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SUBCELLULAR LOCALIZATION OF ENZYMES OF ANTHOCYANIN BIOSYNTHESIS IN PROTOPLASTS

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Abstract—Flavanone synthase, chalcone-flavanone isomerase and UDP-glucose; anthocyanidin-3-*O*-glucosyltransferase activities of protoplasts and subcellular fractions of protoplasts of *Hippeastrum* and *Tulipa* were investigated. Subcellular fractions studied were intact vacuoles, cytosol and particulate components of protoplasts less the vacuole. The cytosol fraction had the highest activity of the three enzymes studied. Results similar to those found for *Hippeastrum* were obtained with fractions from leaves and petals of *Tulipa*. The increase in flavanone synthase activity in the cytosol fraction from petals of *Hippeastrum* during development paralleled the increase in anthocyanin content of the petals.

INTRODUCTION

Hippeastrum petals contain the 3-rutinosides of kaempferol, quercetin, pelargonidin and cyanidin [1]. *Tulipa* petals contain the 7-glucuronide of kaempferol, the 3-glucosides and 3-rutinosides of pelargonidin, cyanidin and delphinidin and the leaves contain 3-rutinoside-7-glucuronides of kaempferol and quercetin [2]. All these compounds are derived by the enzymatic transformation of naringenin, the primary product of the enzyme flavanone synthase.

In recent years elucidation of the pathways of flavonoid biosynthesis has led to speculation on the localization of the enzymic activities involved. It has been suggested [3] that some of the enzymic activities associated with the synthesis of flavonoids may occur on or in the tonoplast, or in the vacuolar sap. The enzymes involved in the biosynthesis of flavonoids, however, have their pH-optima at 7 or above [4] whereas the vacuoles of most plant tissues is acidic. It therefore seems unlikely that synthesis of flavonoid compounds occurs in the vacuolar sap. It is rather expected, that most, if not all enzymes involved in the above process are associated with cytoplasmic fractions or the cytoplasmic face of the tonoplast.

The availability of enzymatically-isolated protoplasts and vacuoles [5] has enabled us to localize the enzymic activities of flavanone synthase, chalcone-flavanone isomerase and UDP-glucose:anthocyanidin-3-*O*-glucosyltransferase in various subcellular fractions obtained from protoplasts of *Hippeastrum* and *Tulipa* petals and *Tulipa* leaves. Data showing a correlation between anthocyanin content of petals and the flavanone synthase activity of protoplasts derived from developing petals of *Hippeastrum* are also presented.

RESULTS

Flavanone synthase

Mature *Hippeastrum* petals contain a relatively high activity of flavanone synthase (Table 1). Similarly, protoplasts obtained from this tissue contain this activity. When protoplasts were fractionated into vacuole, enriched cytosol and particulate cytoplasm fractions most of the activity was associated with the cytosol (Table 1). In addition to naringenin, the product of the flavanone synthase reaction, intermediate products of the enzymatic synthesis, bisnoryangonin, *p*-hydroxybenzalacetone and the 4-hydroxy-5,6-dihydro-6-(4-hydroxyphenyl)-2-pyrone [6] were detected on radiochromatograms after paper chromatographic separation of incubation mixtures. In this respect flavanone synthase of *Hippeastrum* petals was similar to that found in *Petroselinum* [6], releasing the intermediate products of naringenin only *in vitro* in the presence of 2-mercaptoethanol. These data suggest that the site of naringenin biosynthesis is in the cytosol. Quantitatively similar data to those obtained with *Hippeastrum* were found when *Tulipa* petals and leaves were studied.

Table 1. Flavanone synthase activity of subcellular fractions of *Hippeastrum* and *Tulipa* protoplasts

Fraction	*Hippeastrum* petals	*Tulipa* petals	*Tulipa* leaves
	total cpm/preparation*		
Petals	78 160†	—	—
Protoplasts	2220	1600	—
Particulate cytoplasm	3000	0	0
Cytosol	22 200	6000	760
Vacuole	0	0	0
100000 *g* pellet	1000	220	104

* Total cpm per preparation derived from 2×10^6 protoplasts.
† Total cpm in a preparation derived from 700 mg of petal tissue—see Experimental.

