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THE HYDROXYLATION STEP IN THE BIOSYNTHETIC PATHWAY LEADING FROM NORCOCLAURINE TO RETICULINE

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Key Word Index—*Berberis stolonifera*; Berberidaceae; phenolase; *N*-methylcoclaurine hydroxylation, alkaloid biosynthesis.

Abstract—A phenolase was purified to homogeneity (488-fold) from cell suspension cultures of *Berberis stolonifera*. This enzyme in the presence of O_2 and ascorbate introduces a *meta*-hydroxyl group into tyrosine, tyramine, coclaurine, and *N*-methylcoclaurine. No other hydroxylating activity directed towards *N*-methylcoclaurine could be found in this tissue. The enzyme (M_r 60×10^3) is probably a dimer with two identical subunits, it has a pH optimum at 6.0 and a temperature optimum in the range of 20–30°. Evidence is presented that this enzyme catalyses two separate reactions in the formation of reticuline, namely the hydroxylation of tyrosine (tyramine) to DOPA (dopamine) and the 3′-hydroxylation of *N*-methylcoclaurine to 3′-hydroxy-*N*-methylcoclaurine, the penultimate intermediate to reticuline.

INTRODUCTION

In contrast to previous assumptions [1] the trihydroxylated base norcoclaurine (also referred to as demethylcoclaurine or higenamine) but not the tetrahydroxylated alkaloid norlaudanosoline is the firmly established universal precursor of reticuline [2–5]. As a result of the revision of the reticuline pathway, the pertinent enzymes catalysing the steps between L-tyrosine and (*S*)-reticuline have had to be renamed and re-allocated [6]. The lack of substrate specificity displayed by these enzymes enabled bioconversion of the unnatural tetraoxygenated bases of the norlaudanosoline type as well as of the natural trioxygenated precursor norcoclaurine. These results, together with the *in vivo* data [7], led to entirely erroneous conclusions and therefore incorrect assignment of the individual *O*- and *N*-methylation sequences of the reticuline pathway [8]. The enzymes involved in the early steps leading to reticuline are as follows [6]: (*S*)-norcoclaurine synthase (stereospecifically condensing dopamine with *p*-hydroxyphenylacetaldehyde), norcoclaurine-6-*O*-methyltransferase (a SAM specific enzyme catalysing the 6-*O*-methylation of norcoclaurine), tetrahydrobenzylisoquinoline-*N*-methyltransferase (also coclaurine-*N*-methyltransferase, a SAM specific *N*-methylating enzyme acting on various benzylisoquinoline alkaloids). By action of these three enzymes an important branch point intermediate, namely *N*-methylcoclaurine, is formed that can either be channelled directly into the bisbenzylisoquinoline alkaloids of the berbamunine type [9, 10] or can be metabolized via hydroxylation and further peripheral modification to (*S*)-reticuline and its derivatives [5]. In order to accomplish the latter transformation *N*-methylcoclaurine has to undergo one hydroxylation (at C-3′) and one *O*-methylation (at C-4′) step. The sequence of both events became clear upon the recent discovery of the novel intermediate (*S*)-3′-hydroxy-*N*-methylcoclaurine [5]. This metabolite, isolated from cell suspension cultures of *Berberis stolonifera*, *Eschscholtzia californica* and *Peumus boldus*, is subsequently transformed by a stereoselective 4′-*O*-methylation reaction to afford (*S*)-reticuline [11]. The question now remaining is the mechanism by which the hydroxyl group is introduced into *N*-methylcoclaurine to yield 3′-hydroxy-*N*-methylcoclaurine. This paper deals with the elucidation of the enzymatic reaction involved in this key hydroxylation step.

RESULTS

The enzyme reaction

There are several possibilities by which hydroxylation reactions can take place in plants. Hydroxylation can be catalysed by microsomal cytochrome P-450 monooxygenases [12], by α-ketoglutarate dependent dioxygenases [13], by flavin enzymes [14, 15], by enzymes which require dihydroxyfumarate and O_2 as co-substrates, or peroxidases [16]. Incubation of [N-$^{14}CH_3$]-*N*-methylcoclaurine under the above conditions and employing enzyme preparations of *Berberis stolonifera* cell cultures in the presence or absence of 'sparker' molecules [15] (in

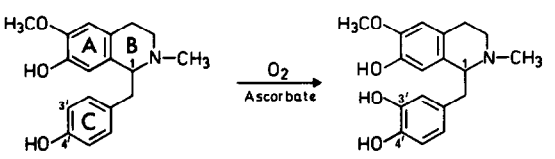

Fig. 1. Phenolase catalysed transformation of *N*-methylcoclaurine to 3′-hydroxy-*N*-methylcoclaurine.

