# Flavin Nucleotide-Dependent 3-Hydroxylation of 4-Hydroxyphenylpropanoid Carboxylic Acids by Particulate Preparations from Potato Tubers

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*Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday*

Potato Tubers, *Solanum tuberosum*, 4-Hydroxyphenylpropanoid Carboxylic Acid 3-Hydroxylation, Phenolase

Particulate preparations from potato tubers, extracted in 4 mM 2-mercaptoethanol, catalyze the 3-hydroxylation of 4-hydroxyphenylpropanoid carboxylic acids, including p-coumaric acid and tyrosine, in the presence of NADH (or NADPH) and FAD (or FMN); ascorbate could not substitute for these electron donors. Among a range of 4-hydroxylated $C_6-C_2$ and $C_6-C_1$ compounds tested, only 4-hydroxyphenylacetic acid and p-cresol were hydroxylated. The hydroxylase was sensitive to KCN and diethyldithiocarbamate and showed some features of phenolase hydroxylation, but no DOPA oxidase nor chlorogenic acid oxidase activity was exhibited under these conditions. It is suggested that the phenolase complex, which is confined to potato tuber particles, in whole or part catalyzes the hydroxylation.

## Introduction

Although isotope studies have shown that the 3,4-dihydroxylation pattern, common to many plant phenolics, is derived from the 3-hydroxylation of 4-hydroxy-substituted phenylpropanoids, the enzymic production of caffeic acid (3,4-dihydroxycinnamic acid) and its derivatives from *p*-coumaric acid under conditions which satisfy any rigid criteria has still to be demonstrated, even though three potential systems are available.

Cytochrome P-450 oxygenases have been shown to catalyze the 5-hydroxylation of ferulic acid [1] and the 3′-hydroxylation of 4′-hydroxyflavonoids [2], but neither could effect the 3-hydroxylation of *p*-coumarate. Evidence has been presented for a mixed function oxidase, requiring NADPH and FAD, in petals of *Silene dioica*, which catalyzes the 3-hydroxylation of *p*-coumaroyl-CoA to caffeoyl-CoA [3], which appears to take part in the biosynthesis of 3′4′-dihydroxylated anthocyanins [4].

A third system which promotes the 3-hydroxylation of 4-hydroxylsubstituted aromatic compounds is the phenolase complex found in many plant tissues [5], requiring ascorbate or other reducing agents, including NADH and NADPH, as electron donors. However, the low specificity and high activity of phenolases from many plants has been held to suggest that their role lies, not in hydroxylations involved in phenolic biosynthesis, but rather in catalyzing the further oxidation of *o*-diphenols to *o*-quinones as a result of their *o*-diphenol oxidase activity. Nevertheless, phenolases which catalyze the hydroxylation of *p*-coumarate to caffeate without any accompanying *o*-diphenol oxidase activity have been extracted from *Sorghum* internodes [6] and spinach beet (*Beta vulgaris* cv.) seedlings [7]. The latter was distinguished from other phenolase forms present by its specificity and its specific induction by light, when it accompanied increases in phenylalanine ammonia-lyase and cinnamate 4-hydroxylase.

The biosynthesis of chlorogenic acid in potato tubers involves the intermediate 3-hydroxylation of *p*-coumaric acid, or its CoA or quinic esters, and is stimulated by illumination [8, 9]. A particulate enzyme system from illuminated potato tuber discs, which catalyzes the 3-hydroxylation of 4-hydroxyphenylpropanoid acids, showing some phenolase characteristics but also a requirement for NADPH (or NADH) and FAD (or FMN), similar to that of the enzyme from the petals of *Silene dioica* [3], is described here.

## Materials and Methods

### *Plant material*

Potato tubers (*Solanum tuberosum* cv. Maris Piper) were purchased from the local market, and

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stored at 15 °C in darkness. The excision of tuber discs and the conditions of incubation and illumination were exactly as described already [9].

*Chemicals*

*p*-Coumaroyl-CoA was prepared by the N-hydroxysuccinimide ester exchange method [10], and purified by acetone precipitation to give a chromatographically pure product. All other substrates used were the purest available commercially.

