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(*Invited paper*)

Characterization of 1-aminocyclopropane-1-carboxylate (ACC) oxidase in broccoli florets and from *Escherichia coli* cells transformed with cDNA of broccoli ACC oxidase

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Abstract. During senescence of broccoli (*Brassica oleracea* L. var. *italica*) florets (flower buds), the rate of ethylene production increased, almost paralleling the rapid increase in 1-aminocyclopropane-1-carboxylate (ACC) oxidase activity. The increase in ACC oxidase activity was strongly suppressed by the administration of cycloheximide, suggesting that it may have resulted from the de novo synthesis of enzyme protein. ACC oxidase extracted from broccoli florets exhibited the requirements for ferrous ion, ascorbate, and CO_2 in its activity besides ACC and O_2 as substrates. ACC oxidase was isolated and purified from *Escherichia coli* cells transformed with cDNA of broccoli ACC oxidase. The recombinant bacterial enzyme was very similar to that of native broccoli enzyme in its enzymatic properties. The molecular mass of the purified enzyme from the transformed cells was estimated to be 37 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The polyclonal antibody was raised in a rabbit against the purified enzyme isolated from the transformed bacteria, which was found to cross react with the enzyme of broccoli florets. Western blot analysis showed that ACC oxidase protein was detected at an almost similar level over the progressive stages of senescence.

Keywords: 1-Aminocyclopropane-1-carboxylate (ACC) oxidase; *Brassica oleracea*; Broccoli florets; *Escherichia coli*; Senescence; Transformant.

Introduction

Broccoli florets (flower buds) senesce rapidly after harvest at ambient temperatures. Symptoms include a rapid yellowing of florets accompanied by degradation of ascorbic acid and chlorophyll. That endogenous ethylene is involved in the postharvest senescence of florets has been reported (Lieberman and Hardenburg, 1954; Wang, 1977; Aharoni et al., 1985; Makhlouf et al., 1989; Tian et al., 1994). We demonstrated that the rate of ethylene production in broccoli florets increased to a maximum and then declined during senescence, almost paralleling the increasing pattern of ACC oxidase activity (Kasai et al., 1996). It was suggested that the induction of ACC oxidase activity was regulated by endogenous ethylene produced during senescence (Kasai et al., 1996).

In higher plants ethylene is synthesized via methionine-ACC pathway (Yang and Hoffman, 1984; Abeles et al., 1992). In this pathway two crucial enzymes regulate the rate of ethylene synthesis, i.e. ACC synthase and ACC oxidase (Yang and Hoffman, 1984; Abeles et al., 1992; Kende, 1993; Prescott and John, 1996). The enzymology of ACC oxidase has been studied extensively (Ververidis and John, 1991; Dong et al., 1992; McKeon et al., 1995; Prescott and John, 1996; John, 1997).

We extracted ACC oxidase from broccoli florets and investigated its enzymatic properties (Kasai et al., 1998). In the present study we isolated and purified ACC oxidase from *E. coli* cells transformed with cDNA of broccoli ACC oxidase, and we compared the enzymatic properties between recombinant bacterial enzyme and native broccoli enzyme. The rabbit antibody has been raised against the purified enzyme from the trnasformed cells, which was used for immunological studies with the broccoli enzyme. We also examined if the increase in ACC oxidase activity in broccoli florets during senescence is derived from de novo synthesis of enzyme protein.

Materials and Methods

Extraction and Assay of ACC Oxidase from Broccoli Florets

Broccoli heads were obtained from a local market in Shizuoka city. Those heads were held at 21±1°C under humidified conditions. Senescence of florets was visualized by the advance of yellowing of florets (Kasai et al.,

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1996). Florets were excised from heads with a scalpel and used for determination of ethylene production by gas chromatography (Kasai et al., 1996). The ACC oxidase was extracted from samples of florets and assayed according to the procedures described previously (Hyodo et al., 1993; Kasai et al., 1996; Kasai et al., 1998). Florets were homogenized with a mortar and pestle in 0.1 M Tris-HCl buffer, pH 7.2, containing 30% glycerol, 10 mM sodium ascorbate, and 5 mM dithiothreitol. The homogenate was centrifuged at 14,000 *g* for 20 min. The supernatant was assayed for ACC oxidase activity in a reaction medium consisting of 0.1 M Tris-HCl buffer, pH 7.2, 30% glycerol, 1 mM ACC, 10 mM sodium ascorbate, 50 μM $FeSO_4$, and 10 mM $NaHCO_3$ in a total of 2 ml in a sealed test tube with an atmospheric volume of 14.1 ml. ACC oxidase activity was expressed as the amount of ethylene converted from ACC during the reaction period. ACC oxidase from broccoli florets was partially purified by the procedures of ammonium sulfate fractionation between 0.3 and 0.7 saturation and gel filtration through a Sephadex G-25 column.