An additional fraction was obtained by centrifuging the enriched cytosol fraction at 100000*g* for 30 min to

Table 2. Anthocyanin content and flavanone synthase activity of developing floral buds of *Hippeastrum*

Bud petal length (cm)	Anthocyanin in μmol/100 mg fr wt	dry wt	Flavanone synthase activity in cpm* cytosol	vacuole
(2.4)	0.01	0.21	367	0
(4.9)	0.68	11.39	1310	0
(7.5)	0.80	14.32	2550	0

* Total cpm in preparations derived from 10^6 protoplasts.

remove residual membranous materials. The 100000*g* pellet and the particulate cytoplasm fraction are presumed to be enriched in endoplasmic reticulum and dictyosome membranes. These fractions contained all the recoverable NADH cytochrome *c* reductase activity of *Hippeastrum* and *Tulipa* protoplasts (G. J. Wagner, unpublished).

To determine if flavanone synthase activity was correlated with anthocyanin production during flower development, the anthocyanin content of *Hippeastrum* petals of varying age was determined, protoplasts were prepared, fractionated, and their cytosol and vacuoles assayed (Table 2). As shown, the increase in flavanone synthase activity in the cytosol fraction paralleled the increase of anthocyanin in petals during development from the early bud (2.4 cm) stage until immediately before opening (7.5 cm). Throughout the expansion process, no enzymatic activity was detected in the vacuole. Clearly, the site of naringenin biosynthesis is in the cytosol.

Table 3. Chalcone-flavanone isomerase activity of subcellular fractions of *Hippeastrum*

Fraction	*Hippeastrum* petals total ΔA/preparation*
Protoplasts	8.82
Particulate cytoplasm	0.762
Cytosol	12.88
Vacuole	0
100000*g* pellet	0

* Total activity (ΔA_{370}) in preparations derived from 2×10^6 protoplasts.

Table 4. UDP-Glucose:anthocyanidin glucosyltransferase activity of subcellular fractions of *Hippeastrum* and *Tulipa*

Fraction	*Hippeastrum* petals	*Tulipa* petals	*Tulipa* leaves
	total cpm/preparation*		
Protoplasts	16000	360	640
Particulate cytoplasm	4000	0	
Cytosol	75000	14200	1700
Vacuole	0	0	0
100000*g* pellet	300	0	0

* Total cpm in preparation derived from 2×10^6 protoplasts

Chalcone-flavanone isomerase

This enzyme, similar to flavanone synthase, could only be detected in the cytosol fraction of *Hippeastrum* (Table 3). Its presence could not be established in any of the *Tulipa* cell fractions presumably due to inhibition by the relatively high levels of phenolic compounds remaining in preparations from petals and leaves, which could not be removed with PVP and Dowex 1 × 2 treatment during and after homogenization.

UDP-Glucose·anthocyanidin-glucosyltransferase

This enzyme was not detected in vacuole preparations from *Hippeastrum* petals or from leaves or petals of *Tulipa* (Table 4). Maximum glucosyltransferase activity was found in cytosol fractions of all three tissues, similar to both other enzymes studied.

The percent distribution in subcellular fractions of the three enzymes investigated is summarized in Table 5. The negligible amount of activity present in the particulate cytoplasm fraction and in the 100000*g* pellet is probably due to contamination of these fractions with cytosol, since no further efforts were made to purify these fractions.