*Extraction of particles*

Extracts were prepared by grinding twenty freshly-blotted tuber discs with 12 ml ice-cold 0.05 M sodium phosphate buffer pH 7.6 containing 0.3 M sucrose and 4 mM 2-mercaptoethanol with sand and insoluble polyvinylpyrrolidone in a chilled mortar. After straining through muslin, the extract was centrifuged at 1000 × *g* for 5 min to remove coarse debris. The supernatant was centrifuged at 100000 × *g* for 2 h in a MSE Superspeed 50 Centrifuge. The pellet was resuspended in 4 ml extraction buffer, and the suspension used at once for enzyme assays.

*Enzyme assays*

Phenylalanine ammonia-lyase [11], cinnamate 4-hydroxylase [9], chlorogenic acid oxidase [6] and hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase [12] were assayed by standard procedures. DOPA oxidase was assayed spectrophotometrically at 470 nm when the extract (0.05 ml) was added to 1 ml sodium phosphate buffer pH 6.4 containing 10 mM L-DOPA.

*p*-Coumarate 3-hydroxylase activity was assayed by incubating the enzyme preparation (0.05 ml) with FMN (0.1 μmol) and NADH (2 μmol) in sodium phosphate buffer pH 7.5 in a total volume of 0.32 ml at 30 °C for 30 min. The reaction was then started by adding caffeate (0.2 μmol) and *p*-coumarate (4 μmol) to give a final volume of 0.4 ml. After further incubation for periods up to 70 min, usually 30 min, the reaction was terminated by adding 0.2 ml 25% trichloracetic acid. After centrifugation, the caffeate content of the supernatant was determined colorimetrically [13], and compared with controls in which trichloroacetic acid was added before *p*-coumarate. To 0.5 ml supernatant were added successively 0.5 ml M HCl, 0.5 ml 20% Na tungstate and 0.5 ml 0.5% $NaNO_2$. After standing at room temperature for not less than 10 min, 1.0 ml 2 M NaOH was added and 15 min later the absorbance at 510 nm was measured. Suitable calibration curves for caffeate and other 3,4-dihydroxylated compounds were prepared.

*Conversion of [3-$^{14}$C]cinnamate to p-coumarate and caffeate*

Cinnamate (0.8 μmol, labelled with 0.5 μCi [3-$^{14}$C]cinnamate, Amersham) was incubated with glucose 6-phosphate (0.16 μmol), NADP (0.08 μmol) and glucose-6-phosphate dehydrogenase (Sigma; 0.5 units), and FMN (0.1 μmol) in 20 μmol sodium phosphate buffer pH 7.5 in a total volume of 0.3 ml at 30 °C. The reaction was started by adding extract (0.1 ml), containing 2 mM 2-mercaptoethanol. After 2 h, the reaction was stopped by addition of 0.2 ml trichloroacetic acid.

The mixture was extracted with ethyl acetate (2 × 0.8 ml), each time centrifuged for 30 s to assist the separation of layers. The combined extracts, containing more than 99% of the radioactivity added, were evaporated to dryness *in vacuo* at room temperature. The residue was dissolved in 0.1 ml 90% ethanol, and 10 μl spots of this applied to a silica gel TLC plate (Polygram SIL G; Machery-Nagel). After developing the chromatogram in toluene/acetic acid/water (40:15:0.7, v/v) over about 14 cm, the plates were dried, examined under U.V. light and cut into 0.5 cm segments. Each segment was immersed in scintillation fluid (80 g naphthalene and 7 g butyl-PBD dissolved in 0.4 l 2-methoxyethanol and 0.6 l toluene) and the radioactivity counted.

## Results

When potato particulate preparations were incubated with *p*-coumarate under the standard conditions, caffeate was formed (Table I). NADPH and NADH were equally effective as electron donors, and FAD could be substituted by FMN without loss of activity. However, when NADH or NADPH were omitted, or when FMN or FAD were omitted, no caffeate was formed. Ascorbate was a weak substitute for NADH or NADPH; tetrahydrofolate and dimethyltetrahydropteridine were inactive as electron donors.

