated by column chromatography (Sephadex LH-20, 1.8 x 100 cm, solvent : 80% EtOH, flow rate : 0.4 ml/min., fraction : 5 ml).

**Isolation of flavonoid aglycones**

The mixture of fractions I, II, and III contained in butyl alcohol (ButOH) and aqueous fractions shown in Fig. 2 were hydrolyzed with 2.0 M HCl for 40 min. at 100°C and the aglycones were then extracted with ethyl acetate. The extract was evaporated to dryness under reduced pressure and dissolved in a small volume of EtOH. The aglycones in the EtOH solution were isolated by thin layer chromatography.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Chlorophyll degradation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll a</td>
<td>0</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>0</td>
</tr>
<tr>
<td>Parsley extract*</td>
<td>46</td>
</tr>
</tbody>
</table>

* Chlorophyll degradation as percent of initial.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Chlorophyll degradation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate fraction*</td>
<td>2</td>
</tr>
<tr>
<td>ButOH fraction*</td>
<td>34</td>
</tr>
<tr>
<td>Aqueous fraction*</td>
<td>100</td>
</tr>
</tbody>
</table>

* Distribution of the unknown substances involved in chlorophyll degradation among fractions, as percent of the largest activity.
CHLOROPHYLL DEGRADATION BY PEROXIDASE IN PARSLEY LEAVES

Results

Table 1 shows the results of chlorophyll degradation by the peroxidase-hydrogen peroxide system. Purified chlorophylls a and b were not degraded by the system. On the other hand, chlorophylls contained in the parsley extract were degraded, and the degree of degradation of chlorophyll a was appreciably higher than that of chlorophyll b.

This fact seemed to indicate that the chlorophylls were not degraded directly by the peroxidase-hydrogen peroxide system, but that peroxidase oxidized some unknown substances in the parsley extract which then degraded the chlorophylls. Consequently, the identification of the unknown substances

layer chromatography (silica gel, solvent: toluene : ethyl formate : formic acid = 5 : 4 : 1).

Fig. 2. Isolation of the substances having chlorophyll a degradation activity from ButOH and aqueous fractions by using of column chromatography.

Column: Sephadex LH-20 (1.8 x 100 cm), Solvent: 80% EtOH, Flow rate: 0.04 ml/min, Fraction: 5 ml.

Upper: Optical density at 250 nm (substances including flavonoids) and 450 nm (substances including carotenoids).

Lower: Chlorophyll degradation activity

The reaction mixture contained 0.3 ml of each fraction, 30 μg chlorophyll a, 0.04% Triton X-100, 0.012% hydrogen peroxide, 20 μg peroxidase, and 68 mM phosphate buffer (pH 6.0) in a total volume of 2.5 ml. Chlorophyll degradation was assayed spectrophotometrically by reading the fall of absorbance at 668 nm, the spectral maximum of chlorophyll a in this reaction solution, as chlorophyll a was degraded.
was carried out as follows.

By using different solvents shown in Fig. 1, the unknown substances were fractionated from the EtOH extracts of parsley leaves. The chlorophyll degradation activities of each fraction were determined. As shown in Table 2, chlorophyll a was degraded by the peroxidase-hydrogen peroxide system in the presence of some substances in the ButOH and aqueous fractions, and the degradation by the aqueous fraction was more significant than that by ButOH fraction. Fig. 2 shows the isolation by column chromatography of the substances involved in chlorophyll degradation. The substances in the ButOH fraction showed activity in 2 fractions (I and II) of the chromatogram, while those in the aqueous fraction, chiefly, in one fraction (III). The substances in fraction III markedly degraded chlorophyll as compared with others. The spectral maxima of the fractions (I, II, and III) in methyl alcohol (MeOH) solution are shown in Table 3. The maxima of UV absorption spectrum occurred at 268 and 330-333 nm in all fractions, corresponding well to that of apigenin glycosides (26). The color reactions of the 3 fractions with diagnostic reagents are shown in Table 4. These results also indicated that flavonoid pigments were contained in the 3 fractions because the fractions exhibited yellowing with AlCl₃ and H₂SO₄, browning with FeCl₃, and bluing with FeCl₃ plus K₃Fe(CN)$_₆$. As is apparent in Table 3, fraction I contained not only flavonoid but also carotenoid pigments, showing spectral maxima in the region of 420-470 nm. The carotenoids were isolated by thin layer chromatography (silica gel, solvent : benzene : ethyl acetate : MeOH=75 : 20 : 5), and the chlorophyll degradation by the peroxidase–hydrogen peroxide system in the presence of carotenoids was studied. It was found that the carotenoids were not involved in the reaction of chlorophyll degradation (data not shown).