Extraction and Assay of ACC

Florets were homogenized with 80% ethanol with a mortar and pestle. The homogenates were centrifuged, and the supernatant was cencentrated to dryness in a rotary evaporator. The residue was taken up in water, and an aliquot (0.5 ml) was assayed for ACC by the method of Lizada and Yang (1979).

Treatment of Florets with Cycloheximide

Segments (curds) excised from broccoli heads were immersed in three different concentrations of cycloheximide, 0.1, 0.5, and 1.0 mM, respectively, containing 0.1% Tween 20 (polyoxyethylene (20) sorbitan monolaurate) for 15 min at a room temperature. Control segments were dipped in 0.1% Tween 20 without cycloheximide in the same way. After treatment with the solution, the segments were lightly blotted with tissue paper and incubated at 21±1°C under a humidified condition until used for measurement of ethylene evolution and enzyme extraction.

Transformation of E. coli with cDNA of Broccoli ACC Oxidase

cDNA of broccoli ACC oxidase was amplified by reverse transcription (RT)-polymerase chain reaction (PCR). Two oligonucleotide primers were designed for PCR based on the DNA sequence of broccoli ACC oxidase (ACCOx1) determined by Pogson et al. (1995); primer 1, 5'-GCGCGGATCCATGGAGAAGAACATTAAGTT-3'; primer 2, 5'-GTAACTCGAGTTAGAAAGTCTCTACGGC TG-3'. DNA sequence of the obtained product (963 bp) was found to be almost the same as ACCOx1 (99.8% identical). The cDNA insert was ligated into the expression vector pGEX-4T-1 (Pharmacia) between the galactose-inducible promotor and the terminator of the plasmid. *Escherichia coli* strain XL1-blue competent cells were transformed with the recombinant plasmid.

Expression and Purification of Recombinat ACC Oxidase

Expression was induced in transformed *E. coli* cells cultured in a growing medium (1% yeast extract, 0.2% bactotryptone, 0.5% NaCl) containing ampicillin by the addition of isopropyl-*β*-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. The induced cells were collected and centrifuged. ACC oxidase was expressed in the transformants as a fusion protein with glutathione S-transferase (GST). Pelleted cells were suspended in phosphate buffered saline containing 0.1% Nonidet P-40 and 5 mM DTT, and sonicated. The sonicates were centrifuged and the resultant supernatant was loaded on the column of glutathione-Sepharose 4B. After washing, the conjugated protein of ACC oxidase with GST on the column was digested by the addition of thrombin. The ACC oxidase eluted from the column was further purified by high performance liquid chromatography (HPLC) (Pharmacia) with a column of Superdex 75. The elution buffer was 0.05 M phosphate buffer, pH 7.0, containing 0.15 M NaCl. The purification steps were followed by SDS-PAGE.

Western Blot Analysis

Samples of broccoli florets were frozen in liquid nitrogen and stored at -80°C until used for protein extraction. The frozen tissue was macerated in SDS-loading buffer (0.02 M Tris-HCl, pH 6.8, 2% SDS, 20% glycerol) and centrifuged at 15,000 *g* for 60 min. The extract was separated by SDS-PAGE with a Pharmacia Phast system. The proteins were then electrotransferred from the gels to nitrocellulose membranes (Amersham). Membranes were blocked by incubation with a 10% BSA blocking reagent. Then nitrocellulose membranes were incubated with a 1:500 dilution of rabbit polyclonal antibodies raised against the purified ACC oxidase obtained from transformed *E. coli* cells. The membranes were washed to remove unbound antibody and then incubated with a solution containing 1:5000 dilution of antirabbit IgG conjugated to horseradish peroxidase (Amersham). The peroxidase was detected by enhanced chemiluminescence (Amersham) and autoradiography on ECL-Hyperfilm (Amersham).

