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Characterization of a Product (Esculetin; 6,7-Dihydroxycoumarin) from the Phenolase–Caffeic Acid Reaction System

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Esculetin (6,7-dihydroxycoumarin) was isolated in crystalline form from a reaction mixture containing phenolase and *cis*-caffeic acid. The oxygen atom in the lactone ring of esculetin thus formed was not derived from molecular oxygen or water, and it is suggested that the carboxyl group of caffeic acid may be involved.

Keywords——phenolase (tyrosinase, polyphenol oxidase); caffeic acid; esculetin; coumarin

Introduction

Esculetin is a dihydroxycoumarin (6,7-dihydroxycoumarin), which occurs in several families of higher plants in the free state or as glucosides.[1] This substance is structurally related to a cinnamic acid, caffeic acid (3,4-dihydroxycinnamic acid), being formed by the lactonization of the side chain of the latter compound. It was found by Butler and Siegelman that in an "old" solution of *trans*-caffeic acid, this coumarin could arise non-enzymically *via* the *cis*-isomer as shown in Fig. 1.[2] We further found that this reaction also took place enzymically in a mixture consisting of *cis*-caffeic acid and an oxidoreductase, phenolase (there are several trivial names for this enzyme, such as tyrosinase, polyphenol oxidase, *etc.*) and presented the scheme shown in Fig. 2.[3]

One of the problems remaining in relation to these schemes is the origin of the oxygen atom in the lactone ring of esculetin. This was assumed to be either molecular dioxygen or the carboxyl group of caffeic acid in the non-enzymic reaction by Butler and Siegelman, and water in our enzymic system. In the present report, examination of our previous assumption was carried out with a product which was isolated from the enzymic system and clearly identified as esculetin.

Results and Discussion

Identification of the Product as Esculetin

The product, obtained in crystalline form, was neutral, lacking the carboxyl group of caffeic acid. An infrared (IR) absorption peak (dioxane) at 1738 cm^{-1} showed the presence of a δ-lactone (characteristic of coumarins in general[4]). In the mass spectra (MS), M^+ with a relative intensity of 100% was found at m/z 178 and this gave the molecular formula $C_9H_6O_4$ for esculetin. Several fragments could be accounted for as originating from esculetin; m/z: 150 (M^+-CO), 132 (M^+-CO-H_2O), 121 ($M^+-CO-COH$) and 104 ($M^+-CO-CO-H_2O$). Four protons of the esculetin nucleus could be assigned by proton nuclear magnetic resonance (^{1}H-NMR) (in DMSO-d_6 (TMS), δ: 6.17 (1H, d, $J=10$ Hz, H-3), 6.74 (1H, s, H-5), 6.98 (1H,

s, H-8), 7.86 (1H, d, $J=10$ Hz, H-4)). Carbon-13 nuclear magnetic resonance (^{13}C-NMR) analysis confirmed that the signals of the nine carbon atoms of the product were identical with those of esculetin as reported by Chang and Floss.[5] Comparisons of all the spectral data with those obtained for authentic esculetin further established the identity of our product.

It can therefore be concluded that the product, which was previously tentatively identified on the basis of an impure sample,[3] is certainly esculetin.

Examination of the Origin of the Oxygen Atom in the Esculetin Lactone Ring

Butler and Siegelman suggested the following mechanism for their non-enzymic esculetin formation: in the presence of heavy metal ions, *cis*-caffeic acid which had been produced from the *trans*-isomer by prolonged exposure to light (ultraviolet (UV)), was either hydroxylated at the *ortho*-position to give rise to an *ortho*-hydroxylated compound or was dehydrogenated to an *ortho*-quinone, and esculetin was subsequently formed by dehydration or direct cyclization (Fig. 1). The oxygen atom was assumed to originate either from molecular dioxygen or from the carboxyl group of caffeic acid.

The former route is now generally accepted for the first step of the biogenesis of several natural coumarins in higher plants.[6] However, this is not the case for our enzymic esculetin formation, because the enzyme used here (mushroom tyrosinase; Sigma Grade III) had no *ortho*-hydroxylating activity; the first step catalyzed by this enzyme is not the production of an *ortho*-hydroxylated substance, but the formation of an *ortho*-quinone.[3]

We considered a scheme in which water was regarded as being involved in the ring formation (Fig. 2), based upon the suggestion of previous authors[7,8] that phenolase oxidizes phenols of catechol type to *ortho*-quinones, and the latters are then converted to hydroxyhydroquinones through the intervention of water. To examine this possibility, we carried out esculetin formation in ^{18}O-enriched water. The MS of the product isolated, however, did not give a molecular ion peak at m/z 180, and was essentially identical with that of the product obtained in ordinary water (data not shown).

Hence the above assumption is untenable. The third possibility, *i.e.* direct ring cyclization as suggested by Butler and Siegelman as an alternative (Fig. 1), thus seems most plausible,

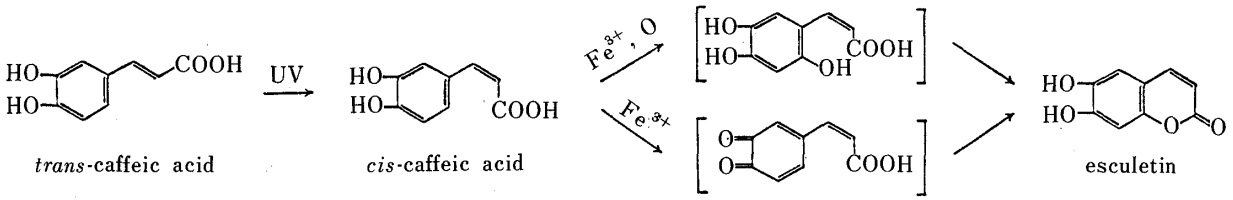

Fig. 1. Proposed Scheme for the Non-enzymic Conversion of *trans*-Caffeic Acid into Esculetin (by Butler and Siegelman[2])

