Enzymatic Catabolism of Ascorbate in Florets of Harvested Broccoli during Senescence

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Summary
Broccoli (Brassica oleracea L. var. italica) florets senesced rapidly after harvest at ambient room temperatures. Whole plants of broccoli were harvested in the field and brought to the laboratory, then the stem was excised from the root. Ethylene production in the first cross-sectional layer (2 mm thick) initially increased markedly from a trace amount at 0 hr to a peak at 12 hr, then decreased gradually. The rate of ethylene production in florets increased gradually after harvest to reach a maximum at 24 hr. A high amount of ascorbate in florets at harvest, just after separation from the stem, rapidly declined to a low level during senescence. However, in the stem tissue including the cut surface, the ascorbate level which was much less than that of florets, remained almost unchanged during the experimental period. Ascorbate peroxidase (APX), which may be responsible for the first step in ascorbate oxidation retained high activity in broccoli florets after harvest, then decreased slightly during senescence. These results suggest that some additional factors (components) besides high APX activity could be involved in the rapid breakdown of ascorbate.

Key Words: ascorbate, ascorbate peroxidase, Brassica oleracea, ethylene production, senescence.

Introduction
Heads of broccoli (Brassica oleracea L. var. italica) are harvested in the immature stage so that their florets (flower buds) senesce rapidly when kept at room temperatures. One visible symptom of senescence is the yellowing of sepals, accompanied by degradation of chlorophyll. Concurrently, endogenous ethylene is evolved in postharvest senescence of broccoli florets (Lieberman and Hardenburg, 1954; Ku and Wills, 1999); the rate of ethylene production by florets significantly increased to a maximum then declined in a pattern almost parallelizing that of ACC oxidase activity (Makhlof et al., 1989; Kasai et al., 1996). Chlorophyll loss was induced by the increased amount of ethylene (Aharoni et al., 1985; Tian et al., 1994). It has also been reported that total soluble protein, lipooxygenase, soluble sugars and starch declined in senescing broccoli (King and Morris, 1994; Zhuang et al., 1995).

A large amount of ascorbate (vitamin C) is present in broccoli florets. It decreases rapidly after harvest as in many vegetables; the rate of decrease depends on the storage temperature (Lee and Kader, 2000; Yamauchi et al., 2000). In plants, ascorbate can accumulate to millimolar concentrations in both photosynthetic and nonphotosynthetic tissues (Rautenkranz et al., 1994; Noctor and Foyer, 1998). Ascorbate is one of the most important antioxidants that scavenge free radicals and reactive oxygen species (ROS) (Asada, 1992; Smirnoff, 1996; Noctor and Foyer, 1998). It also plays a role as a cofactor for hydroxylase enzymes, such as ACC oxidase (Verderis and John, 1991). The physiological role of ascorbate in plant cell development has been proposed (Citterio et al., 1994). The recycle pathway of ascorbate from the oxidized form has been widely reported. Ascorbate utilization always leads to ascorbate-free radical (AFR) which normally has a short life span that, if not rapidly reduced by AFR reductase, disproportionates ascorbate and dehydroascorbate (DHA). DHA is reduced to ascorbate by the action of DHA reductase, utilizing glutathione as the reducing substrate (Foyer and Halliwell, 1976). Synthesis of ascorbate in plants is not well-established, but it is proposed that L-galactono-1,4-lactone (GL) is the terminal precursor in the synthesis of ascorbate and that this last reaction is catalyzed by GL dehydrogenase (Wheeler et al., 1998). In spite of the
well-established roles and synthetic pathway of ascorbate, the regulation of its metabolism after harvest is poorly understood.

A normal component of plant metabolism is hydrogen peroxide, whose formation is accelerated under stress conditions such as chilling, high temperature, intensive lighting and wounding (Okuda et al., 1991; Foyer et al., 1994, 1997; Dat et al., 1998; Watanabe and Sakai, 1998). It must be rapidly scavenged because it can form molecular species that damage essential cell structure and function (Bowler et al., 1992). Ascorbate peroxidase (APX) reacts with ascorbate to reduce hydrogen peroxide to water, with concomitant generation of ascorbate−free radical (AFR). It is thought that most of the ascorbate is oxidized by this method in harvested broccoli where high activity of APX occurs.

Ethylene production is induced by various kinds of environmental stress, such as wounding, physical load, disease, exposure to low temperature and chemicals, and water deficiency in plants. Hence, these stress factors play a role in the induction of enzymes, such as peroxidase and phenylalanine ammonia-lyase in wounding tissue (Yang and Pratt, 1978; Hyodo, 1991; Hyodo et al., 1991; Kato et al., 2000). The activation of APX by ethylene has also been documented (Mehlhorn, 1990; Levinsh et al., 1995).

