Purification and Characterization of ACC Oxidase and Increase in its Activity during Ripening of Pear Fruit

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Summary

Pear (Pyrus communis L. cv. La France) fruit induced to ripen by low-temperature treatment was used for this study. The activity of ACC (1-aminoacyclopropane-1-carboxylic acid) oxidase and the rate of ethylene production increased rapidly during fruit ripening at 20°C. ACC oxidase, isolated from pear fruit at the climacteric stage, was purified to near homogeneity. Its molecular mass was estimated to be 40 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Pear fruit ACC oxidase was recognized by the antibody raised against ACC oxidase, purified from Escherichia coli cells transformed with cDNA of broccoli ACC oxidase by western blotting. The apparent Km values for their substrates ACC and O_2 of ACC oxidase were estimated to be 42.2 μM and 0.53 %, respectively. The optimal concentration of ascorbate, co-substrate, for ACC oxidase was 15 mM and the apparent Km for ascorbate was estimated to be 1.9 mM. The concentrations of cofactors Fe^{2+} and HCO_3^- for maximum activity were 25 μM and 30 mM, respectively; the Km for HCO_3^- was estimated to be 3.8 mM. These enzymatic properties were found to be similar to ACC oxidase derived from other plants.

Key Words: ACC oxidase, enzyme characterization, ethylene, fruit ripening, Pyrus communis.

Introduction

Ethylene is a plant hormone which regulates many aspects of plant growth, development, and senescence. It promotes fruit ripening and senescence, and thus plays an important role in the ripening of climacteric fruits. In higher plants, the ethylene biosynthesis pathway has been established as follows: methionine → S-adenosylmethionine (SAM) → 1-aminocyclopentane-1-carboxylic acid (ACC) → ethylene (Adams and Yang, 1979; Yang and Hoffman, 1984; Abeles et al., 1992). There are two crucial enzymes associated with this pathway, ACC synthase and ACC oxidase. ACC synthase catalyzes the cleavage of SAM to ACC and S'-methylthioadenosine, and ACC oxidase catalyzes the oxidation of ACC to ethylene.

Low-temperature treatments are essential and commercially beneficial to synchronize the onset of ripening of pear fruits after harvest. 'Bartlett' pear fruits were promoted to produce ethylene in response to low-temperature treatments either on the tree (Wang et al., 1971) or after harvest (Looney, 1972). Similarly, the low-temperature treatments stimulated autocatalytic ethylene production in 'Conference' pear and 'Golden Delicious' apple fruits (Knee et al., 1983). Murayama et al. (1995) reported that ethylene production and ACC content increased rapidly after low-temperature treatment in 'Le Lectier' pear. The same treatments induced ACC oxidase activity in 'Granny Smith' apple fruit (Lelièvre et al., 1995). ACC synthase activity and ethylene production were also induced by exposure to low temperatures and these increases were inhibited by cycloheximide in 'Conference' pear (Knee, 1987). The expression of ACC oxidase gene and its activity were strongly induced by both low-temperature and propylene treatments in pears (Lelièvre et al., 1997).

ACC oxidase catalyzes the oxidation of ACC to ethylene: ACC + ascorbate + O_2 → C_2H_4 + CO_2 + HCN + 2H_2O + dehydroascorbate (Dong et al., 1992), in which Fe^{2+} is required for its activity (Veraverbig and John, 1991). This enzyme, which is CO_2-activated (Dong et al., 1992) has been studied in melon (Smith et al., 1992), avocado (McGarvey and Christoffersen, 1992), apple (Kuai and Dilley, 1992; Fernández- Maculet and Yang, 1992; Dong et al., 1992; Dupille et al., 1993), winter squash (Hyodo et al., 1993), pear (Vioque and Castellano, 1994), and banana (Moya–Leon and John, 1995). The enzyme was purified to homogeneity from apple (Dong et al., 1992; Dupille et al., 1993) and banana fruits (Moya–Leon and John, 1995). The expression of the ACC oxidase gene was studied in broccoli.