DISCUSSION

Flavanone synthase, the first enzyme in the flavonoid biosynthetic pathway, is responsible for the establishment of the C_{15} skeleton [7] of flavonoid compounds. Other

Table 5. Distribution of enzymic activities in subcellular fractions of *Hippeastrum* and *Tulipa*

Tissue	Fraction	% of total activity recovered: flavanone synthase	chalcone-flavanone isomerase	UDP-glucose anthocyanidin glucosyltransferase
Hippeastrum petal	Particulate cytoplasm	5–15	5–6	6–12
	Cytosol	80–95	94	88–96
	Vacuole	0	0	0
	100000*g* pellet	1–4	0	<1
Tulipa petal	Particulate cytoplasm	0	—	0
	Cytosol	97	—	100
	Vacuole	0	0	0
	100000*g* pellet	3.5		0
Tulipa leaf	Particulate cytoplasm	0	—	3
	Cytosol	88	—	87
	Vacuole	0	—	0
	100000*g* pellet	12	—	0

enzymes in specific flavonoid pathways modify (e.g. hydroxylate, methylate, oxidize and glycosylate) the reaction product of the flavanone synthase, the 4',5,7-trihydroxyflavanone naringenin. Until recently it was thought [7] that the next enzyme in flavonoid metabolism was chalcone-flavanone isomerase and that this enzyme was associated with the synthesis of naringenin. Recent investigations [8, 9] have revealed that the end product of the flavanone synthase reaction is naringenin, and not its chalcone isomer, and that the chalcone-flavanone isomerase activity is not required for the closure of the heterocyclic ring. Wong [10] showed that chalcones are used as substrates for a garbanzo bean (*Cicer arietinum*) peroxidase producing flavonols and their isomeric benzoxepinone-spiro-cyclohexadienones. A possible role of chalcone-flavanone isomerase may be in providing substrates for this reaction. Thus, the isomerase can be considered as a central enzyme in the biosynthetic pathway of flavonoids involved in the conversion of flavanones to flavanonols and flavonols. It has been suggested that glycosylation is the last step in the metabolism of flavonoids and that glycosyltransferases may be localized on the inner face of the tonoplast, or in the vacuole [3]. Glycosylation may either assist in transport of anthocyanins across membranes or provide necessary solubility properties. Data presented here indicate that enzymes involved in the three stages of flavonoid synthesis, e.g. beginning (flavanone synthase), central (chalcone-flavanone isomerase), and end (UDP-glucose:anthocyanidin-3-*O*-glucosyltransferase) are associated with the cytosol and not the vacuole. These observations are consistent with a hypothesis in which flavonoid biosynthesis takes place on closely-clustered enzymes in the cytosol. The pH optima of enzymes involved in flavonoid biosynthesis support this view.

It is not likely that the method used to prepare vacuoles and other fractions results in dissociation from the tonoplast of the enzymes studied. Available evidence indicates that treatments such as sonication, exposure to high ionic strength or metal ion chelating agents are required to solubilize peripheral proteins from membranes [11]. Further, if the enzymes studied here were associated with the cytoplasmic face of the tonoplast, and were released during isolation, one would expect to find some residual activity to be retained. No vacuolar associated activities were detected. The anthocyanin and ion content of isolated vacuoles is virtually the same as that of intact protoplasts [12]. Thus, loss of sap proteins during vacuole isolation is not expected. The low enzyme activities recovered from whole protoplasts relative to protoplast fractions suggests that disruption of whole protoplasts subjects sensitive enzymes to more extensive destruction by phenolic compounds than do results during fractionation by isolation of intact vacuoles. A similar phenomenon was observed when the ATPase of isolated vacuoles was compared with that of whole protoplasts [12].

There is suggestive evidence that some flavonoids may be synthesized on the endoplasmic reticulum (ER). Dark-staining droplets of condensed tannins in the shoot apex of *Oenothera* [13] and in cell cultures of white spruce [14] and slash pine [15] have been found to be first associated with the smooth ER. Nearly all of the cinnamate hydroxylase and a portion of phenylalanine ammonia lyase of *Solanum tuberosum* L. were reported to be associated with microsomal preparations from this tissue [16]. The overall conversion of phenylalanine to anthocyanins may occur near the ER by cytoplasmic and ER bound enzymes acting in close proximity to each other and to the tonoplast.