No reaction was observed without the addition of "sparker" caffeate [14] nor at mercaptoethanol concentrations exceeding 1 mM. In 0.5 mM mercapto-

Table I. Nucleotide requirements for enzymic hydroxylation of *p*-coumaric acid. The incubation conditions are described in Materials and Methods, using 2 μmol NADH or NADPH or 4 μmol ascorbate, and 0.1 μmol FMN or FAD where indicated.

| Expt. | | Rate of hydroxylation[a] | | |
|---|---|---|---|---|
| | | + NADH | + NADPH | + ascorbate |
| 1 | + FAD | 1.16 | 1.30 | 0.04 |
| | − FAD | 0 | 0 | 0 |
| 2 | + FAD | 1.74 | − | 0.08 |
| | + FMN | 1.82 | − | 0.09 |

[a] μmol/h/disc.

ethanol, neither DOPA oxidase nor chlorogenic acid oxidase activities could be detected.

The hydroxylation of *p*-coumarate was completely inhibited by 2 mM diethyldithiocarbamate, 1 mM azide and 1 mM salicylhydroxamate, partially by 5 mM cyanide, but less than 50% by 1 mM 2,2′-bipyridyl and 10 mM metyrapon (Table II); the last has been used as an inhibitor of cytochrome P-450 hydroxylases [15]. These results suggest that the hydroxylation is catalyzed by a copper enzyme, similar to phenolase, rather than a flavin or cytochrome P-450 hydroxylase.

Caffeate was established as the product of *p*-coumarate hydroxylation (otherwise determined only colorimetrically) by spotting the product on TLC silica gel plates, and developing the chromatogram as described in the Methods section. The caffeate spot ($R_f$, 0.35) was detected under U.V. illumination, and ran with a marker spot, clearly separated from *p*-coumarate ($R_f$, 0.53). In the same way, using n-butanol/acetid/water (5:2:3, v/v) as solvent on cellulose TLC plates (Polygram CEL 400; Machery-Nagel), caffeoyl-CoA was established as the product of *p*-coumaroyl-CoA hydroxylation. The third potential substrate in chlorogenic acid biosynthesis, *p*-coumaroyl 5-quinate, was not available for test.

Using direct colorimetric assay, the preparations catalyzed the hydroxylation of a range of 4-hydroxyphenylpropanoid carboxylic acids, 4-hydroxyphenylpropionate and 4-hydroxyphenyllactate being more effective substrates than *p*-coumarate (Table III). Among a range of $C_6$-$C_2$ and $C_6$-$C_1$ acids tested, only 4-hydroxyphenylacetate was hydroxylated; *p*-cresol was highly active. The hydroxylation was specific for 4-hydroxyphenyl substrates, the 2-hydroxy analogues being inactive. A greater range of substrates was hydroxylated when the particles were prepared and assayed in the absence of 2-mercaptoethanol, as well as hydroxylation proceeding at appreciably greater rates. These preparations were indifferent to the presence of FMN.

The data presented here have been calculated from measurements of the amounts of product after a period of incubation, usually 30 min. The reaction course (Fig. 1) shows the unreliability of this type of assay. Even with the addition of sparker caffeate, an initial lag period was observed. This lag was largely eliminated by preincubation with a raised concentra-

Table II. Effect of inhibitors. The reaction was carried out under standard conditions using NADH and FMN, the final concentrations of inhibitor as indicated.

| Inhibitor | Rate of hydroxylation | |
|---|---|---|
| | μmol/h/disc | % Control |
| None | 1.24 | 100 |
| 10 mM metyrapon | 0.91 | 73 |
| 1 mM 2,2′-bipyridyl | 0.69 | 56 |
| 5 mM NaCN | 0.52 | 42 |
| 1 mM Na azide | 0 | 0 |
| 2 mM Na diethyldithiocarbamate | 0 | 0 |
| 1 mM Na salicylhydroxamate | 0 | 0 |