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(Pogson et al., 1995), melon (Yamamoto et al., 1995),
tomato (Nakatsuka et al., 1997), pear (Lelièvre et al.,
1997), and kiwifruit (Whittaker et al., 1997). In tomato
plants the antisense pTom13, which encodes ACC
oxidase, inhibited synthesis of ethylene (Hamilton et al.,
oxidase from pear fruit (cv. Blanquilla) and investigated
its enzymatic properties. They used an extract without
purification for characterizing ACC oxidase in pear
fruit.

In this work, we studied the changes in the activity of
ACC oxidase during the ripening of pear fruits in
relation to ethylene production; we tried to purify ACC
oxidase to almost homogeneity from pear fruit to char-
acterize its enzymatic properties.

Materials and Methods

Plant material and postharvest treatment

Pear (Pyrus communis L. cv. La France) fruit were
harvested at commercial maturity in an orchard in
Kamiyama-shi, Yamagata prefecture and stored at 2°C
for 2 weeks. After the low-temperature treatment, fruit
were held at 20°C. For the purification of ACC oxidase,
fruit were allowed to ripen at 20°C and reach a climac-
teric and then the accessory/receptacle tissue (edible
pulp) was stored at -80°C until used for enzyme
extraction.

Assay of ethylene production

A pear fruit was placed in a 920-ml jar and sealed
with a plastic screw cap and vinyl tape for 1 hr at 20°C.
A 1-ml gas sample was withdrawn from the internal
atmosphere and injected into a gas chromatograph
(Hitachi 163) fitted with an alumina column at 70°C and
a flame ionization detector for ethylene determination.

Extraction of ACC oxidase

For the time-course experiments to assay ACC
oxidase activity during ripening, the pear pulp was
homogenized with a mortar and pestle in 10 times
volume (v/w) of 0.1 M Tris-HCl buffer, pH 7.2, con-
taining 30 mM sodium ascorbate, 5 mM dithiothreitol
(DTT), 10% glycerol (w/v) at 2°C. The homogenate
was centrifuged at 14,000 x g for 20 min at 2°C. The
supernatant was filtered through Miracloth and the crude
extract was used for measuring ACC oxidase activity.
For enzyme purification, the frozen tissue was homoge-
ized with a mortar and pestle in 2 times volume of 0.1
M Tris-HCl buffer, pH 7.5, containing 30 mM sodium
ascorbate, 5 mM DTT, 10% glycerol, 10 μM 1,10-
phenanthroline (PA), and 2% polyvinylpolypyrrolidone
(PVPP) at 2°C. The homogenate was centrifuged at
14,000 x g for 20 min at 2°C. The supernatant was
filtered through Miracloth and the filtrate subjected to
further purification.

Concentration and desalting of ACC oxidase

To 420 ml crude extract, 21 g (5%) of polyeth-
ylenyglycol 4,000 (PEG) were added and the mixture
stirred for 10 min. Then 163.8 g (0.55 saturation)
of ammonium sulfate (As) were added and the mixing
continued for 30 min longer. After centrifugation at
14,000 x g for 20 min at 2°C, the bottom layer was
removed and the upper layer containing PEG was stirred
in 210 ml of the medium, consisting of 20 mM Tris-
HCl buffer, pH 7.2, 30 mM sodium ascorbate, 5 mM
DTT, 10% glycerol (w/v), 10 μM PA, and 1.4 M As
with the aid of mortar and pestle. After re-centrif-
ugation at 14,000 x g for 20 min, the bottom layer,
which includes the enzyme solution, was withdrawn
with a pipet and filtered through Miracloth. By these
procedures the enzyme was concentrated and partially
purified.

The concentrated extract was passed through a col-
umn of Sephadex G-25 (bed volume 700 ml), pre-
equilibrated with buffer A (20 mM Tris-HCl buffer, pH
7.2, 30 mM sodium ascorbate, 5 mM DTT, 10% glycerol
(w/v), 10 μM PA) for desalting. A fraction of
240 ml was collected and assayed.