As the substances involved in chlorophyll degradation seemed to be flavonoid glycosides having sugars at various position (26), fractions I, II, and III were hydrolyzed by HCl solution and the resultant aglycones were identified. Fig. 3 shows the thin layer chromatogram of the aglycones; the Rf value of the main aglycone was about 0.63, agreeing well with that of apigenin. The absorption spectrum of the aglycone was also determined; as shown in Table 5, the spectral maxima of the aglycone in MeOH solution as well as the shift of the maxima by the addition of sodium methylate (NaOMe) and AlCl₃ coincided completely with those of apigenin glycosides.
CHLOROPHYLL DEGRADATION BY PEROXIDASE IN PARSLEY LEAVES

Degradation of chlorophyll by the peroxidase-hydrogen peroxide system in the presence of apigenin was also determined. As expected, chlorophyll was degraded in the system, while no change occurred without apigenin, as shown in Table 6.

From these results, the mechanism of chlorophyll degradation by the peroxidase-hydrogen peroxide system in parsley leaves is assumed to be that peroxidase first oxidizes an apigenin, and then chlorophylls are degraded by the oxidized apigenin.

Discussion

It has been reported that chlorophyllase catalyzes the removal of phytols from chlorophylls to form chlorophyllides(8, 10). Looney and Patterson observed that chlorophyllase activity in apple and banana fruits increased following climacteric rise(13). It was also reported that in ethylene-treated citrus fruits, chlorophyll in peels was degraded with the enhancement of chlorophyllase activity(2, 25).

On the other hand, it was thought that chlorophyllase combines chlorophyllides and phytols to form chlorophylls(24). Purvis observed that chlorophyllase in green calamondin fruits showed higher activity than that in calamondin fruits which have lost approximately half of their chlorophyll(21). In addition, Aljuburi et al. suggested that chlorophyllase was closely related to chlorophyll biosynthesis since chlorophyllase activity increased following the increase of chlorophyll content during the regreening of valencia oranges(1). In detached leaves of tobacco and radish, chlorophyllase activity decreased with yellowing(20, 23). We reported in the previous paper that chlorophyllase activity showed a sharp decline with yellowing in stored parsley leaves (30), suggesting that chlorophyllase may not be involved in chlorophyll degradation associated with leaf yellowing during storage.

It has been further reported that peroxidase activity increased with the progress of senescence in many species of plants(6, 19). We also found that peroxidase activity increased with yellowing of stored parsley leaves and that the enzyme was supposed to relate to chlorophyll degradation(30). In this study, it is concluded that peroxidase degrades chlorophylls: peroxidase first oxidizes an apigenin, a major flavonoid of parsley leaves (29), and then chlorophylls are degraded by the oxidized apigenin. It was also recognized by Matile(14) and Huff(11) that chlorophylls were degraded by a peroxidase-hydrogen peroxide system in the presence of phenolic compounds, such as 2, 4-dichlorophenol and resorcinol. Ferguson et al. demonstrated that the hydrogen peroxide content increased in parallel with chlorophyll degradation when discs of cucumber cotyledon were floated(5). Frenkel and Eskin observed that the hydrogen peroxide content increased in the course of ripening of tomato fruits after harvest (7). In addition, it was noticed that the chlorophyll content declined in rice seedlings treated with hydrogen peroxide as compared with non-treated ones (18). These facts seem to indicate that the chlorophyll degradation by the peroxidase-hydrogen peroxide system would occur in many fruits and vegetables as well as in parsley leaves.