We report changes of the rate of ethylene production and ascorbate content in various parts of stem tissue and florets during postharvest senescence. In addition, we describe changes in gene expression and activity of APX in those tissues in response to wounding the stem tissue. We aimed to investigate the mechanism of ascorbate catabolism in harvested broccoli florets.

**Materials and Methods**

**Plant materials**

Whole, intact broccoli (Brassica oleracea L. var. italica) plants with roots were harvested and transported to the laboratory where the stem ends were cut. Harvested broccoli heads were incubated at 20 °C under humidified condition. Samples of stem tissue (2 mm thick) for the first and second layers, and the basal portion (ca. 2 mm thick) of curds, and florets were excised from broccoli every 12 hr (Fig. 1A). The outer portion (green part) of the stem tissue was excised (Fig. 1B) and assayed for ethylene production because this section evolves nearly 3-fold more ethylene than the inner zone. The excised stem tissue and florets were immediately frozen in liquid nitrogen, except the sample for ethylene production assay, and stored at −80 °C until ready for use.

**Assessment of yellowing broccoli and assay of ethylene production**

Color scores of florets were assessed visually from green to yellow as follows: 5, all green; 4, 20% yellow; 3, 40% yellow; 2, 60% yellow; 1, 80% yellow; 0, completely yellow. The decrease in the scores almost paralleled the decline in chlorophyll which was extracted with ethanol and determined spectrophotometrically according to Hyodo et al. (1995).

A 0.5-g sample was placed in a 17-ml vial and enclosed with a silicon rubber cap for 30 min at 20 °C. The headspace gas in the vial was sampled with a 1-ml hypodermic syringe and injected into a gas chromatograph (Hitachi 163) fitted with an alumina column at 70 °C and a flame ionization detector according to Kasai et al. (1996). The amount of ethylene was calculated and the rate expressed as nmol ethylene per hr per g FW.

**Extraction and assay of ascorbate**

Assay for the reduced and oxidized forms of ascorbate was performed using HPLC. Each frozen sample (0.5 g) was homogenized with a mortar and pestle in 5 ml of 2% metaphosphoric acid. The homogenate was centrifuged at 3000 rpm for 15 min, then the supernatant was filtered through Miracloth (Calbiochem). The pH of the filtrate was adjusted by adding an equal volume of 0.2 M K−phosphate buffer (pH 7.5). The total ascorbate was assayed by adding 1 ml of 1 mM dithiothreitol (DTT) to an aliquot of filtrate and incubating the mixture for 15 min (Masuda et al., 1988). After the sample was filtered through 0.2-μm cellulose acetate filter (Advantec), a 20-μl aliquot was injected onto a TSK−GEL (Amide−80) column (TOSOH) attached to a LC−10AD pump.
(Shimadzu). The column kept at 20 °C was eluted with 80% acetonitrile: 0.04% phosphoric acid at a flow rate of 1.0 ml·min⁻¹. Ascorbate was monitored at 245 nm (retention time 5.3 min) with a SPD-10A spectrophotometric detector (Shimadzu) attached to a chart recorder (C-R6A, Shimadzu). Peaks were converted to concentrations by using dilution of stock ascorbate to construct a standard curve. Ascorbate content was determined in a similar manner without the addition of DTT. Dehydroascorbate content was calculated by subtracting the ascorbate value from total ascorbate.

**Extraction and assay of APX**

APX was determined, using the method of Nakano and Asada (1981). Frozen sample (0.5 g) was homogenized in 5 ml of extraction buffer consisting of 50 mM K-phosphate, pH 7.5, 1 mM EDTA, 1 mM Na-ascorbate at 2 °C. After centrifugation at 14,000 × g for 20 min at 4 °C, the supernatant was assayed for APX. The assay mixture (3 ml) contained 50 mM K-phosphate, pH 6.0, 0.5 mM Na-ascorbate, and 0.1 ml of extracted enzyme. The reaction was initiated with the addition of 0.4 mM H₂O₂. Activity was determined by following the H₂O₂-dependent decomposition of ascorbate at 290 nm. Ascorbate oxidase activity was assayed in a same manner except that H₂O₂ was not added to the assay medium.

**RNA extraction and RT–PCR**

The extraction of total RNA from florets and stem tissue was performed as described by Kato et al. (2000). The first strand cDNA was synthesized from 5 μg of total RNA with Ready-To-Go T-Primed First Strand Kit (Amersham Pharmacia Biotech). cDNA encoding for APX was amplified by a PCR procedure with GeneAmp PCR System 9600 (Perkin Elmer). The primers (ACCC[AT]/GG[ACT]/AGGAGGCAA as the upstream primer and AGCAGAT[ACT]/CC[AGT]/AG/[CT]/TC/[AG]/GA AA as the downstream primer) were designed by the common sequences based on Brassica napus (accession no. Y11461), Brassica juncea (accession no. AF038839), Raphanus sativus (accession no. X78452), Glycine max (accession no. L10292), Cucumis sativus (accession no. D88649) and Spinacia oleracea (accession no. D85864) APX that have been reported. The PCR procedure started with 10 min at 95 °C and was carried out for 35 cycles of 30 sec at 95 °C, 30 sec at 52 °C and 30 sec at 72 °C, and then ended with 10 min at 72 °C. The PCR products were confirmed by agarose gel electrophoresis.

