Monodehydroascorbate Reductase Gene from Blueberries and the Impact of CA Storage on Its Expression and on the Level of Antioxidants

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Abstract

Blueberry is considered as one of the richest fruit types in ascorbic acid (AA), and is highly recommended for a healthy diet. In plant tissues mono-dehydro-ascorbate reductase (MDAR) is the enzyme involved in the regeneration of oxidized ascorbate, which is produced after the detoxification of free radicals. Taking into account the importance of this enzyme and using the gene fishing technique, a partial PCR-product of the gene encoding MDAR was isolated. Subsequently the 5-RACE PCR technique was employed to complete the characterization of this gene, and a sequence of 1551 bp was identified with a deduced protein that contained 433 amino acids. The sequence showed high homology to MDARs of *Psium sativum* and *Vitis vinifera*. Northern blot hybridization was employed to assess the gene expression of this gene upon storage of blueberries under various controlled atmosphere (CA) conditions. Results clearly showed differential expression between freshly harvested versus stored fruit as well as among fruit stored under various CA conditions. Quantitative assessments of ascorbic acid and the antioxidative capacity of water soluble antioxidants (ACW) revealed a dramatic loss in ascorbic acid under all storage conditions, even after three weeks in storage. The ACW decreased under all storage conditions and low O_2 concentrations did not enhance preservation of ACW. However, 2% O_2 combined with 6-12% CO_2 gave significantly better preservation of ACW than cold air storage. Northern blot hybridization results were in general agreement with the quantitative assessments of AA and ACW.

INTRODUCTION

Ascorbic acid is one of the most important water-soluble antioxidants for a healthy diet in humans (FAO/WHO, 2002). Humans, plus other primates and some birds and bats, are unable to synthesize AA, and plants are considered as the major source of AA (Noctor and Foyer, 1998). In this respect, fruits and vegetables comprise the major dietary sources of AA for humans and recent studies suggest that an intake of 60 to 200 mg d^{-1} may provide significant health benefits (Carr and Frei, 1999). In plant cells, AA is one of the major antioxidants which can act as a direct free radicals scavenger (Halliwell and Gutteridge, 2000). Plant tissues show an increased production of harmful reactive oxygen species (ROS) on exposure to environmental stress (Yoon et al., 2004) and plant cells will suffer oxidative injury, if the generation rates of ROS exceed their detoxification rates (Foyer and Noctor, 2000). The primary components of plant defense mechanisms required to detoxify ROS are antioxidants like ascorbate, glutathione, carotenoids and flavonoids, and enzymes involved in the ascorbate-glutathione cycle such as monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase, glutathione reductase and ascorbate peroxidase (Asada, 1996).

The objectives of this research were to isolate and characterize the MDHAR gene in blueberry, cv. 'Bluecrop', and to assess the effect of depressed oxygen and elevated carbon dioxide on its expression and on AA and hydrophilic antioxidant contents.

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MATERIALS AND METHODS

Fruit Harvest and Storage Treatments

Blueberries (cv. 'Bluecrop') were obtained in two consecutive years (2003 and 2004) from a cooperative packing house in the Lake Constance area, Southwest Germany. Fruit were picked, selected for uniformity and freedom from decay and external injuries, cooled to 1°C±0.5°C within 12h, and then placed into CA chambers under one of the following conditions (% CO_2+% O_2): 0+18 (regular air storage), 6+18, 12+18, 18+18, 24+18, 6+02, 12+2, 18+2 and 24+2. Storage temperature was 1°C±0.5.

Ascorbic Acid Content and Water Soluble Antioxidative Capacity (ACW)

Ascorbic acid was determined under cold and dark conditions. At each sampling date, samples were obtained from at least 15 fruit per replicate, two replicates for each treatment, and quickly immersed in liquid N_2. The frozen samples were ground to a powder while immersed in liquid N_2, and 6 g was added to 15 ml of 3% HPO_3 (w/v) solution and homogenized (Ultra Turrax, T25 basic; IKA Labortechnik, Staufen, Germany) for 30 s, and analyzed using HPLC. The antioxidative capacity of water soluble compounds (ACW) was analyzed according to PHOTOCHEM system (Analytik Jena AG, Jena, Germany).

