Vol. 93, No. 1, 1980
March 13, 1980

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 113-117

CAROTENOID BIOSYNTHESIS IN VITRO CONVERSION OF VIOLAXANTHIN TO CAPSORUBIN BY A CHROMOPLAST ENRICHED FRACTION OF CAPSICUM FRUITS

B. CAMARA

Laboratoire
de
Régulations Métaboliques et Différenciation des Plastes
Institut de Biologie Végétale
Université Paris VI
Tour 53, 4, place Jussieu, 75230 Paris Cedex 05 (France)

Received December 11, 1979

SUMMARY

This paper reports for the first time the conversion of [^{14}C] violaxanthin and [^{3}H] violaxanthin into capsorubin by a chromoplast enriched fraction prepared from semi-ripened pepper fruits. The conversion is enzymatic since no radioactivity was detected in the control experiment where the boiled chromoplast preparation was used.

Capsanthin (I) and capsorubin (II) are the main carotenoids of red pepper (Capsicum annuum) fruits. Their occurrence has also been shown in other plants (1-4).

Cholnoky et al. (5-7) proposed the first hypothesis for the biosynthesis of these carotenoids as being in relation to the transport of photosynthetic oxygen by epoxy-xanthophylls. Later studies about the evolution pattern of the carotenoids in different varieties of pepper fruits argued in favour of the key role played by epoxy-xanthophylls : antheraxanthin and violaxanthin (8,9). Similar conclusions were also obtained in pulse-chase experiments with disks excised from the pericarp of pepper fruit and incubated in the presence of [2-^{14}C] acetate (10).

Based on structural studies, a mechanism involving a pinacolic rearrangement of cyclohexenyl epoxides has been proposed for the conversion of violaxanthin into capsorubin (11).

I Capsanthin (3R,3'S,5'R)-3,3'-Dihydroxy-β,κ-caroten-6' one
II Capsorubin (3S,5R,3'S,5'R)-3,3'-Dihydroxy-κ,κ-carotene-6,6'-dione
III Violaxanthin (3S,5R,6S,3'S,5'R,6'S)-5,6,5',6'-Diepoxy-5,6,5',6'-tetrahydro-β,β-carotene-3,3'-diol

0006-291X/80/050113-05$01.00/0

In this study, we have investigated the possibility of this conversion in a chromoplast enriched fraction prepared from semi-ripened pepper fruits.

MATERIALS AND METHODS

Capsicum annuum fruits were obtained from BUD Agriculture Plant in Senegal. They were at a semi-ripened stage (orange color).

The pericarp was cut into small pieces, infiltrated for 30 min at 0-4°C in a buffered solution containing 10 mM $MgCl_2$, 10 mM KCl, 1 mM β-mercaptoethanol, 1 mM EDTA, 0,2 % BSA, 0,4 M sucrose, 50 mM Tris/HCl, pH 8. Two methods were used. In method A, the infiltrated pieces were homogenized in a Waring Blendor for 5 sec. The homogenate was filtered through 4 layers of Blutex (50 µ mesh). The cellular debris were pelleted after 5 min centrifugation at 150 x g. The chromoplast enriched fraction was obtained after 30 min of centrifugation at 7000 x g. In method B, the infiltrated pieces were ground in a chilled mortar. The chromoplast enriched fraction was recovered after 10 min centrifugation at 20 000 x g.

[^{14}C]-violaxanthin (433 dpm/µg) was biosynthetized by dark grown wheat seedlings incubated for 24 h at 25°C and 4300 lux in the presence of $^{14}CO_2$ produced from barium ^{14}C carbonate (53 mCi/mmole, CEA France). [^{3}H]-violaxanthin 5500 dpm/µg was obtained from the pericarp disks of green fruits to which sodium ^{3}H-acetate (7 Ci/mmole, CEA France) was directly applied. The incubating conditions were the same as those above. The resulting freeze-dried material was extracted with acetone in the presence of $CaCO_3$ at 0-4°C. The extract was saponified overnight in a nitrogen atmosphere. A preliminary separation of the carotenoids was performed by TLC on silica gel adjusted to pH 7. The chromatogram was developed with petroleum ether : acetone (60:40). Details about the identification of these carotenoids have already been described (12). The violaxanthin band was purified by TLC on MgO-Kieselgur plate with petroleum ether : acetone (60:40). A final purification step was carried out on silica gel at pH 7, with benzene : ethyl acetate : methanol (75:20:5) described by Davies et al. (8). In order to ascertain the purity of violaxanthin, a fraction of the latter was acid-isomerized into auroxanthin. The latter was purified on MgO-Kieselgur chromatoplates with petroleum ether : acetone (60:40) and its specific radioactivity compared to that of violaxanthin.

The chromoplast preparation, equivalent to 100 g fresh weight of pericarp tissue, was incubated in the presence of 0,7 mM $NADP^+$, 0,7 mM $NADPH_2$, 8 mM ATP, 50 mM tris/HCl buffer pH 7.6 and labelled violaxanthin dissolved in 200 µl methanol containing 5 mg tween-80. The mixture was gently shaken for 5 or 10 h at 25°C in a dim fluorescent light. In control experiments the chromoplast preparation was heated at 90°C for 30 min. The reaction was stopped by the addition of acetone : ethanol (2:1) and the carotenoids were extracted with acetone.

After a preliminary TLC on Silicagel with petroleum ether : acetone (60:40) the capsorubin band was eluted and purified res-

pectively on MgO-Kieselgur and Silicagel TLC with the same solvent system. This purified capsorubin was acetylated overnight or reduced with Na BH_4 for 100 min, before radioactivity determination in a Packard 3375 spectrometer. Corrections for quenching were made with an automatic external standardization method, using [^{14}C]-hexadecane (1.10^6 dpm/g) and [^{3}H]-hexadecane (4.10^6 dpm/g) purchased from the radiochemical Center, Amersham, England.

