Pergamon

PII: S0031-9422(97)00057-5

Phytochemistry, Vol. 45, No. 4, pp. 671–674, 1997

0031-9422/97 $17.00+0.00

ANTIOXIDANT ABILITY OF ANTHOCYANINS AGAINST ASCORBIC ACID OXIDATION

ANNAMRAJU D. SARMA, YELLAMRAJU SREELAKSHMI and RAMESHWAR SHARMA*

Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad-500046, India

(*Received in revised form* 20 *November* 1996)

Key Word Index—Anthocyanin; antioxidant; ascorbic acid; metal chelation; copigmentation.

Abstract—Oxidation of ascorbic acid by metal ions like copper was prevented by addition of anthocyanin (cyanidin derivative). Previous reports attributed the ability of flavonoids to protect ascorbate oxidation to the metal complexing properties of these compounds. We provide evidence that anthocyanin not only chelates metal ions, but also forms an ascorbic acid (copigment)–metal–anthocyanin complex, which could be the possible protection mechanism. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Ascorbic acid (AsA) is a ubiquitous antioxidant present in both animal and plant cells. It plays a key role in the detoxification of activated oxygen. It acts as an antioxidant either by reducing superoxide, H_2O_2 and hydroxyl radicals, or by quenching singlet oxygen. Alternatively, it helps in regenerating α-tocopherol from chromotoxy radicals [1]. However, under stress conditions like high light irradiance, low temperature or exposure to heavy metals, the levels of this antioxidant decrease [2–5]. In view of the importance of AsA in cellular metabolism, systems associated with the recycling and affording direct protection of ascorbic acid may play an important role in the overall tolerance against these stress conditions.

In higher plants flavonoids are the naturally occurring pigments, which are shown to be induced during stress conditions [6]. Additionally, they have been implicated for their various antioxidant activities such as scavenging of hydroperoxy radicals, nitric oxide radicals [7, 8], etc. In this study we show that anthocyanin pigments acts as potential antioxidants against ascorbic acid oxidation. This finding may also have an *in vivo* significance, as ascorbic acid concentration is proportional to anthocyanin content in plant cells [9].

RESULTS AND DISCUSSION

Effect of cyanidin derivative on copper-induced oxidation of AsA

The absorbance of AsA does not change significantly on incubation in air for a period of 30 min. However, addition of copper potentiates the oxidation of AsA by acting as a strong oxidizing agent and rapidly converts the reduced form to the oxidized form, resulting in a decrease in the absorbance of AsA. Prior addition of small amounts (4–6 μM) of anthocyanin to AsA protected it against copper-induced oxidation. Even after 30 min of incubation almost 80–90% of ascorbate was present in reduced form in the presence of anthocyanin. It is likely that anthocyanin addition may have modified AsA and/or anthocyanin itself, thereby offering protection against copper-mediated oxidation.

Spectral analysis

The above possibility was examined by UV-visible spectroscopy to ascertain structural changes in the AsA and anthocyanin moieties under specified conditions, similar to the *in vivo* conditions. However, the UV spectra (200–300 nm) for AsA remained unchanged (Fig. 1) in the absence and presence of anthocyanin, indicating that the enediol moiety of

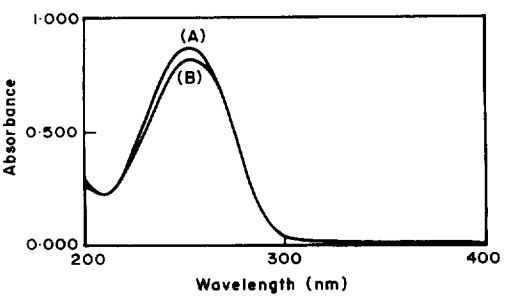


Fig. 1. Absorption spectra of AsA (100 μM) in (A) presence of and (B) absence of cyanidin derivative (6 μM) in 50 mM NaOAc buffer pH 4 at room temperature.

* Author to whom correspondence should be addressed.