Results were subjected to analysis of variance (ANOVA) using the CoStat-software (CoHort Software, Monterey, USA, 1998), and mean separations were calculated by Duncan's Multiple Range Test at $P \leq 0.05$.

Gene Expression Analysis

RNA was extracted according to the protocol of Chang et al. (1993). Gene Fishing Kit from Seegene, Inc. (Seol, Korea) was used to assess the differentially expressed genes, after which the differentially expressed fragments were cloned using the GeneJET™ PCR Cloning Kit from Fermentas (Fermentas GmbH, St. Leon-Rot, Germany). Resultant DNA was purified using the Wizard® Plus SV Minipreps (DNA Purification System) from Promega (Promega GmbH, Mannheim, Germany) and sequenced by the GATC Company (Constance, Germany). Following sequencing, the RACE PCR of blueberry monodehydroascorbate reductase (MDHAR) cDNA was carried out according to Zhu et al. (2001) using the BD Smart RACE cDNA Amplification Kit (Clontech). Subsequent PCR reactions were performed using the UPM Primer-Mix supplied in combination with gene specific primer derived from the target of *MDHAR* mRNA. Full products were excised from the gel, cloned and sequenced. Following full sequencing of the designated gene, Northern blot hybridization was carried out.

RESULTS

Characterization of the MDHAR and Alignments

The blueberry cDNA with highest similarity to the MDHAR cDNA was cloned as full-length cDNA, sequenced and termed *MDHAR* of *Vaccinium corymbosum* (Acc. No. EU327873). The amino acid sequence deduced from the nucleotide sequence of the ORF was aligned with those of MDHAR from other plant species. A substantial degree of homology was found to amino acid sequences of MDHAR from *Pisum sativum* (84.5%), *Vitis vinifera* (83.6%), *Lycopersicon esculentum* Mill. (78.8%), and *Cucumis sativus* (75%) (Fig. 1). In contrast, the amino acid sequence of the *V. corymbosum* showed lower than 50% homology to those from *Chlamydomonas reinhardtii* (38.8%) and *Spinacia oleracea* (39.3%). The prediction of protein domains in the Pfam database identified all functional domains present in the *MDHAR* of *Vaccinium corymbosum*. The analysis of the sequence (Table 1) shows two putative conserved domains: Domain 1 is the pyridine nucleotide-disulphide oxidoreductase. This family includes both class I and class II oxidoreductases and also NADH oxidases and peroxidases, which is actually a small NADH binding domain within a larger FAD binding domain. Domain 2 is also pyridine nucleotide-disulphide oxidoreductase, with similar characteristics as the first domain.

Quantification of AA and ACW

A dramatic loss in ascorbic acid content occurred under all storage conditions, even after three weeks in storage (Table 2). However, fruit stored under 2% O_2 plus high CO_2 (up to 18%) showed the best preservation of ascorbic acid. However, 24% CO_2 was injurious and caused significant losses in ascorbic acid content. Storing fruit for a short period (up to four weeks) ensured minimal losses of AA. Correlation analysis revealed a negative correlation between CO_2 concentrations and ascorbic acid content, either at high (r=-0.66) or low O_2 (r=-0.59). The ACW decreased under all storage conditions, even after three weeks storage (Table 2). Extending the storage period for another three weeks resulted in a further loss in ACW, in particular with fruit stored in air (0+18) and 24% CO_2 plus 18 O_2. Low O_2 did not preserve ACW, although 2% O_2 plus 6-12% CO_2 resulted in significantly better preservation than air storage. The best preservation was obtained with CO_2 up to 12% plus 18% O_2. Correlation tests over the entire storage period revealed a negative correlation (r=-0.5) between O_2 and ACW, but a slightly positive correlation (r=+0.35) between CO_2 and ACW. Correlation tests revealed negative correlations between CO_2 and ACW, either under high O_2 (r=-0.28) or low O_2 concentrations (r=-0.85). Northern blot hybridization results were generally in agreement with the quantitative assessments of AA and ACW (Fig. 2).