RESULTS AND DISCUSSION

The capsorubin fraction recovered after preliminary TLC on Silicagel was contamined by violaxanthin. The purification by TLC on MgO-Kieselgur gave a good separation of these carotenoids as revealed by their polarities (Rf : 0.7 for violaxanthin and 0.1 for capsorubin). After acetylation followed by TLC on a Silica gel plate with petroleum ether : acetone (90:10), the specific radioactivity of the diacetylated capsorubin was not reduced.

The results shown in table I display the transformation of violaxanthin into capsorubin. This reaction is enzymatic since in the boiled chromoplast preparation this conversion failed. The prolongation of the incubation time did not significantly improve the conversion rate. Thus we limited the incubation time to 5 h in the subsequent experiments.

The low spectific radioactivity of [^{14}C] - violaxanthin prompted us to synthetize [^{3}H]-violaxanthin with a higher specific radioactivity. The samples incubated in the presence of the latter showed a significant conversion into capsorubin. The enzymatic nature of the reaction was also evident (table I). A portion of capsorubin was reduced after a purification procedure similar to that described above. The resulting capsorubol appeared after TLC on Silica gel with petroleum ether : acetone (60:40) as being a mixture of three isomers having the same absorption characteristics in ethanol (absorption maxima 419-439-468 nm) but differing with respect to their polarities (Rf 0.23, 0.13 and 0.02). This was probably a consequence of steric hindrance between the newly formed hydroxyl group and the dimethyl group in the cyclopentane ring of capsorubin. A similar fact was noted for capsanthin (13). The isomers obtained retained 72% of the radioactivity initially present in capsorubin. In a test experiment based on the recovery of radioactivity in the reaction products after [^{14}C]-capsorubin and [^{14}C]-capsanthin reduction, we noted in all cases the presence of radioactive

TABLE I

In vitro conversion of [^{14}C]-violaxanthin and [^{3}H]-violaxanthin into capsorubin by red pepper chromoplasts

	Chromoplasts (method A)*				Chromoplasts (method B)**			
	experiment (dpm)	control (dpm)	experiment*** (dpm)	control*** (dpm)	experiment (dpm)	control (dpm)	experiment (dpm)	control (dpm)
Capsorubin recovered	503	0	680	0	8571	0	9934	0
Percent conversion	3.87	0	5.23	0	5.17	0	6.00	0

* Chromoplasts prepared according to method A (see text) were incubated with [^{14}C]-violaxanthin 13 000 dpm

** Chromoplasts prepared according to method B (see text) were incubated with [^{3}H]-violaxanthin 165 500 dpm

*** Incubation was carried out for 10 h.

degradation products, which explains the loss of radioactivity during the reduction of [^{3}H]-capsorubin.

The results presented in this study demonstrate for the first time that direct conversion of violaxanthin into capsorubin occurs in pepper chromoplasts. The role of cofactors (ATP, $NADPH_2$, NADP) added to the incubation medium has not yet been well established so far. However these cofactors may act as allosteric regulators (14,15). Work to get more information on this point as well as on the biosynthesis of the companion carotenoid of capsorubin (capsanthin) is in progress.

ACKNOWLEDGMENT - we wish to thank Mr Avignant for his helpful reading of the manuscript.

REFERENCES

1. HOWARD, G.E. (1925) Ann. mo. Bot. Gard., 12, 145-211.
2. KARRER, P., and JUCKER, E. (1950) Carotenoids of known or largely known structure containing one or more carbonyl groups pp. 218-255, Elsevier Publishing Company, New York, Amsterdam, London and Brussels.
3. BUBICZ, M., and WIERZCHOWSKI, Z. (1960) Bull. Acad. Pol. Sci., 8, 323-327.
4. NEAMTU, G., ILLYES, G., and BODEA, C. (1969) St. Cerc. Biochim., 12, 77-82.
5. CHOLNOKY, L., GYÖRGYFY, C., NAGY, E., and PÁNCZÉL, M. (1955) Acta Chim. Acad. Sci. Hung., 6, 143-170.
6. CHOLNOKY, L., GYÖRGYFY, C., NAGY, E., and PÁNCZÉL, M. (1956) Nature, 178, 410-411.
7. CHOLNOKY, L., GYÖRGYFY, K., NAGY, E., and PÁNCZÉL, M. (1958) Acta Chim. Acad. Sci. Hung., 16, 227-246.
8. DAVIES, B.H., MATTHEWS, S., and KIRK, J.T.O. (1970) Phytochemistry, 9, 797-805.
9. VALADON, L.R.G., and MUMMERY, R.S., (1977) Z. Pflanzenphysiol., 82, 407-416.
10. CAMARA, B., (1978) Thèse de Doctorat 3° Cycle-Université Paris VI. 80 p.
11. WEEDON, B.C.L., (1967) Chem. Britain, 3, 424-432.
12. CAMARA, B., and MONÉGER, R. (1978) Phytochemistry, 17, 91-93.
13. PHILIP, T., and FRANCIS, F.J. (1971) J. Food Sci., 36, 823-827.
14. MAUDINAS, B., BUCHOLTZ, M.L., PAPASTEPHANOU, C., KATIYAR, S.S., BRIEDIS, A.V., and PORTER, J.W. (1975) Biochem. Biophys. Res. Commun., 66, 430-436.
15. PORTER, J.W., and SPURGEON, S.L. (1979) Pure Appl. Chem., 51, 609-622.