Table III. Substrate specificity of hydroxylase in particles extracted in 4 mM mercaptoethanol and without mercaptoethanol, and incubated with and without mercaptoethanol respectively. Each incubation mixture included 0.02 μmol caffeate.

| Substrate[a] | Rate of hydroxylation[b] | |
|---|---|---|
| | + mercapto-ethanol | − mercapto-ethanol |
| *p*-Coumarate | 1.18 | 2.81 |
| 4-Hydroxyphenylpropionate | 1.85 | − |
| 4-Hydroxyphenyllactate | 2.16 | − |
| 4-Hydroxyphenylpyruvate | 0.43 | 1.48 |
| Tyrosine | 0.55 | 1.36 |
| 4-Hydroxyphenylacetate | 1.36 | 3.98 |
| 3-Hydroxyphenylacetate | 0 | − |
| 4-Hydroxyphenylglycine | 0 | 0.05 |
| 4-Hydroxyphenylglyoxylate | 0 | − |
| *p*-Cresol | 6.5 | − |
| 4-Hydroxybenzoate | 0 | 0.11 |
| *o*-Coumarate | 0 | − |
| Salicylate | 0 | − |

[a] 10 mM.
[b] μmol/h/disc.

tion of NADH for 20 to 30 min; increasing the concentrations of FMN, FAD or caffeate during this preincubation had no effect. The lag was especially persistent with low rates of hydroxylation.

The lag period was followed by a period of linearity, before the reaction rate rapidly declined to zero, even though the phenolic substrate was far from exhausted. This decline could be arrested by addition of further quantities of NADH, and appears to be due to exhaustion of the reductant (Fig. 1). This was confirmed by spectrophotometric assay of NADH at this stage. Attempts to assay the reduction of FMN or FAD spectrophotometrically at 420 nm failed. It was concluded that rapid autoxidation of reduced FMN and FAD exhausted the supply of NADH, necessary for further hydroxylation.

Conditions for the extraction of the active particles are similar to those used for cinnamate 4-hydroxylase [9]. The successive conversion of [3-$^{14}$C]cinnamate to *p*-coumarate and caffeate was demonstrated, using a NADPH-generating system, NADP and FMN, followed by chromatographic separation of the products. Small quantities of *p*-coumarate and caffeate were produced with the complete system, but only *p*-coumarate was produced in the absence of FMN, almost exactly increased by the amounts of caffeate and its oxidation products in the complete system (Table IV). The conversion of *p*-coumarate was also inhibited by 2 mM diethyldithiocarbamate.

Active particles could be extracted from freshly-cut tuber discs, their activity being about 20% higher after 16 h illumination (Table V). Both phenylalanine ammonia-lyase and cinnamate 4-hydroxylase are almost inactive in fresh tuber discs but increase rapidly upon illumination. The pattern of change found for hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase is more similar to this enzyme. The changes in *p*-coumarate 3-hydroxylase activity are of the same order of magnitude, suggesting that at least part of this activity is inducible and perhaps physiologically related to the changes in the other enzymes and to the biosynthesis of chlorogenic acid.