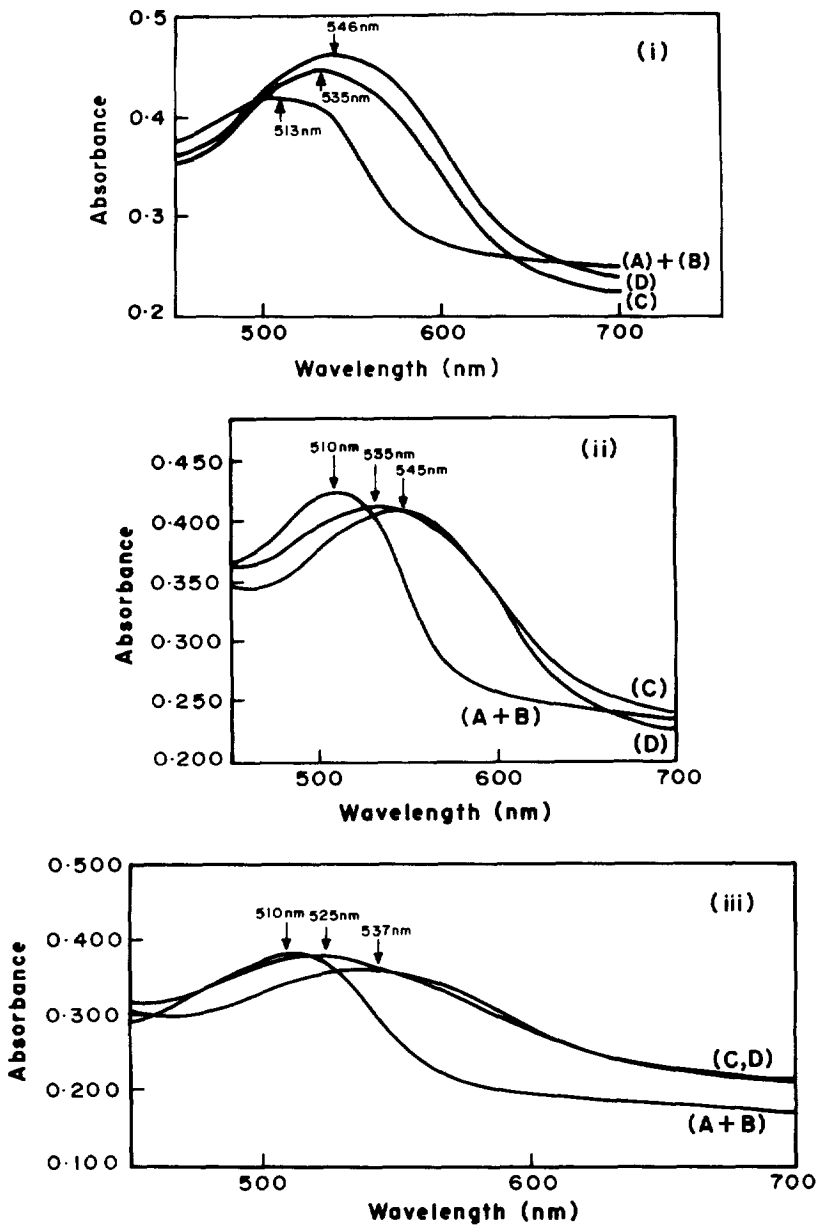


Fig. 2. Absorption spectra of cyanidin derivative from (i) *Oryza sativa*, (ii) *Zea mays*, and (iii) garden roses: (A) 6 μM cyanidin derivative; (B) 6 μM cyanidin derivative and 15 μl of 10 mM AsA; (C) 6 μM of cyanidin derivative and 10 μl of 5% of $AlCl_3$; and (D) 6 μM cyanidin derivative, 15 μl of 10 mM AsA and 10 μl of 5% of $AlCl_3$ in 50 mM NaOAc buffer pH 4. The spectra were recorded after incubating the mixtures for 1 hr at room temperature.

AsA remains unaffected. Likewise, the effect of various concentrations of AsA (0.1–0.4 mM) in the buffered solutions of pH 3–4 on the visible spectra of the cyanidin derivative was examined. Figure 2 shows that addition of AsA had no measurable effect on the position or the stability of the λ_{max} of the anthocyanin.

It is well known that flavonoids containing an ortho-dihydroxy group can chelate metal ions to form a stable metal–anhydro chelate with a concomitant bathochromic shift in the visible spectra [10]. Moreover, these metal-chelated flavonoids associate with various organic compounds at physiological pH ranges to form stable anthocyanin–metal–copigment complexes [11]. Figure 2 shows that cyanidin with its 3′,4′-dihydroxy group rapidly chelates aluminium with a 20–25 nm shift in the visible spectra. When AsA was added to the above metal-anthocyanin complex, a further shift of 10–15 nm was observed, indicating that apparently AsA complexes with metal-chelated anthocyanin [Fig. 2(i)]. A similar observation was also made for cyanidin derivative extracted from *Zea mays* [Fig. 2(ii)], and garden roses [Fig. 2(iii)]. In addition to the shift in the visible spectrum of metal–anthocyanin complex, a constant decrease in the intensity of the spectra was observed on addition of AsA with time (Fig. 3), which is probably due to a shift in the equi-