this case 3′-hydroxy-*N*-methylcoclaurine), upon chromatographic analysis, failed to yield any evidence for the formation of 3′-hydroxy-*N*-methylcoclaurine. However, an assay for phenolase activity in this tissue led to the expected hydroxylation reaction. The formation of 3′-hydroxy-*N*-methylcoclaurine was observed with *N*-methylcoclaurine as substrate and in the presence of ascorbate that is indispensable in this reaction and serves to reduce the *o*-quinone back to the diphenolic product [17]. A preliminary examination showed that the phenolase from *Berberis* has a broad substrate specificity in that not only *N*-methylcoclaurine but also coclaurine, tyramine and tyrosine could serve as substrates, yielding in each case the respective dihydroxylated product. The phenolase is obviously a monophenol-monooxygenase (EC 1.14.18.1). The lack of substrate specificity, however, proved advantageous for the assay procedure. [N-$^{14}CH_3$]-*N*-methylcoclaurine and [3,5-T]-L-tyrosine were used interchangeably as substrates. Phenolase activity could be measured easily and precisely by monitoring tritium release from the substrate resulting from the hydroxylation reaction.

Purification of the phenolase

Berberis stolonifera cell suspension cultures, which contain large amounts of both protoberberine and bisbenzylisoquinoline alkaloids [18], served as the enzyme source. In the early phases of the purification, we observed that the phenolic alkaloids present in the cell homogenate apparently exert a strong inhibitory effect on the phenolase activity. Addition of polyvinylpyrrolidone to the crude extracts and subsequent chromatography on Sephadex G-25 removed most of the interfering compounds. The resulting, almost colourless, extract was then subjected to standard fractionation procedures: hydroxylapatite, DEAE-Sephacel, Q Sepharose Fast Flow, Mono Q, and isoelectric focusing. The purified enzyme showed only one protein band on disc electrophoresis. The first two purification steps reflect a drastic increase in total enzyme yield, probably due to the removal of low M_r compounds during the purification procedure. The final isoelectric focusing step led to a considerable loss of catalytic activity brought about by the acid instability of the enzyme. This is clearly visible in the sharp drop of the purification factor. However, this terminal step was necessary to obtain the homogeneous enzyme and also made possible the separation of a second enzyme activity, representing approximately 25% of the final activity and having an isoelectric point (IEP) of 3.95. The purification scheme is summerized in Table 1.

Properties of the phenolase

The homogeneous enzyme had a pH optimum at 6.0 with half maximal activity at 4.5 and 7.5. The temperature optimum lay between 20 and 30°. The activation energy for tritium release over the temperature range from 4 to 20° was determined to be 53 kJ mol^{-1}. The IEP of the homogeneous enzyme was at pH 4.25. The M_r of the protein was determined by gel permeation HPLC on a calibrated TSK 3000 SW column. Assuming a globular shape of the phenolase, a M_r of 60×10^3 was determined. The homogeneous enzyme was also subjected to SDS gel electrophoresis and showed a M_r of 36×10^3. These results infer that the phenolase is composed of two identical subunits.

The product of the enzymatic hydroxylation reaction was determined by employing the radiolabelled substrate [N-$^{14}CH_3$]-(*S*)-*N*-methylcoclaurine. The reaction product was isolated by TLC and then incubated with SAM and the highly substrate and stereoselective enzyme (*S*)-3′-hydroxy-*N*-methylcoclaurine-4′-*O*-methyltransferase [11], yielding a compound that co-migrated with reticuline. Further biotransformation by addition of the berberine bridge enzyme [19] furnished a compound identical to (*S*)-scoulerine. As the latter enzyme is also extremely stereo- and substrate-selective, there is absolutely no doubt that the original product formed by the phenolase is (*S*)-3′-hydroxy-*N*-methylcoclaurine. The kinetic properties of the phenolase reaction are shown in Table 2 using five different substrates.

Phenolases (tyrosinases) are copper-containing enzymes [17, 20]. The activity of the *Berberis* enzyme should, therefore, also be inhibited by chelating agents. The enzyme with tyrosine as substrate is strongly impaired by the copper chelators diethyldithiocarbamate, KCN and bathocuproine. NaN_3, thiourea and dipicolinic acid were inhibitory at only high concentrations, but EDTA was ineffective even at a concentration of 10 mM.

DISCUSSION

The trioxygenated alkaloid *N*-methylcoclaurine, isolated on several occasions from alkaloid-containing plants [21, 22], is the firmly established intermediate *en route* to

Table 1. Purification to homogeneity of phenolase from cell suspension cultures of *B. stolonifera*

Purification step	Volume (ml)	Total activity (nkat)	Total protein (mg)	Specific activity (pkat mg^{-1})	Yield (%)	Purification (fold)
Crude extract	565	0.79	712	—*	—	—
Sephadex G-25	60	1.67	308	5.4	100	1
Hydroxylapatite-DEAE-Sephacel	26	3.50	9.4	373.3	210	69
Q Sepharose Fast Flow	12.5	3.74	2.1	1782.9	224	329
Mono Q	7.3	1.58	0.6	2637.5	95	488
Isoelectric focusing	0.2	0.31	0.28	1089.0	19	202

*Because of contaminating phenolic alkaloids no phenolase activity could be detected in the crude extract.