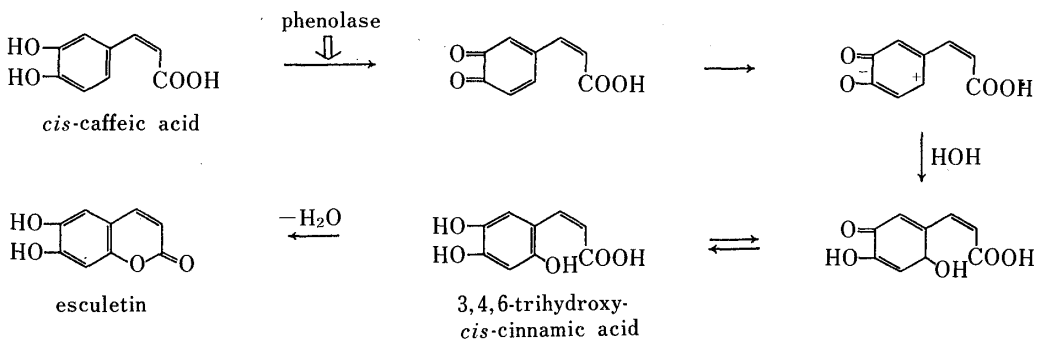

Fig. 2. Proposed Scheme for the Enzymic Transconversion of *cis*-Caffeic Acid into Esculetin (by Satô[3])

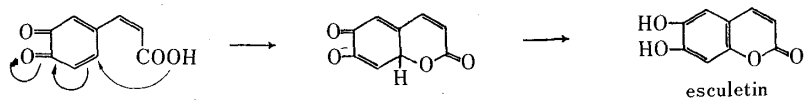

Fig. 3. Probable Scheme for the Conversion of the *ortho*-Quinone of *cis*-Caffeic Acid into Esculetin

although decisive evidence is not yet available. The *ortho*-quinone which is produced from *cis*-caffeic acid by the action of phenolase may be spontaneously converted into esculetin as indicated in Fig. 3.

The enzyme phenolase occurs widely in the plant kingdom,[9] but no comprehensive understanding has been reached of its physiological role.[9-11] Like the enzyme, one of its substrates, *trans*-caffeic acid, is also ubiquitous in plants and its *cis*-isomer may be produced in plant tissues by UV irradiation in sunlight. Thus, a great number of plants possessing both phenolase and *trans*-caffeic acid could produce esculetin. However, in fact esculetin is only found in a few species belonging to a limited number of families such as Hippocastanaceae, Oleaceae, Solanaceae and Asteraceae.[1] This is consistent with the view that the plant phenolase is often in an inactive latent form and does not always operate in cells *in situ*,[9-11] and consequently the present enzymic esculetin formation may have no general significance for plant coumarin biosynthesis.

Experimental

Isolation of the Product——*trans*-Caffeic acid was synthesized according to the literature[12] and recrystallized from dilute EtOH before use. Esculetin (mp 260 °C) was obtained from commercial esculin (mp 205 °C) by acid hydrolysis and purified by recrystallization from dilute EtOH.

trans-Caffeic acid (500 mg) was dissolved in 100 ml of EtOH and the alcoholic solution was irradiated for 30 min under a 20 W UV lamp (maximal output at 360 nm) from a distance of 10 cm. The resulting solution, which contained about 35% of the *cis*-isomer,[3] was brought to dryness and the residue was dissolved in 250 ml of 20 mM sodium phosphate buffer, pH 6.6. To this solution, 0.2 ml of phenolase solution (Sigma Grade III mushroom tyrosinase; total units: 2500) were added, and the mixture was stirred at room temperature for 40 min until a faint reddish color developed. The mixture was boiled for 3 min to stop the reaction, then concentrated and the product was extracted from the residue five times with 100 ml each of boiling EtOH. The combined extracts were evaporated and the residue was taken up in 20 ml of water, then applied to a diethyl aminoethyl (DEAE) cellulose column (Cl^- form, 3 × 5 cm). The product was eluted with water; caffeic acid remained adsorbed on the column. After concentration of the eluate, the product was crystallized once from water, then twice from dilute EtOH. Yield 40 mg (mp 260 °C; UV λ_{max}^{EtOH} nm: 230, 260, 302, 353; IR $\nu_{max}^{Dioxane}$ cm^{-1}: 3250 (OH), 2810 (CH), 1738 (δ-lactone), 1618 and 1577 (C=C)). No esculetin was formed under the same conditions in the absence of phenolase.

Experiment Using ^{18}O-Enriched Water——The residue of the *trans*–*cis*-caffeic acid mixture obtained from 100 mg of *trans*-isomer as described above was taken up in 52.2 g of $H_2{}^{18}O$ (atomic percent excess of ^{18}O: 3.09, purchased from YEDA R and D Co., Ltd.), then 1 g of a finely ground solid mixture of equimolar NaH_2PO_4 and Na_2HPO_4 was dissolved in the solution to bring the pH to 6.6. The reaction was started by adding 0.04 ml of phenolase solution (total units: 500) and the product was subsequently isolated by the same procedures as above. Yield 8.1 mg.

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