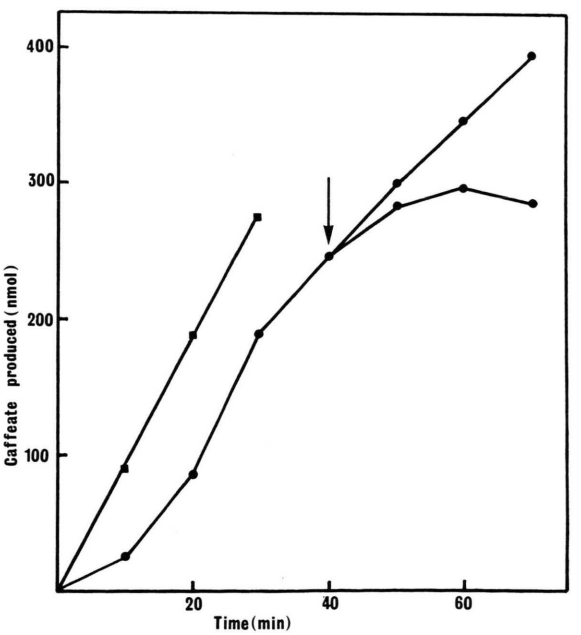


Fig. 1. Reaction course of caffeate formation from *p*-coumarate. Incubation conditions are as described under Methods. *p*-Coumarate and caffeate added immediately after mixing enzyme with FMN and NADH, ●——●; further NADH (2 μmol) added at the arrow. *p*-Coumarate and caffeate added after 20 min pre-incubation of the enzyme with FMN and NADH, ■——■.

## Discussion

Evidence is presented here for a flavin nucleotide-dependent hydroxylase in particulate preparations from illuminated potato tuber discs, which catalyzes the 3-hydroxylation of *p*-coumarate to caffeate, requiring specifically NADPH or NADH as electron donor, and the presence of FMN or FAD. This is an essential stage in the biosynthesis of chlorogenic acid, though its ability to catalyze the 3-hydroxylation of *p*-coumaroyl-CoA may be significant.

The inhibition of both oxidase and hydroxylase functions of phenolase by 2-mercaptoethanol has long been recognised [16]. However, the sensitivity of this hydroxylase to diethyldithiocarbamate, cy-

Table IV. Incorporation of [3-$^{14}$C]cinnamate into *p*-coumarate and caffeate by potato preparation. The results are presented as c.p.m. in four radioactive peaks corresponding to the products shown. Two incubation mixtures are compared, one without and one with FMN (0.1 μmol); NADPH (2 μmol) was the electron donor.

| Compound | Radioactivity | |
|---|---|---|
| | + FMN | − FMN |
| Cinnamate | 76322 | 73838 |
| *p*-Coumarate | 726 | 1152 |
| Caffeate | 310 | 21 |
| Caffeate oxidation products | 120 | 0 |

Table V. Activities of enzymes of chlorogenic acid biosynthesis in freshly-cut potato discs and after 16 h illumination.

| Treatment | Phenylalanine ammonia-lyase[a] | Cinnamate 4-hydroxylase[a] | *p*-Coumarate 3-hydroxylase[a] | CQT[a,b] |
|---|---|---|---|---|
| Freshly-cut | 0 | 0.04 | 1.17 | 0.12 |
| After 16 h light | 0.77 | 0.29 | 1.38 | 0.29 |

[a] μmol/h/disc.
[b] Hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase.

anide and azide, and the requirement for trace quantities of an *o*-diphenol, suggest that some part of the phenolase has survived the extraction and assay conditions, or has undergone some change which confers the flavin nucleotide requirement, and from which the characteristic oxidase activity has been lost. The relative insensitivity to metyrapon suggests that cytochrome P-450 is not involved. The hydroxylase activity surviving mercaptoethanol extraction attacks a somewhat more restricted spectrum of substrates, though too much significance should not be attached to this, since the supply of *p*-coumarate from cinnamate in these particles under conditions of active chlorogenic acid synthesis probably determines its natural substrate.

The specific requirement for flavin nucleotides bears comparison with the mammalian mitochondrial NADPH-cytochrome P-450 reductase, which contains both FAD and FMN [17]. With the suppression of the necessary oxidase activity [18], NADPH and the flavin nucleotides may play a similar role here.

The potato enzyme shows some similarities with the *p*-coumaroyl-CoA hydroxylase from petals of *Silene dioica* [3]. This enzyme was presumably membrane-bound, since Triton X-100 was included in the homogenization mixture. It required NADPH and catalytic quantities of FAD. NADH could be substituted for NADPH when *p*-coumarate was used, but although it was argued that *p*-coumarate was only a substrate effector [19], no evidence was recorded that caffeate was not produced. The potato hydroxylase has a much wider specificity, though the substrate concentrations used here are much higher, and unlike the *Silene* enzyme, it is sensitive to cyanide and azide.

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