Results and Discussion

Increase in ACC Oxidase Activity during Senescence of Broccoli Florets and Effect of Cycloheximide

During senescence of broccoli florets, the rate of ethylene production increased to a peak and then declined. In a previous paper we demonstrated that ACC oxidase activity in florets increased almost in parallel with an increasing ethylene production rate (Kasai et al., 1996). It was also shown that in the presence of 2,5-norbornadiene, an inhibitor of ethylene action, the increase in ACC oxidase activity was strongly suppressed, suggesting that endogenous ethylene produced in florets is involved in promoting the induction of ACC oxidase activity (Kasai et al., 1996).

In the present study we investigated the effect of cycloheximide on the increase in ACC oxidase activity. The results show that by the administration of cycloheximide at a concentration ranging from 0.1 to 1.0 mM to broccoli florets with Tween 20, the increase in the activity was strongly suppressed (Figure 1). These results suggest that the enhanced level of ACC oxidase activity results from de novo synthesis of enzyme protein. Asano and Furuta (1983) reported that the treatment of broccoli with cycloheximide greatly retarded the progress of senescence. We also found that the administration of cycloheximide markedly inhibited ethylene production and retarded the rate of degradation of ascorbic acid and chlorophyll in florets. These results indicate that newly formed proteins are required and involved in the development of senescence of broccoli florets including ACC oxidase as a key enzyme of ethylene biosynthesis.

Characterization of ACC Oxidase in Broccoli Florets

Broccoli ACC oxidase was extracted from florets and partially purified by ammonium sulfate fractionation and gel filtration, although its activity was significantly lost during these procedures. The enzyme required for its activity ferrous ion, ascorbate, and CO_2 (Kasai et al., 1998). For routine experiments we have used bicarbonate in the reaction mixture instead of CO_2 applied to the gas phase in the reaction tube. In Table 1, Km value for ACC and concentrations of ferrous, ascorbate, and bicarbonate for the maximal enzyme activity are shown. These values determined with broccoli ACC oxidase are comparable to those determined with ACC oxidase isolated from other plant sources (Ververidis and John, 1991; Dong et al., 1992; Hyodo et al., 1993; Kende, 1993; Prescott and John, 1996).

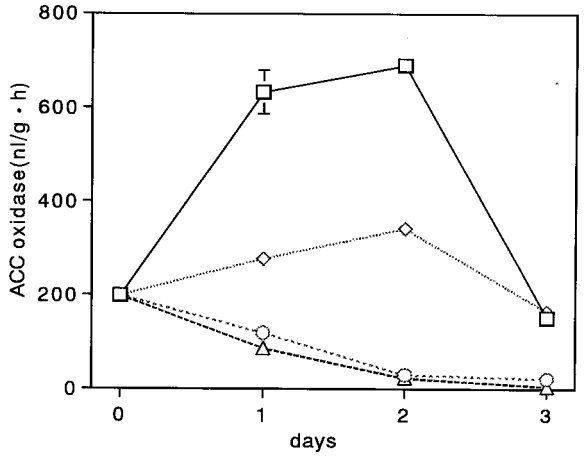

Figure 1. Effect of cycloheximide on increase in ACC oxidase activity during senescence of broccoli florets. (○), 0 (control); (◊), 0.1 mM; (¡), 0.5 mM; (Δ), 1.0 mM cycloheximide. Data are the means of three replicates. Bars represent SDs when larger than symbols.

We tried to purifiy ACC oxidase to a high extent from broccoli florets, but this has so far been unsuccessful, mostly due to the high instability of the enzyme activity. In fact we have experienced a great loss of enzyme activity in vitro during the purification processes. Thus we tried to isolate and purifiy ACC oxidase from bacterial cells transformed with the broccoli ACC oxidase gene.

Purification and Characterization of Recombinant ACC Oxidase

We constructed transformed *E. coli* cells by the recombinant plasmid with cDNA of ACC oxidase from broccoli. From the transformants ACC oxidase was expressed as a fusion protein with glutathione S-transferase (GST). Recombinant cells induced by IPTG were sonicated and centrifuged. The supernatant was purified by passing through an affinity column of glutathione Sepharose 4B. And the GST-ACC oxidase conjugate was hydrolyzed by thrombin to yield ACC oxidase free from GST. Finally, the ACC oxidase was purified by HPLC. The SDS-PAGE patterns indicate that by these procedures ACC oxidase from recombinant *E. coli* was highly purified as a 37 kDa protein (Figure 2).