Anion exchange chromatography

A DEAE-Sephalcel column (bed volume 100 ml) was
pre-equilibrated with buffer A, to which the pooled
fraction from Sephadex G-25 was applied. After wash-
ing with buffer A, ACC oxidase was eluted with a linear
gradient of KCl (0 to 2 M) at a flow rate of 1.2 ml
min⁻¹ at 4°C. ACC oxidase fraction of 24 ml was
collected.

Gel filtration chromatography

A Sephadex G-75 column (bed volume 700 ml) was
pre-equilibrated with buffer A. The pooled fraction
from DEAE-Sephalcel was passed through the column
at 4°C. An 80-ml fraction which contained ACC
oxidase activity was collected and assayed.

Assay of ACC oxidase

ACC oxidase activity was assayed in the medium
consisting of 0.1 M Tris-HCl buffer, pH 7.2, 30%
glycerol (w/v), 1 mM ACC, 10 mM sodium ascorbate,
50 μM FeSO₄, and 10 mM NaHCO₃ in a total volume
of 2 ml. The enzyme reaction was performed in a 16.1-
ml test tube which was sealed with a rubber serum cap
and gently shaken at 30°C. After incubation for 20 min,
a 1-ml gas sample was withdrawn from the atmosphere
of the tube and analyzed for ethylene by gas chromato-
graphy as described above. ACC oxidase activity was
expressed as nL ethylene per hr per g fresh weight and its
specific activity as nL ethylene per hr per mg protein.

Protein assay

Protein was measured by the method of Bradford
using bovine serum albumin as a standard.

**Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE)**

SDS-PAGE was performed in 12.5% polyacrylamide gel (Atto) in the presence of 0.1% SDS according to the method of Laemml (1970). After electrophoresis, proteins on the gel were stained with the silver staining kit (Pharmacia) according to the manufacturer’s instruction.

**Western blotting**

After SDS-PAGE, proteins were electrotransferred from the SDS-PAGE gel to a nitrocellulose membrane (Pharmacia Phast system). Free binding sites of nitrocellulose membrane were blocked by incubation with 10% bovine serum albumin in the buffer containing 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween-20. The membrane was incubated with a solution (1:100 dilution) of a rabbit polyclonal antibody raised against recombinant *E. coli* ACC oxidase (Kasai et al., 1998). Then the membrane was incubated with a solution (1:5000 dilution) of an anti-rabbit IgG antibody conjugated to horseradish peroxidase (Amersham). The membrane was exposed to blue-light sensitive autoradiography film for the detection.

**Results and Discussion**

*Increases in the activity of ACC oxidase and the rate of ethylene production in pear fruit during storage at 20 °C*

The activity of ACC oxidase reached a level over 250 nl·g⁻¹·hr⁻¹ on the 3rd day at 20 °C after cold storage. Subsequently, the activity increased rapidly, reaching a peak on the 11th day, then decreasing sharply (Fig. 1). The rate of ethylene production also began to increase gradually after the 3rd day, reaching a peak on the 11th day, then declining sharply (Fig. 2). Because ACC oxidase activity and ethylene production rate remained almost constant from the day of transfer to the 3rd day in the separate experiments, ACC oxidase activity and ethylene production rate started from the 3rd day (Fig. 1 and 2). The rate of ethylene production was much slower than that of ACC oxidase activity, which may have resulted from the low ACC content (data not shown) or because in vivo and in vitro ACC oxidase activities may differ. We demonstrated that the level of in vitro ACC oxidase activity in ‘La France’ was much higher than in vivo activity of ‘Anjou’ (Gerasopoulos and Richardson, 1997).