Table 5. Spectral maxima in UV of the isolated aglycone, and their shift by addition of sodium methylate (NaOMe) and AlCl3.

<table>
<thead>
<tr>
<th>Aglycone</th>
<th>MeOH</th>
<th>NaOMe</th>
<th>AlCl3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apigenin</td>
<td>268, 235, 384, 318, 383, 277, 302, 346, 384</td>
<td>277, 302, 346, 384</td>
<td></td>
</tr>
</tbody>
</table>

*= 9% in MeOH solution.

*= 1% in MeOH solution.

The pure apigenin was purchased from Sigma.

Table 6. Chlorophyll degradation by peroxidase-hydrogen peroxide system in the presence of apigenin.

<table>
<thead>
<tr>
<th>Chlorophyll a degradation*</th>
<th>Apigenin (0.02 μ mole)</th>
<th>Control*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25</td>
<td>8</td>
</tr>
</tbody>
</table>

*= Chlorophyll degradation as percent of initial.

*= Apigenin was not contained in the reaction mixture of control.

The pure apigenin was purchased from Sigma.
While we have recognized the effect of L-ascorbic acid in retarding the chlorophyll degradation by peroxidase(30), it is thought that L-ascorbic acid would reduce the amount of oxidized apigenin produced by peroxidase.

Martinoia et al. demonstrated that chloroplast thylakoids showed a chlorophyll-bleaching activity in the presence of hydrogen peroxide and monophenol, such as a 2,4-dichlorophenol (15). Takahama also found that flavonols, such as quercetin and quercitrin, were oxidized by peroxidase-like activity in spinach chloroplasts in the presence of hydrogen peroxide (27). Nakano and Asada observed that hydrogen peroxide was generated from thylakoids in illuminated spinach leaves (16). Sanuders and McClure found that many kinds of flavonoid were contained in the chloroplasts of many species of vascular plants (22). Consequently, it is inferred that both peroxidase and apigenin are contained in parsley chloroplast, and that the oxidation of apigenin by the peroxidase-hydrogen peroxide system occurs in the chloroplast. Conformation of this is the subject for a future study.

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CHLOROPHYLL DEGRADATION BY PEROXIDASE IN PARSLEY LEAVES


パセリーにおけるペルオキシダーゼによるクロロフィルの分解

山内 直樹*2・南田 隆久
大阪府立大学農学部 591 堺市百舌鳥梅町

要

本研究は、パセリーにおけるペルオキシダーゼによるクロロフィルの分解について追及した。パセリー葉柄のエタノール抽出物にペルオキシダーゼ並びに過酸化水素を添加すると、クロロフィルの分解が認められた。しかしながら、精製したクロロフィルはペルオキシダーゼ・過酸化水素系で分解されなかった。この結果から、パセリーのエタノール抽出物中に含有される未知物質がペルオキシダーゼ・過酸化水素系によって酸化され、その酸化生成物がクロロフィルを分解しているものと思われたので、以下未知物質の検討を行った。

未知物質は溶媒分画並びにカラムクロマトグラフィーによって分離され、紫外外部吸収极大位置からアビゲニン配糖体であると推定した。さらに、塩酸による水分解によりアグルロンを抽出し、薄層クロマトグラフィーでのRf値並びにスペクトル特性の検討により、未知物質はパセリーの主要フラボノイドのアビゲニンであることを見出した。

以上の結果から、パセリーにおけるクロロフィルの分解は、ペルオキシダーゼがアビゲニンを酸化し、生成したアビゲニンの酸化物がクロロフィルを分解することを認め、収穫後におけるパセリーの黄化にペルオキシダーゼが関与しているものと推察した。

*1 収穫された葉菜類のクロロフィル分解機構. 第1報
*2 現在 兵庫県立姫路短期大学