The enzyme exhibited the Michaelis-Menten kinetics as a function of ACC concentration (Figure 3). From the Lineweaver-Burk reciprocal plot, the Km value for ACC was calculated to be 81.6 μM (Figure 3, inset). We compared the properties between native broccoli ACC oxidase, and recombinant *E. coli* ACC oxidase (Table 1). Both enzymes displayed a close similarity in enzymatic nature.

Expression of recombinant ACC oxidase has been made in *E. coli* (Zhang et al., 1995; Lay et al., 1996; Shaw et al., 1996), *Saccharomyces cerevisiae* (Hamilton et al., 1991; Wilson et al., 1993), and *Xenopus laevis* oocytes (Spanu et al., 1991). These expressed products of the inserted ACC oxidase genes exhibited characteristics similar to the native ACC oxidase originating from plants.

Time Courses of Ethylene Production Rate, ACC Oxidase Activity, ACC Level, and Western Blot Analysis

The polyclonal antibody was raised in a rabbit against the purified recombinant ACC oxidase isolated from trans-

Table 1. Comparison of enzymatic properties of ACC oxidase between native broccoli enzyme and recombinant *E. coli* enzyme.

Substrate/cofactor	Native ACC oxidae	Recombinant ACC oxidase
Km for ACC	55.6 μM	81.6 μM
Ferrous ion	10 μM	50 μM
Ascorbate	5 mM	5 mM
Bicarbonate	10 mM	10 mM

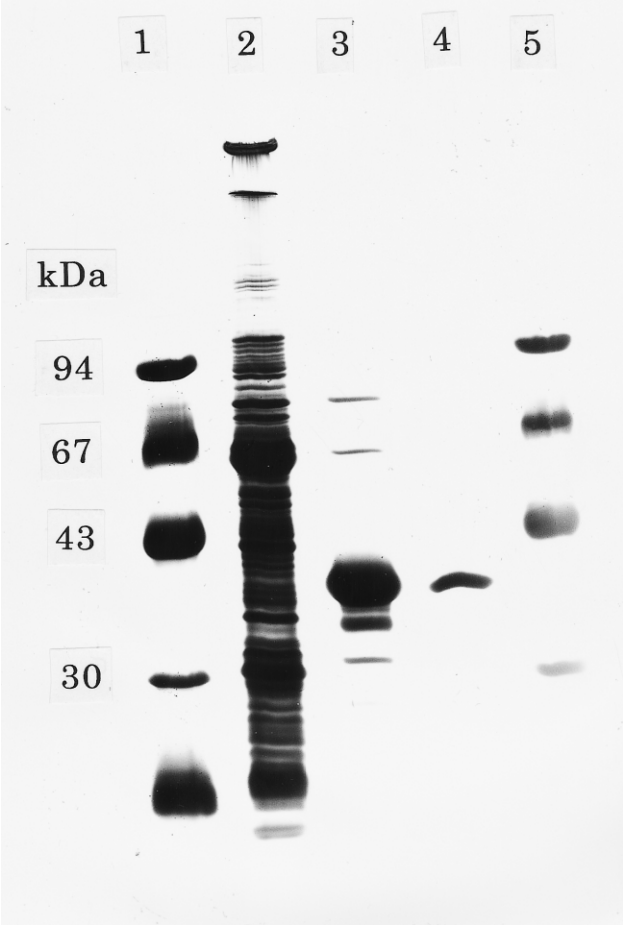

Figure 2. SDS-PAGE of recombinant ACC oxidase at the various stages of enzyme purification. Proteins in the gel were stained with the silver staining reagent (Pharmacia). Lane 1 and 5, marker proteins; lane 2, supernatant after sonication; lane 3, ACC oxidase obtained after glutathione Sepharose 4B column chromatography and thrombin hydrolysis; lane 4, ACC oxidase after HPLC.