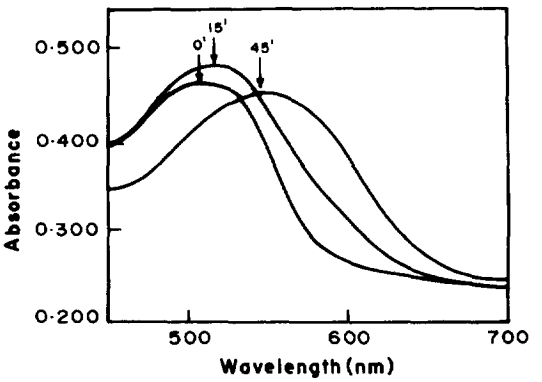


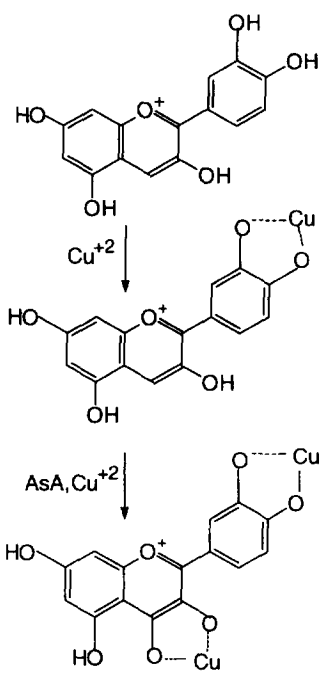
Fig. 3. Absorption spectra of 6 μM cyanidin derivative in the presence of AsA (15 μl of 10 mM) and 10 μl of 5% $AlCl_3$ in 50 mM NaOAc buffer pH 4. The spectra were recorded at various time intervals at room temperature.

librium between the anhydro base to colourless carbinol base.

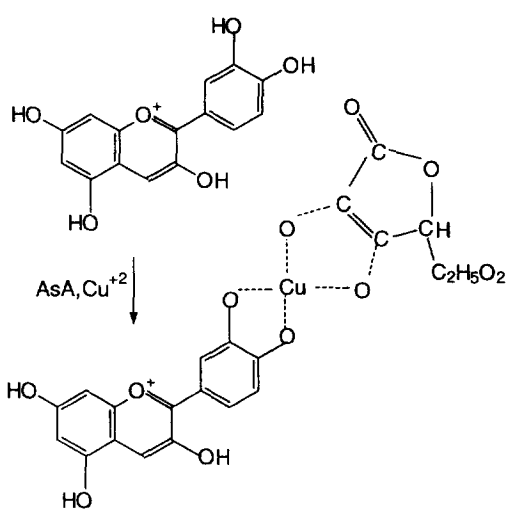
Almost five decades ago Hooper and Ayers [12] found that blackcurrants, in which AsA was remarkably stable, contain substances which inhibited the oxidation of AsA. The protective action was found to be associated with a red pigment (anthocyanin) and with a yellow pigment (flavanone). Later reports from various investigators suggested that the sparing effect of flavonoids on ascorbate oxidation was due to chelation of metal ions, which was dependent on the 3-hydroxy, 4-carbonyl and 3′,4′-dihydroxyl groups of the flavonoids [13, 14].

We propose the following two possible ways to account for the protection of AsA oxidation by anthocyanin.

(*a*) *Metal chelation.* AsA in the presence of oxygen and metal ions is known to hydroxylate the aromatic rings in a non-enzymatic reaction [15]. Anthocyanins at low acidic pH ranges 2–4 mostly exist in the form of flavylium cations and because of the charge distribution, they are susceptible to nucleophilic attack on positions 2 and 4 [16, 17]. It can be postulated that the hydroxylation of an anthocyanin at these positions enhances its chelating ability, thereby protecting AsA from metal-induced oxidation (Scheme 1).



Scheme 1



Scheme 2

Scheme 1. A possible mechanism of enhanced metal chelation by cyanidin in presence of AsA and copper.
Scheme 2. A copigment–metal–anthocyanin coordinate complex formed by AsA, copper and cyanidin.

