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# METABOLISM OF PHENOLIC SUBSTANCES BY THE CHLOROPLASTS—II.*

## CONVERSION BY THE ISOLATED CHLOROPLASTS OF *p*-COUMARIC ACID TO CAFFEIC ACID

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**Abstract**—The isolated chloroplasts of *Saxifraga stolonifera* are able to oxidize *p*-coumaric acid to caffeic acid. This activity is also found in the chloroplasts of several other plants, and its possible role in the biogenesis of polyphenols is discussed.

## INTRODUCTION

*p*-COUMARIC acid is found in plant kingdom both in free state and as combined ester,[1] and is considered as an important intermediate in the biosynthesis of other phenolic substances.[2] It was found by Neish that this acid is formed from L-tyrosine by the action of an enzyme tyrase (L-tyrosine ammonia lyase) present in many grasses.[3] Recently its formation by the hydroxylation of *trans*-cinnamic acid was discovered using an acetone powder obtained from spinach leaves.[4] *p*-Coumaric acid may subsequently be converted by phenolase (*o*-diphenol: $O_2$ oxidoreductase) to caffeic acid, and Walker found that apple polyphenoloxidase converts *p*-coumaric acid to a substance which could be caffeic acid.[5]

The present author reported that an enzyme of the phenolase type is able to oxidize arbutin, a monohydric phenol similar to *p*-coumaric acid, to the corresponding *o*-dihydric phenol.[6] The main concern of the present study is to examine whether *p*-coumaric acid can be converted to caffeic acid by similar enzymes present in the chloroplasts of several plants.

## RESULTS

The chloroplast sediment corresponding to 20 g fresh leaves of *S. stolonifera* (250–300 mg, dry wt.) was suspended in 10 ml of 0·05 M tris buffer (pH 6·7; a preliminary experiment showed that the pH optimum of the reaction was in the range from 6·5 to 7·0) and to the suspension was added *p*-coumaric acid in a final concentration of 0·01 M. The mixture was shaken for 3 hr at 30°, and then filtered to remove chloroplasts. The coloured filtrate was acidified with 0·5 M $H_2SO_4$ to pH 2·0, and extracted five times with ether. The combined ether extracts were evaporated to dryness and the residue dissolved in an appropriate volume of water or alcohol for further analysis.

In Table 1 are shown some properties of a product of the above reaction. From the $R_f$

* Part I—*Phytochem.* **2**, 385 (1963).

[1] E. SONDHEIMER, *Botan. Rev.* **30**, 667 (1965).
[2] D. R. MCCALLA and A. C. NEISH, *Can. J. Biochem. Physiol.* **37**, 537 (1959).
[3] A. C. NEISH, *Phytochem.* **1**, 1 (1961).
[4] P. M. NAIR and L. C. VINING, *Phytochem.* **4**, 161 (1965).
[5] J. R. L. WALKER, *Australian J. Biol. Sci.* **17**, 360 (1964).
[6] S. HATTORI and M. SATÔ, *Phytochem.* **2**, 385 (1963).

TABLE 1. SOME PROPERTIES OF THE PRODUCT AS COMPARED WITH *p*-COUMARIC ACID AND CAFFEIC ACID

| Test | | Compound | | |
|---|---|---|---|---|
| | | *p*-coumaric acid | caffeic acid | product |
| $R_f$ values in solvent | (*a*) | 0·89 | 0·84 | 0·84 |
| | (*b*) | 0·47 | 0·29 | 0·29 |
| | (*c*) | 0·82 | 0·63 | 0·63 |
| Colour with reagent | (*a*) | orange | dark green | dark green |
| | (*b*) | blue | blue | blue |
| | (*c*) | — | pink | pink |
| | (*d*) | — | red | red |
| | (*e*) | brown | orange | orange |
| | (*f*) | — | yellow | yellow |
| Fluorescence in u.v. light | | | | |
| long wave | $-NH_3$ | purple | sky blue | sky blue |
| | $+NH_3$ | bright purple | bright blue | bright blue |
| short wave | | purple | faint blue | faint blue |

Solvents: (*a*) *n*-butanol : acetic acid : water (4 : 1 : 2, by vol.); (*b*) 6% acetic acid; (*c*) water-saturated phenol. Reagents: (*a*) 1% alc. ferric chloride; (*b*) 1% ferric chloride–1% potassium ferricyanide; (*c*) alkaline 0·5% potassium ferricyanide; (*d*) diazotized benzidine; (*e*) Höpfner's test; (*f*) 1% phosphomolybdic acid. Maximal output of the u.v. lamps: long wave, 3650 Å; short wave, 2543 Å.