DISCUSSION

Our results show that the ACW in fruit decreased with prolonged storage and that high CO_2 and/or low O_2 partial pressures within the storage atmosphere did not consistently preserve the water soluble antioxidants, although lower O_2 concentrations had some effect on slowing losses. Stewart et al. (2000) stated that the water soluble antioxidant capacity declines with prolonged storage due to O_2-promoted oxidation of the major antioxidants (AA, anthocyanins and other phenolics). In contrast, Bangerth (1977) and Xuan (2003) found that storage of 'Conference' pears under high CO_2 results in rapid loss of AA. According to our results, we hypothesize that a decrease in the activity of MDHAR during storage will negatively affect the content of AA. However, only limited reports are available about the regulation of MDHAR, which plays a critical role in the ascorbate-glutathione cycle as a direct reducer of oxidized ascorbate (Yoon et al., 2004). Nishikawa et al. (2003) assessed the mRNA abundance of ascorbate-related enzymes in chloroplasts and the cytosol of broccoli, and found that MDHAR transcript level in chloroplasts was strongly suppressed by 24h after harvest.

In conclusion, further work is needed to elucidate the direct effects of elevated CO_2 and/or low O_2 on the biosynthesis, degradation, and stabilization of AA in stored blueberries.

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Tables

Table 1. Domain structure of the *Vaccinium corymbosum MDAR* protein.

Pfam-A	Description	Entry type	Sequence start	Sequence end	HMM from	HMM to	Bits score	E-value
Pyr_redox_2	Pyridine nucleotide-Disulphide oxidoreductase	Domain	7	302	1	271	143.7	5.1e-40
FAE_3-kCoA_syn1	Fatty acid elongase 3-ketoacyl-CoA synthase 1	Family	12	25	316	329	2.2	0.82
Biotin_lipoyl	Biotin-requiring enzyme	Domain	200	258	1	75	-23.7	0.8
Cas_Cas5a	CRISPR-associated protein (Cas_Cas5a)	Family	268	287	168	188	6.8	0.28

Table 2. Changes in ascorbic acid concentration and water soluble antioxidative capacity (ACW) of blueberries ('Bluecrop') following storage periods of 4 and 6 weeks under various atmospheres at 1±0.5ºC.

(%CO_2 + %O_2)	0+18	6+18	12+18	18+18	24+18	6+2	12+2	18+2	24+2
Ascorbic acid content (mg g^{-1}, fresh mass)									
At harvest time				6.3					
3 weeks in store	3.2 ab*	2.5 b	2.4 b	2.9 ab	2.3 b	3.2 ab	3.5 ab	3.9 a	3.1 ab
6 weeks in store	2.2 bc	2.2 bc	2.2 bc	2.3 bc	1.0 d	2.9 ab	-	3.3 a	1.6 cd
Water Soluble Antioxidative Capacity (ACW) (µg g^{-1}, fresh mass)									
At harvest time			5373.9						
3 weeks in store	3852 cd*	4546 abc	4667 ab	3789 d	4583 abc	3634 d	3942 bcd	4906 a	4639 ab
6 weeks in store	3214 a	4526 a	4608 a	3396 a	3121 a	3518 a	3775 a	3112 a	2702 a

*Mean values in the same row that are not followed by the same letter are significantly different using Duncan's Multiple