Ripening of pear fruit progressed rapidly, accompanied by softening (Wang et al., 1985) and degradation of chlorophyll in the peel (Gerasopoulos and Richardson, 1997) after the low-temperature treatment. Pear fruit exhibits a climacteric rise of respiration and rapid increase of ethylene production by the low-temperature (Wang et al., 1985) and ethylene treatments (Wang et al., 1972). Lelièvre et al. (1997) reported that ACC oxidase mRNA accumulated rapidly in the fruit during ripening after the low-temperature treatment; its transcript accumulation was suppressed by the treatment with 1-methylcyclopropene, an inhibitor of ethylene action which is induced by propylene. Our results indicate that the rapid increase in ethylene production rate may correlate with that of ACC oxidase activity during fruit ripening (Fig. 1 and 2), suggesting the possibility that the rise in ACC oxidase activity was induced by an increase in ethylene.

**Purification of ACC oxidase from pear fruits**

For the extraction of ACC oxidase from pear, PVPP, a phenolic-binding compound, was effective (Kuai and Dilley, 1992). ACC oxidase purification has encountered many difficulties because of the instability of the enzyme activity. For instance, within 10 hr after the extraction, ACC oxidase activity in a crude extract
Table 1. Purification steps of ACC oxidase from pear fruit.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (nl·h⁻¹)</th>
<th>Total protein (mg)</th>
<th>Specific activity (nl·h⁻¹·mg protein⁻¹)</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>22463.2</td>
<td>61.5</td>
<td>365.2</td>
<td>--</td>
<td>100.0</td>
</tr>
<tr>
<td>PEG-As, Sephadex G-25</td>
<td>19330.3</td>
<td>26.9</td>
<td>715.3</td>
<td>2.0</td>
<td>86.1</td>
</tr>
<tr>
<td>DEAE- Sephacel</td>
<td>9699.7</td>
<td>10.6</td>
<td>896.6</td>
<td>2.5</td>
<td>43.2</td>
</tr>
<tr>
<td>Sephadex G-75</td>
<td>5751.4</td>
<td>4.2</td>
<td>1364.5</td>
<td>3.7</td>
<td>25.6</td>
</tr>
</tbody>
</table>

decreased rapidly to a half (data not shown). Phenylmethanesulfonyl fluoride, an inhibitor of serine protease, could not prevent its rapid decrease (Dupille et al., 1993). This inactivation could be partially prevented by the addition of catalase, which implies that H₂O₂ is involved (Smith et al., 1994). Barlow et al. (1997) suggested that there are three inactivation processes of ACC oxidase: 1) partial unfolding of the catalytically active conformation; 2) oxidative damage from H₂O₂ in solution, which is catalase protectable; 3) oxidative damage to the active site, which is not catalase protectable. PA, a metal chelating agent, effectively stabilized ACC oxidase activity during the purification (Dupille et al., 1993).

To concentrate ACC oxidase from crude extract, we adopted the method of Moya-Leon and John (1995) with a slight modification, because conventional ammonium sulfate precipitation was not effective. Using this modification, ACC oxidase in the crude extract was purified effectively and concentrated with a high rate of recovery. The purification steps in Table 1 show that its specific activity was increased 3.7 fold with 25.6% recovery from 117-g tissue of pear fruits. This low purification fold is attributed to the rapid loss of ACC oxidase activity during the purification procedures.

The progress of purification is shown on SDS-PAGE in Fig. 3. ACC oxidase from pear fruit was purified to near homogeneity and the major protein band was detected in the fraction eluted from Sephadex G-75. The molecular mass was estimated to be 40 kDa. This value well agrees with ACC oxidase of other plant sources; 35.0 kDa and 40.0 kDa from apple fruit (Dong et al., 1992; Dupille et al., 1993), 41.0 kDa from melon fruit (Smith et al., 1992), and 36.0 kDa from banana fruit (Moya-Leon and John, 1995). The single protein band was recognized by the antibody raised against the recombinant E. coli ACC oxidase (Kasai et al., 1998) (Fig. 3), indicating that the 40 kDa protein has the same antigenic determinant of recombinant E. coli ACC oxidase.