EXPERIMENTAL

Plant material. Hippeastrum (cv Dutch Red Hybrid) was obtained from Jackson and Perkins Co. and *Tulipa* (variety Most Miles) from K. Van Bourgondein and Sons, Inc., Babylon, NY.

Materials. p-Coumaryl-CoA was synthesized as described previously [6], [2-^{14}C]-malonyl-CoA and UDP-^{14}C-D-Glucose was obtained from New England Nuclear, Boston, MA. Naringenin chalcone was prepared according to ref. [17]. Naringenin, malvidin and malvidin-3-glucoside were from our laboratory collection.

Determination of the anthocyanin concentration. Petals were thoroughly extracted at room temp. with 50 ml 0.01% HCl in MeOH. Vacuoles (10^6) were added to 50 ml 0.01% HCl in MeOH where they ruptured immediately. The anthocyanin concentration determined as described previously [18].

Preparation of protoplasts and vacuoles from anthesis stage and developing *Hippeastrum* petals and *Tulipa* petals and mature leaves was carried out as previously described [5]. In brief, vacuoles were released from enzymatically-isolated protoplasts (incubated in enzyme 19 hr) by treatment with 0.2 M K_2HPO_4/HCl pH 8. After removing the particulate materials (particulate cytoplasm), the vacuole-containing suspension was centrifuged at 100 *g* for 3 min. This resulted in a pellet containing intact vacuoles and a supernatant enriched in cytosol. *Tulipa* leaf vacuoles were centrifuged at 110*g* for 3 min. Sedimented vacuoles were washed by resuspending them in 0.7 M mannitol, 1 mM HEPES/NaOH buffer, pH 8, 0,5 mM dithiothreitol and recentifuged. Yields of vacuoles from protoplasts were approximately 20% for *Hippeastrum*, and 40% for *Tulipa* petals and leaves. Vacuoles and protoplasts were counted using a 0.2 mm deep well type slide. Since protoplasts were not generally washed free of isolation medium prior to preparation for enzyme assays, control experiments were performed to determine if the enzyme mixture used in protoplast isolation contained any of the enzymatic activities studied. No activity was found.

Preparation of tissue and subcellular fractions for enzymatic assay. All manipulations were carried out at 4°. Unless otherwise noted homogenates were centrifuged at 12500*g* for 1 min. HCl washed polyvinyl pyrrolidone (PVP) and Dowex 1 × 2 ion exchange resin (PO_4-form, pH8) were used to remove anthocyanin and phenolic contaminants. The buffer mixture contained 0.2 M K_2HPO_4/HCl, pH 8, 2.4 mM mercaptoethanol.

Hippeastrum *petals* (*ca* 700 mg) were ground with 100 mg silica and 200 mg PVP in 2 ml buffer mixture in a chilled mortar for 5 min. The homogenate was centrifuged and the supernatant (*ca* 1.6 ml) stirred with 100 mg Dowex 1 × 2, and centrigufed. The final supernatant was used for the determination of enzymatic activities.

Protoplast. Ca 2 × 10^6 protoplasts in 1 ml buffer mixture were homogenized in a glass tissue grinder with 100 mg PVP. The supernatant, after centrifugation and Dowex 1 × 2 treatment, was used for the enzyme assays.

Particulate cytoplasm Particulate cytoplasm recovered from 2 × 10^6 protoplasts was homogenized with 100 mg PVP in 1.0 ml buffer mixture and was centrifuged for 2 min. The supernatant was treated with Dowex 1 × 2, centrifuged and the resulting supernatant used for enzyme assays.

Cytosol. The K_2HPO_4/HCl from which the vacuoles were originally sedimented was enriched in soluble cytoplasmic constituents, but also contained vacuolar sap and tonoplast derived from vacuoles which lysed during isolation. This fraction was centrifuged at 100000*g* for 30 min to remove residual particulate materials. The resulting pellet (100000*g* pellet) was suspended in 1 ml buffer mixture, homogenized and assayed. The supernatant (cytosol) was concd to *ca* 3 ml using a Diaflow CECI ultrafiltration cell [19] and used for enzyme assays.