**Cloning and sequencing of cDNA**

The amplified cDNA was cloned with TA Cloning Kit (Invitrogen). The sequences were determined using Taq Dye Primer Cycle Sequencing Kit (Perkin Elmer) with 373S DNA Sequencing System (Perkin Elmer).

**Northern blot analysis**

Total RNA was separated in formaldehyde-agarose gels (10 μg per lane). After electrophoresis for 2 hr, RNA was visualized with ethidium bromide under UV light to ensure equal loading of RNA in each lane. RNA was transferred to nylon membranes (Roche) with 20 × SSC, then the blots were heated for 3 hr at 80 °C.

cDNA for APX (BO-APX) was labeled with DIG RNA labeling kit (Roche) as probes. Hybridization and detection procedures were performed according to Kato et al. (2000). The prehybridization was performed for 3 hr at 67 °C in a solution containing 5 × SSC, 50% formamide, 0.1% N-lauroylsarcosine, 0.02% SDS, and 2% Blocking reagent (Roche), followed by hybridization at 67 °C in the same solution with labeled probes. Following hybridization, the blots were washed twice in 2 × SSC and 0.1% SDS at room temperature, then twice again at 67 °C with 0.1 × SSC and 0.1% SDS. The detection was carried out by chemiluminescence with CDP-Star (Roche); blots were exposed to Hyperfilm ECL (Amersham Pharmacia Biotech).

**Results**

**Changes in ethylene production and yellowing of florets in broccoli after harvest**

Senescence of the broccoli head was initiated by severing it from its root, as evident by the degreening of its sepals. The extent of greening began to decline 24 hr after harvest. The rate of ethylene production in florets increased gradually after harvest, reaching a maximum at 24 hr; after a temporary decline, the second rise occurred as senescence progressed (Fig. 2). The results indicate that the degradation of chlorophyll in florets occur in close association with the rise in ethylene evolution. Ethylene production rates in the three portions of stem tissue exhibited different patterns; that

![Fig. 2. Time courses of color score (▼) and ethylene production in the first layer (○), second layer (◇), basal portion of curds (□), and florets (△), of broccoli after harvest. Data are the means of three replicates. Bars represent SE when larger than symbols.](image-url)
in the first layer (0–2 mm) increased markedly from a trace amount at 0 hr to a peak at 12 hr, then decreased gradually. Likewise, ethylene evolution increased in the second layer, although the peak at 12 hr was much less than in the first. Ethylene production in the basal portion of curds was much higher than that of the other three portions at 0 hr; it decreased to a lower level at 24 hr.

Changes in ascorbate in broccoli after harvest

The ascorbate level in florets just after harvest (0 hr) was 175.3 mg per 100 gFW (Fig. 3), which was 20 times more than that of the oxidized form (dehydroascorbate). The high ascorbate level was retained for about 12 hr and then rapidly declined to a low level during senescence (Fig. 3). In the stem samples, the levels of ascorbate were much less than that of florets at 0 hr and remained almost unchanged during the experimental period. Total ascorbate content exhibited the same pattern as the ascorbate.

Fig. 3. Changes in ascorbate in the first layer ( ), second layer ( ), basal portion of curds ( ), and florets ( ) of broccoli after harvest. Data are the means of three replicates. Bars representing SE are included in the side of symbols.

Changes in APX activity and its gene expression in broccoli after harvest

APX, being an enzyme involved in ascorbate catabolism, was highly active in florets at 0 hr but its activity gradually decreased during senescence (Fig. 4A). The stem samples exhibited a steady, low APX activity.

The cDNA isolated for APX was labelled as BO-APX by RT-PCR with the designed primers based on common sequence for APX. The BO-APX was 359 bp long and shared 95.5 and 94.7% nucleotide identities with the

Fig. 4. (A) Time course of APX activity and expression of BO-APX gene in the first layer ( ), second layer ( ), basal portion of curds ( ), and florets ( ) of broccoli after harvest. Data are the means of three replicates. Bars representing SE are included within the size of symbols.

(B) Northern blot analysis for APX transcripts with BO-APX gene fragments as a probe. Equal loading of RNA was confirmed by staining gels with ethidium bromide.