Characterization of ACC oxidase from pear fruits

Using the purified enzyme preparation after passage through Sephadex G-75, ACC oxidase in pear fruit was characterized as follows: 1) ACC oxidase activity depends on ACC and O₂ concentrations; 2) the apparent Km values for ACC and O₂ of ACC oxidase are 42.2 µM and 0.53 %, respectively (Fig. 4 and 5). This value is similar to 57.5 µM for pear fruit (Vioque and
Fig. 5. Michaelis–Menten kinetics of ACC oxidase activity as a function of O₂ concentrations. The vertical bars indicate SE (n=3). Insert: double-reciprocal plot.

Castellano, 1994) and 56 μM for banana fruit (Moya-Leon and John, 1995). The Km value (0.53 %) for O₂ agrees with 0.44 % for pear fruit (Vioque and Castellano, 1994) and 0.4 % for apple fruit (Kuai and Dilley, 1992).

The dependence of ACC oxidase activity on ascorbate which is known to be another substrate (Dong et al., 1992) was also determined. The optimal concentration of ascorbate for ACC oxidase activity was 15 mM and an apparent Km for ascorbate was estimated to be 1.9 mM. At a concentration higher than 30 mM, the enzyme activity was arrested (Fig. 6). The optimal concentration for ascorbate of pear approximates 10 mM for pear fruit (Vioque and Castellano, 1994), and 40 mM for banana fruit (Moya-Leon and John, 1995). The Km value (1.9 mM) for our purified ACC oxidase was much lower than 10 mM for the enzyme from banana fruit (Moya-Leon and John, 1995).

The optimal concentration of Fe^{2+} for ACC oxidase activity was 25 μM (Fig. 7) which is much lower than 0.2 mM for crude extract from pear fruit reported by Vioque and Castellano (1994), but similar to 10 μM for the purified enzyme from apple fruit (Dong et al., 1992). At a higher concentration of Fe^{2+}, the enzyme activity was gradually reduced.

ACC oxidase activity was also greatly enhanced by the addition of sodium bicarbonate (Fig. 8); the optimal concentration of HCO₃⁻ for maximum activity was 30 mM; Km of ACC oxidase was estimated to be 3.8 mM (Fig. 8) which is within the range of 2.9 mM for the purified enzyme from banana fruit (Moya-Leon and John, 1995).
ACC oxidase from pear fruit is considered to belong to the ferrous-ascorbate dioxygenase family (Prescott and John, 1996). Our results revealed that pear ACC oxidase has an absolute requirement for Fe$^{2+}$ and ascorbate (Fig. 6 and 7) and that cross-reacted with the antibody raised against the ACC oxidase purified from the recombinant E. coli transformed with cDNA of broccoli ACC oxidase. We could use the affinity column based on the antibody to further purify ACC oxidase isolated from various plants. Such a highly purified ACC oxidase would allow one to investigate its enzymology and the regulation of ethylene biosynthesis during fruit ripening.

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Literature Cited


セイヨウナシ果実におけるACC酸化酵素の精製と性質および成熟過程における活性増大

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摘 要

低温処理して追熟を誘導させたセイヨウナシ（Pyrus communis L. cv. La France）果実を本研究に使用した。ACC酸化酵素活性とエチレン生成量は、果実の成熟過程（20℃）において急速に増大した。クライマックテリック段階のセイヨウナシ果実から抽出されたACC酸化酵素はほぼ均一にまで精製された。その分子量はSDS-PAGEから40kDaであることか推定された。ウエスタンブロット分析においてセイヨウナシのACC酸化酵素は、形質転換された大腸菌から精製されたACC酸化酵素に対する抗体により認識された。精製したセイヨウナシ果実のACC酸化酵素の基質ACCとO₂に対するKm値は、それぞれ42.2μMと0.53%であった。補基質であるアスコルビン酸に対する最適濃度は15mMであり、Km値は1.9mMであった。補因子であるFe²⁺とHCO₃⁻に対するACC酸化酵素の最適濃度は、それぞれ25μMと30mMであり、HCO₃⁻に対するKm値は3.8mMであった。これらの酵素学的性質は、他の植物由来のACC酸化酵素の性質と類似していた。