(*b*) *Copigmentation.* Another likely explanation could be that AsA acts as a copigment and directly interacts with the metal-chelated anthocyanin, forming a stable anthocyanin–metal–copigment coordinate complex (Scheme 2). The formation of copigment–metal–anthocyanin complexes has been already shown with several organic compounds [11, 18], where it was suggested that factors like metal ions stabilise the copigmentation. Therefore, it is likely that such copigmentation could be the mechanism responsible for protection of AsA from oxidation.

EXPERIMENTAL

Isolation of anthocyanins. Seeds of a cyanic cultivar of *Oryza sativa* 'purple puttu' were used for isolation of cyanidin derivative. The seeds were dehusked and macerated in 2 ml of acidified MeOH (1% HCl). The solution was shaken for 24 hr at 4° to extract the anthocyanins. Aliquots of anthocyanin soln were spotted onto cellulose TLC plates and developed with solvent system *n*-BuOH–HOAc–H_2O (4:1:5, upper phase). The separated anthocyanins were identified on the basis of their R_f values. Individually 10 spots of cyanidin derivative were removed and eluted with H_2O. The amount of cyanidin derivative was quantitatively estimated by measuring the $A_{535}(\varepsilon = 31\,623)$.

Effect of cyanidin derivative on AsA oxidation. To 1 ml of H_2O, AsA (15 μl of 10 mM) and $CuSO_4$ soln (20 μl of 1 mM) were added, both in the absence and presence of cyanidin derivative (6 μM). The rate of oxidation of AsA was monitored for 30 min by recording the decrease of A at 265 nm (λ_{max} for AsA).

Effects of AsA and Al^{3+} on the absorption spectra of cyanidin derivative. Together or individually, each component, AsA (15 μl of 10 mM) and $AlCl_3$ (10 μl of 5% MeOH solution) were mixed with the soln of cyanidin derivative in 50 mM NaOAc buffer pH 4 (0.5 ml). After incubation for 1 hr at room temp, the absorption spectrum was recorded.

Acknowledgements—We thank Prof. D. Rangaswamy, TNAU, Coimbatore, India, for providing rice seeds of purple puttu variety. YSL is a recipient of CSIR-JRF fellowship, New Delhi, India. This work in part was supported by DST (SP/SO/A-30/93) and CSIR Grant (38 (0887)/95/EMR-II).

REFERENCES

1. Foyer, C. H., Lelandias, M., Edwards, E. A. and Mullineaux, P. M., *Current Topics in Plant Physiology*, 1991, **6**, ASPP, Rockville, 131.
2. Mapson, L. W., *Biochemical Journal*, 1962, **85**, 360.
3. Wise, R. R. and Naylor, A. W., *Plant Physiology*, 1987, **83**, 278.
4. Walker, M. A. and McKersie, B. D., *Journal of Plant Physiology*, 1993, **141**, 234.
5. Luna, C. M., Gonzalez, C. A. and Trippi, V. S., *Plant Cell Physiology*, 1994, **35**, 11.
6. Hahlbrock, K. and Scheel, D., *Annual Review of Plant Physiology and Molecular Biology*, 1989, **40**, 347.
7. Husain, S. R., Cillard, J. and Cillard, P., *Phytochemistry*, 1987, **26**, 2489.
8. Van Acker, S. A. B. E., Tromp, M. N. J. I., Haenen, G. R. M. M., Van der Vijgh, W. J. F. and Bast, A., *Biochemical and Biophysical Research Communications*, 1995, **214**, 755.
9. Eddy, B. P. and Mapson, L. W., *Biochemistry Journal*, 1951, **49**, 694.
10. Markham, K. R. and Mabry, T. J., *Phytochemistry*, 1968, **7**, 1197.
11. Jurd, L. and Asen, S., *Phytochemistry*, 1966, **5**, 1263.
12. Hooper, F. C. and Ayres, A. D., *Journal of the Science of Food and Agriculture*, 1950, **1**, 5.
13. Clements, C. A. B. and Anderson, L., *Annals of the New York Academy of Science*, 1966, **136**, 399.
14. Samorodova-Biank, G. B., *Biokhimiya*, 1965, **30**, 213.
15. Udenfriend, S., Clark, C. T., Axelrod, J. and Brodie, B. B., *Journal of Biological Chemistry*, 1954, **208**, 731.
16. Brouillard, R., *Phytochemistry*, 1983, **22**, 1311.
17. Jurd, L., In *Chemistry of Plant Pigments*, ed. C. O. Chichester. Academic Press, New York, p. 123.
18. Takeda, K., Kaviuda, M. and Itoi, H., *Phytochemistry*, 1985, **24**, 2251.