Fig. 5. Comparison of partial nucleotide sequences of BO-APX, Brassica napus-APX and Raphanus sativus-APX. The identical residues between three alignments are represented by the shaded areas.
corresponding region of APX genes from Brassica napus (accession no. Y11461) and Raphanus sativus (accession no. X78452), respectively (Fig. 5). The DIG-labeled probe with the BO-APX which we constructed for northern blot analysis was found to have a level of transcripts in florets which remained relatively constant in florets during the senescing phase similar to the expression pattern exhibited by the second layer and the basal portion of curds (Fig. 4B). However, in the first layer, wounding resulted in a significant increase in APX mRNA.

Discussion

In this paper, the effect of harvesting (cutting) of broccoli on the senescence of florets is described and correlations between changes in ascorbate content, APX activity, APX mRNA abundance, and ethylene synthesis in the stem tissues and florets were investigated.

Yellowing of florets after harvest, a symptom of the postharvest senescence of broccoli, is induced by ethylene (Wang, 1977; Aharoni et al., 1985; Ku and Wills, 1999). Changes in the rate of ethylene production exhibited different patterns in all four portions examined (Fig. 2), e.g. a dramatic increase in ethylene production in response to wounding was observed in the first layer. It is assumed that the increased rate of ethylene production in florets was affected by ACC and/or ethylene produced in the first layer and the basal portion of curds (Kato et al., 2001).

The ascorbate level in florets is much higher than in the stem of broccoli; this is consistent with a previous observation that organs which have abundant chlorophyll contain considerable amounts of ascorbate (Fig. 3, Foyer et al., 1983; Grantz et al., 1995). The ascorbate content in the outer green part of the stem was almost the same as that in the inner whitish part. Ascorbate functions not only as an antioxidant but also as a cofactor in certain enzymatic reactions that are related to plant cell development and senescence, suggesting that a high level of ascorbate in florets may serve as a large source of antioxidants in this tissue.

A rapid loss of ascorbate occurs only in florets accompanied by a high, continuous APX activity and gene expression (Fig. 4). It may be that high APX activity is one of the factors inducing ascorbate breakdown in florets after harvest. In spite of rapid catabolism of ascorbate in florets, dehydroascorbate content remained relatively low during senescence, indicating that most of the dehydroascorbate formed was further metabolized without reduction to ascorbate. In broccoli florets the activity of ascorbate oxidase was hardly detectable when the enzyme activity was determined in the presence of ascorbate and O₂ (data not shown). Therefore, APX may be the major route of ascorbate oxidation.

In higher plants the existence of several types of APX with different cellular localization has been reported (Kubo et al., 1992; Mittler and Zilinskas, 1992; Yamaguchi et al., 1995, 1996; Caldwell et al., 1998). Isolated cDNA, used as the probe in this study, was assumed to be cytosolic APX so that the APX activity we assayed could have included those from other organelles. Recent studies, which have focused on the changes in APX under environmental stress, have demonstrated that cytosolic APX can be induced by oxidative stress in rice and spinach (Morita et al., 1999; Yoshimura et al., 2000). A significant rise in APX mRNA level in the first layer may be the result of oxidative stress caused by wounding.

In conclusion, ascorbate content declined rapidly in broccoli florets after harvest; no significant changes in APX activity or expression of BO-APX in florets were observed. These results suggest that wounding stem tissue at harvest and increased ethylene production in florets have little effect on the activity and expression of BO-APX in florets. Other factors, such as the presence of hydrogen and organic peroxides, besides the high APX activity, could be related to the rapid breakdown of ascorbate in the broccoli florets.

Literature Cited


収穫されたブロッコリー小花の老化におけるアスコルビン酸の酵素的分解

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摘 要

ブロッコリー (*Brassica oleracea* L. var. *italica*) は根を付けたまま農場で収穫し実験室に持ち帰った後、茎・葉を切断して上部を実験に用いた。ブロッコリーの小花は室温で急速に黄化し、茎切断直後からエチレン生成量は切断面で急速に増大し、12時間後にピークに達してから緩やかに減少した。小花のエチレン生成量は、緩やかに増大し切断後24時間でピークに達した。その後一時的にエチレン生成量は減少したが、再び増大した。小花にはアスコルビン酸が豊富に含まれており、茎切断後急速に減少した。一方、切断面を含む茎組織のアスコルビン酸含量は、切断後0時間では小花より少なく、貯蔵期間中はほとんど変化しなかった。小花のアスコルビン酸ベリオキシダーゼ活性は他のいずれの茎組織よりも高かったが、その活性・遺伝子発現とも老化の過程で著しい変化はみられなかった。これらの結果は、茎組織においての傷害および小花におけるエチレン生成量の増大が、小花におけるアスコルビン酸ベリオキシダーゼの活性と遺伝子発現の変化にはあまり影響を及ぼさないことを見唆している。また、アスコルビン酸の急速な減少は、アスコルピン酸ベリオキシダーゼの高い活性とともに、その他の要因（例えば過酸化水素の生成等）によって引き起こされていることが考えられる。

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