Vacuoles. Vacoules from *ca* 2 × 10^6 protoplasts were homogenized with 100 mg PVP in 1 ml buffer mixture. The homogenate was centrifuged, the supernatant treated with Dowex

1 × 2, centrifuged and the final supernatant used for the enzyme assays.

Determination of enzyme activities. Flavanone synthase. Samples (100 μl) were incubated with 5 μl *p*-coumaryl-CoA (1 nmol) and 5 μl [2-^{14}C] malonyl-CoA (1.5 nmol, 1.1 × 10^5 dpm) for 20 min at 30°. The ^{14}C-naringenin was separated and counted as described in ref. [8].

Chalcone-flavanone isomerase. Determined according to ref. [20]

UDP-glucose anthocyanidin glucosyltransferase Samples (100 μl) were incubated with 5 μl [^{14}C]-UDP-glucose (423 pmol 1.1 × 10 dpm) and 5 μl (14 nmol) malvidin in ethylene glycol monomethyl ether (EGME) for 20 min at 30°. The reaction was stopped by the addition of 20 μl conc HOAc and 20 μg of malvidin-3-glucoside in 20 μl EGME was added as carrier. Malvidin-3-glucoside was separated on Whatman no. 1 paper strips (4 × 40 cm) in H_2O–HOAc–HCl (82:15.3), cut out and counted by liquid scintillation spectrometry in toluene (2.5 g PPO/1.).

REFERENCES

1. Asen, S. Private communication
2. Harborne, J. B (1967) *Comparative Biochemistry of the Flavonoids*. Academic Press, London
3. Fritsch, H and Grisebach, H (1975) *Phytochemistry* **14**, 2437.
4. Grisebach, H. and Hahlbrock, K (1974) in *Metabolism and Regulation of Secondary Plant Products* (Runecles, V C and Conn, E. eds) pp. 22–51. Academic Press, New York.
5. Wagner, G. J. and Siegelman, H. W. (1975) *Science* **190**, 1298.
6. Hrazdina, G., Kreuzaler, F., Hahlbrock, K. and Grisebach, H. (1976) *Arch. Biochem. Biophys.* **175**, 392
7. Kreuzaler, F and Hahlbrock, K (1972) *FEBS Letters* **28**, 69.
8. Kreuzaler, F. and Hahlbrock, K (1975) *European J. Biochem.* **56**, 205
9. Kreuzaler, F. and Hahlbrock, K (1975) *Arch. Biochem Biophys* **196**, 84.
10. Wong, E. and Wilson, J. M. (1976) *Phytochemistry* **15**, 1325
11. Singer, S. J. (1974) *Ann. Rev. Biochem* **43**, 805.
12. Lin, W., Wagner, G. S., Siegelman, H W. and Hind, G. (1977) *Biochim. Biophys. Acta* **465**, 110.
13. Dyer, T. A. and Payne, P. I (1974) *Planta* **117**, 259.
14. Chafe, S. C. and Durzan, D. J. (1973) *Planta* **113**, 251.
15. Baur, P. S. and Walkinshaw, C. H. (1974) *Can. J. Botany* **52**, 615
16. Czichi, U. and Kindl. H. (1975) *Planta* **125**, 115.
17. Moustafa, E. and Wong, E. (1967) *Phytochemistry* **6**, 625.
18. Wellman, E., Hrazdina, G. and Grisebach, H. (1976) *Phytochemistry* **15**, 913.
19. Butcher, H. C., Wagner, G J. and Siegelman, H. W. (1977) *Plant Physiol.* in press.
20. Hahlbrock, K., Wong, E., Schill, L. and Grisebach, H. (1970) *Phytochemistry* **9**, 945.