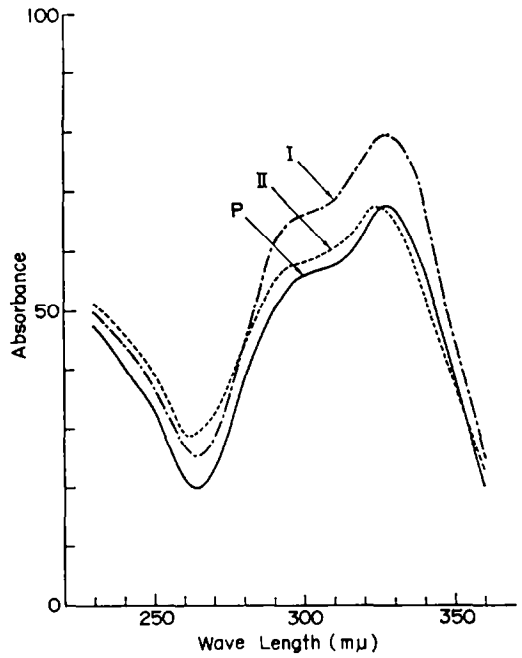


FIG. 1. ABSORPTION SPECTRA OF THE PRODUCT (P), CAFFEIC ACID ELUATED FROM THE CHROMATOGRAM (I) AND AN AUTHENTIC SAMPLE OF CAFFEIC ACID (II). ALL THE SAMPLES WERE TAKEN IN 50% METHANOL.

values in different solvent systems, its reaction to various reagents and fluorescence under u.v. light, the product appears to be caffeic acid.

The ether extract was purified by paper chromatography with 6% acetic acid, and eluted with 50% methanol. The resulting solution was filtered, and the filtrate examined spectrophotometrically. The absorption curves of the product (P), caffeic acid eluated from the paper as above (I), and untreated caffeic acid (II), are shown in Fig. 1. As can be seen, the absorption spectrum of the product coincides well with (I).

To characterize the enzyme involved in this hydroxylation, experiments were carried out using inhibitors. The production of caffeic acid was completely inhibited by $10^{-3}$ M 8-oxyquinoline or sodium diethyldithiocarbamate, but was scarcely affected by the addition of even $10^{-3}$ M of $\alpha,\alpha'$-dipyridyl. These results suggest that the enzyme contains copper, not iron, as the prosthetic group. Since the chloroplasts lack laccase activity,[6] the reaction seems to be catalysed by an enzyme of the phenolase type.

The chloroplast activity of plants other than *S. stolonifera* was examined by means of paper chromatography. The results in Table 2 show that the chloroplasts of several plants, especially of woody angiosperms, are able to oxidize *p*-coumaric acid.

TABLE 2. DISTRIBUTION IN PLANT WORLD OF *p*-COUMARIC ACID OXIDIZING ACTIVITY OF THE ISOLATED CHLOROPLASTS

| Species | Activity* | Species | Activity* |
|---|---|---|---|
| Gymnospermae | | Angiospermae—*cont.* | |
| *Podocarpus macrophylla* | − | Dicotyledoneae—*cont.* | |
| *Taxus cuspidata* | − | Herbaceous plants—*cont.* | |
| *Cedrus deodora* | − | *Primula sieboldi* | − |
| *Pinus thunbergii* | − | *Polygonum longisetum* | − |
| *Chamaecyparis pisifera* | − | *Chenopodium album* | − |
| | | *Macleaya cordata* | ++ |
| Angiospermae | | *Ambrosia artemisiifolia* | − |
| Dicotyledoneae | | *Geranium thunbergii* | − |
| Trees and shrubs | | *Rumex acetosa* | − |
| *Castanopsis cuspidata* | ++ | *Aster tataricus* | − |
| *Rhododendron pulchrum* | +++ | *Taraxacum officinale* | − |
| *Camellia sinesis* | − | Monocotyledoneae | |
| *Camellia japonica* | ++ | *Narcissus tazetta* | + |
| *Fatsia japonica* | − | *Allium tuberosum* | − |
| *Rubus trifidus* | + | *Mathiola incana* | − |
| *Citrus aurantium* | − | *Zingiber mioga* | − |
| *Ilex integra* | + | *Convallaria majalis* | − |
| *Ternstroemia japonica* | +++ | *Liriope graminifolia* | − |
| *Forsythia suspensa* | − | *Rohdea japonica* | − |
| Herbaceous plants | | *Hosta japonica* | − |
| *Saxifraga stolonifera* | ++ | *Tradescantia reflexa* | − |
| *Stellaria media* | − | *Agave americana* | − |

* The reactions were carried out under the same conditions as in *S. stolonifera*. −, no activity was found; +, activity weaker; ++, similar; +++, stronger than that of *S. stolonifera*, as determined from the area of spot of the product.