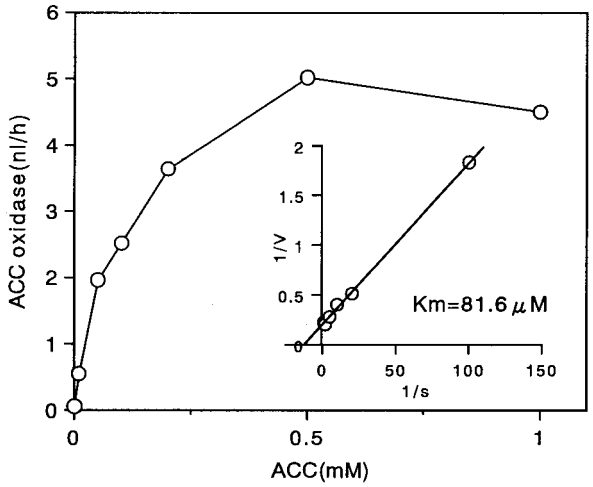

Figure 3. Michaelis-Menten kinetics of recombinant ACC oxidase as a function of ACC concentration. Inset, Lineweaver-Burk double-reciprocal plot.

formed *E. coli* cells. The antiserum obtained from the immunized rabbit was purified with protein A or by ammonium sulfate fractionation for isolating IgG fraction, which was used for western blotting.

At daily intervals during storage after harvest, broccoli florets were regularly sampled, and the rate of ethylene production and ACC oxidase activity were measured. The florets were frozen in liquid nitrogen and stored at -80°C until used. Frozen samples were extracted and assayed for ACC content and ACC oxidase protein by western blot analysis. During senescence of broccoli florets, ACC oxidase activity rapidly increased in association with the ethylene production rate (Figure 4b). ACC content in the florets increased gradually and reached a high level at the later stage of senescence (Figure 4c). In contrast to the rise and fall of ACC oxidase activity, ACC oxidase protein appeared to remain at a similar level (Figure 4a). Pogson et al. (1995) demonstrated by northern blot analysis that abundance of gene transcripts (mRNA) of ACC oxidase began to increase in broccoli florets after harvest in association with the rise in ethylene production rate. The reasons for the discrepancy between these results are not known. The change of ACC oxidase protein in relation to ACC oxidase activity remains to be investigated.

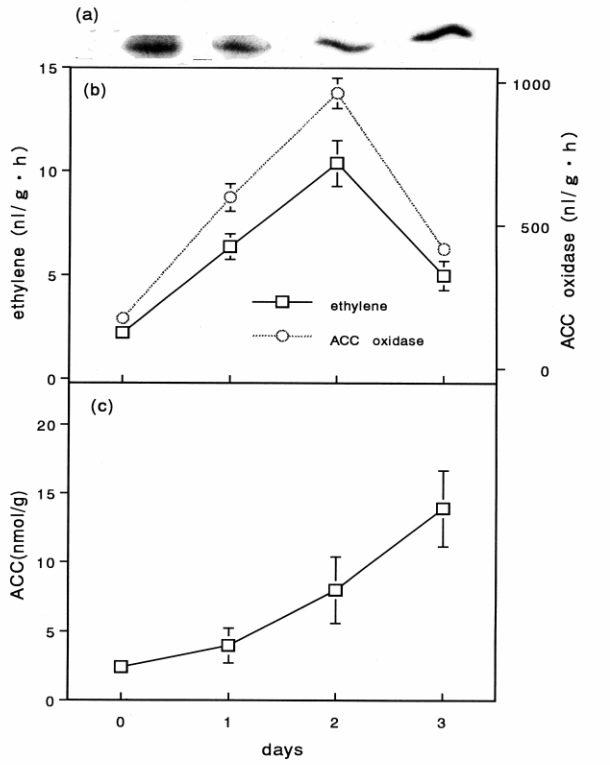

Figure 4. Time courses of rate of ethylene production, ACC oxidase activity, ACC content, and ACC oxidase protein (antigen) as observed by western blotting during senescence of broccoli florets. (a), western blot analysis (10 μg proteins were separated for immunoblotting); (b), ethylene production rate (○) and ACC oxidase activity (¡); (c), ACC content. Data are means of three replicates. Bars represent SDs when larger than symbols.

Conclusions

Ethylene is involved in the progress of senescence of postharvest broccoli florets. The induction of ACC oxidase activity rapidly occurred during senescence, which is correlated with the increased level of endogenous ethylene. The increase in ACC oxidase activity is likely to have resulted from de novo synthesis of enzyme protein. Broccoli ACC oxidase exhibited the characteristics of ferrous-ascorbate dioxygenase. Recombinant ACC oxidase isolated and purified from *E. coli* cells transformed with the broccoli ACC oxidase gene was quite similar in enzymatic properties to the native broccoli enzyme. The use of the antibodies raised against the purified recombinant enzyme will be effective for studying the enzymology of ACC oxidase induced in broccoli florets during senescence.

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