Figures

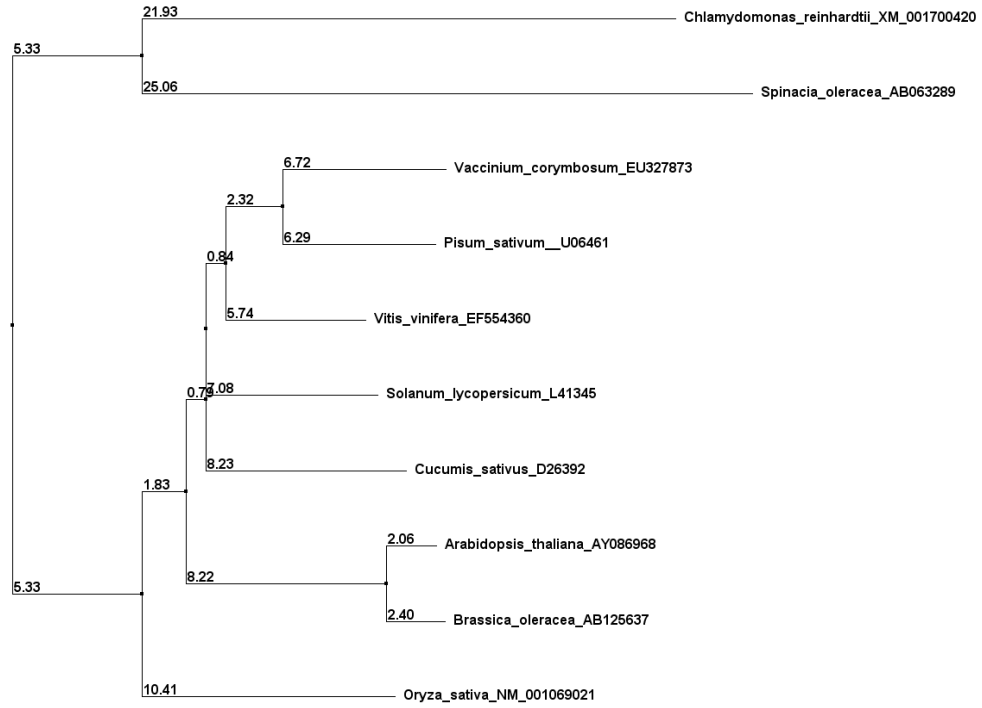

Fig. 1. Multiple alignment of the deduced amino acid sequence of MDAR from *Vaccinium corymbosum* 'Bluecrop' (accession no. EU327873) with MDAR from *Pisum sativum* (Q40977), *Vitis vinifera* (EF554360.1), *Lycopersicon esculentum* (L41345.1), *Cucumis sativus* (D26392.1), *Arabidopsis thaliana* (AY086968.1), *Oryza sativa* (D85764.1), *Brassica oleracea* (AB125637.1), *Chlamydomonas* (XM_001700420), and *Spinacia oleracea* (AB063289.1).

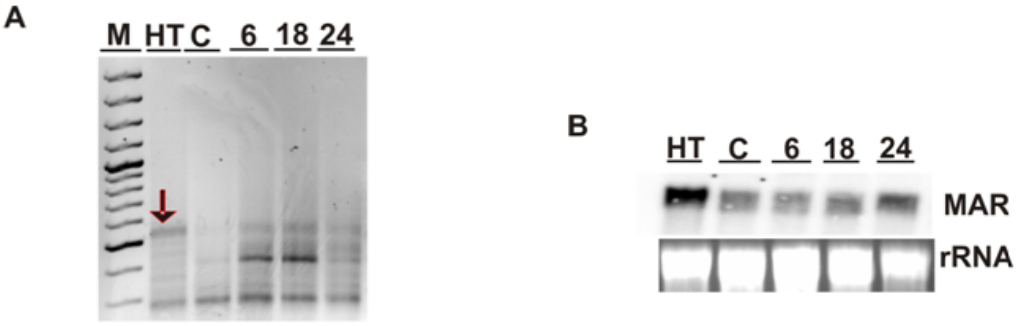

Fig. 2. Molecular analysis of the Blueberry monodehydroascorbate reductase (MDHAR). A. Amplification of differentially expressed cDNAs by GeneFishing^{TM} PCR (Arbitrary ACP/ dT-ACP2). B. RNA gel blots from the Blueberry fruit after hybridisation with *MDHAR* probe. The ethidium bromide stained gel below indicates equal loading. Treatments were HT= harvest time, Control = air storage, 6 = 6% CO_2 + 18% O_2, 18 = 18% CO_2 + 18% O_2, and 24 = 24% CO_2 + 18% O_2.