## DISCUSSION

From the results shown above, it can be concluded that the isolated chloroplasts of *S. stolonifera* convert *p*-coumaric acid to caffeic acid by an enzyme of the phenolase type. Similar activity could also be detected in the chloroplasts of several other plants.

Levy and Zucker demonstrated that chlorogenic acid is formed from 3-O-*p*-coumaryl-quinic acid by an enzyme preparation from potato tuber. In this case hydroxylation did not proceed with free *p*-coumaric acid, but with its ester.[7] McCalla and Neish, however, found in Salvia cuttings that cinnamic acid is converted through *p*-coumaric acid to caffeic acid by a series of hydroxylations. They suggested that, although *p*-coumaric and caffeic acids could be detected only as esters, the interconversion occurs at the free acid level before esterification.[2] The *in vitro* conversion of cinnamic acid to *p*-coumaric acid and of the latter to caffeic acid have been shown using spinach acetone powder[4] and apple phenolase,[5] respectively. The present results support the assertion of McCalla and Neish,[2] and it appears that an enzyme of the phenolase type in the chloroplasts plays a role in the biogenesis of polyphenols.

## MATERIALS AND METHODS

### *Isolation of the Chloroplasts*

Fresh leaves of *S. stolonifera* (40 g) were homogenized for 2 min in 500 ml of a medium consisting of 0·35 M sucrose and 0·06 M tris (hydroxymethyl aminomethane) buffer (pH 7·0). The homogenate was squeezed through silk, and the filtrate centrifuged at 200 *g* for 2 min to remove cell debris and nuclei. The supernatant was further subjected to a centrifugation at 2000 *g* for 10 min. The residue was suspended in 200 ml of 0·3 M sucrose, and the suspension was passed through double-folded silk. The filtrate was again centrifuged at the above speed, the sediment suspended in 200 ml of 0·3 M sucrose, centrifuged, and this procedure was repeated three times to isolate the chloroplasts from other cell components. The preparation was finally washed with water to remove sucrose. The preparation obtained consisted of chloroplasts or chloroplast fragments.

The procedure for obtaining the chloroplast preparation from other plants was essentially the same as above. In some plants, a high-speed centrifugation (3000 *g* for 20 min) was required for sedimenting the chloroplasts.

### *Paper Chromatography and Reagents*

Whatman No. 1 filter paper was used with the following solvents: (*a*) *n*-butanol : acetic acid : water (4 : 1 : 2, by vol.), (*b*) 6 % acetic acid, (*c*) water-saturated phenol. Reagents for detecting phenolic substances were: (*a*) 1 % alcoholic solution of ferric chloride as spray, (*b*) equal volume of 1 % aq. potassium ferricyanide and 1 % alcoholic ferric chloride as a dip (all the phenolic substances give a blue colour),[8] (*c*) 0·5 % potassium ferricyanide of pH 8·0 as spray (with *o*- or *m*-dihydric phenols this gives a pink or red colour), (*d*) Höpfner's test as a dip (with caffeic acid this reagent produces a red colour),[9] (*e*) diazotized benzidine reagent as a dip (caffeic acid reacts with this reagent to give an orange colour),[10] (*f*) 1 % phosphomolybdic acid in a test tube (with *o*- or *p*-dihydric phenols this reagent generally gives a blue colour, but in the case of caffeic acid a yellow colour is obtained).[11]

### *Inhibitors*

Following inhibitors of heavy metal enzymes were used:[12] 8-oxyquinoline inactivates all the heavy metal enzymes; $\alpha,\alpha'$-dipyridyl combines with the prosthetic group of iron-

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[9] W. HÖPFNER, *Chemiker Ztg.* **56**, 991 (1932).
[10] G. LINDSTEDT, *Acta Chim. Scand.* **4**, 448 (1950).
[11] L. REIO, *Chromatog. Rev.* **1**, 39 (1959).
[12] W. BRUCKER and W. A. K. SCHMIDT, *Ber. Deut. Botan. Ges.* **72**, 321 (1959).

containing enzymes and inhibits their action; sodium diethyldithiocarbamate in a relatively low concentration suppresses the activity of copper enzymes. Chloroplast suspension containing 1/100 M *p*-coumaric acid was incubated with these inhibitors at various concentrations ($10^{-3}$, $10^{-4}$, or $10^{-5}$ M).

*Fluorescence*

The u.v. lamps having maximal output of 365 and 254 m$\mu$, respectively, were used for detecting fluorescent substances.

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