Table 2. Relative rates of conversion, K_m and V_{max} values of five different substrates as well as identification of the reaction products of the phenolase catalysed reaction

Substrate	Rate of transformation (%)	K_m value ($mmol\,l^{-1}$)	V_{max} ($pmol\,sec^{-1}$)	Product of reaction
Tyramine	100	0.29	30	Dopamine
(*R,S*)-*N*-Methylcoclaurine	80	0.58	16	(*R,S*)-3′-OH-*N*-Methylcoclaurine
(*R*)-Coclaurine	71	0.63	20	(*R*)-3′-OH-Coclaurine
L-Tyrosine	67	0.83	36	L-DOPA
(*S*)-Coclaurine	60	0.75	21	(*S*)-3′-OH-Coclaurine

The reaction mixture contained 10 nmol substrate, 30 μmol ascorbate, 50 μmol K-Pi pH 6.0 and 0.76 pkat (0.83 μg) homogeneous enzyme. The incubation was for 30 min at 30°.

Table 3. Inhibition of the *Berberis* phenolase by different compounds

Compound	Concentration ($mmol\,l^{-1}$)	Inhibition (%)
Diethyldithiocarbamate	0.005	50
	0.3	100
KCN	0.07	50
	1.0	100
Bathocuproine	0.5	18
NaN_3	12	50
Thiourea	13	50
Dipicolinic acid	10	22
Bathocuproine disulphonate	10	0
Bathophenanthroline disulphonate	10	0
Na_2EDTA	10	0

The inhibition rate was measured at the given concentration in the ^3H-release assay employing [3,5-$^3H_2$]-L-tyrosine as the substrate.

reticuline and reticuline-derived bases [5]. Further transformation to reticuline embraces a 3′-hydroxylation reaction of the benzylic ring to furnish 3′-hydroxy-*N*-methylcoclaurine, that has recently been found in cell cultures that elaborate the benzylisoquinoline nucleus [5]. This observation led to the search for an enzyme capable of hydroxylating *N*-methylcoclaurine at the C-3′ position. Our attention focussed on cell suspension cultures of *Berberis stolonifera* as the ideal enzyme source not only because of their immense biosynthetic capacity regarding alkaloid production, but also because of the presence of the novel intermediary base 3′-hydroxy-*N*-methylcoclaurine [5]. Initial attempts to elucidate the catalytic nature of the reaction were more or less centered on the search for specific hydroxylase activity that could, theoretically, involve either a cytochrome P-450, NADPH, α-ketoglutarate, dihydroxyfumarate, or flavin-dependent enzyme.

However, it has already been noted [23] that *Berberis* cell cultures display ascorbate-dependent phenolase activity in the conversion of L-tyrosine into L-dihydroxyphenylalanine (DOPA) and tyramine into dopamine. Administration of the monohydroxylated intermediates *in vivo* led to the expected entry of the resulting catecholamines into the 'upper' portion of the protoberberine skeleton [23]. The homogeneous phenolase isolated here shows a relatively broad substrate specificity hydroxylating tyrosine, tyramine, (*R*)- and (*S*)-coclaurine, and (*R,S*)-*N*-methylcoclaurine with similar efficiency as expressed by the respective K_m values. Furthermore, the lack of both substrate specificity and stereoselectivity is above all reflected by feeding experiments with (*R*)-coclaurine that result in the metabolic accumulation of both (*R*)-*N*-methylcoclaurine and (*R*)-3′-hydroxy-*N*-methylcoclaurine in *Eschscholtzia californica* cell cultures [5]. As anticipated, these 'unnatural' (*R*)-configurated bases could not be metabolized any further to reticuline by the tissue due to the highly stereoselective nature of the 4′-*O*-methylating enzyme. Comparison of the properties of the phenolases/phenol oxidases from different plant species such as *Papaver* [24, 25], *Mucuna* [26] or *Berberis* [23], clearly demonstrates that these enzymes are species specific and not uniform. We assume that the phenolase described here has a dual function in alkaloid metabolism. This enzyme is responsible, firstly, for the hydroxylation of tyrosine to DOPA as well as tyramine to dopamine that furnishes the 'upper' isoquinoline portion of these alkaloids after condensation with *p*-hydroxyphenylacetaldehyde. The second function entails the site-specific introduction of a 3′-hydroxyl group into the benzylic moiety of *N*-methylcoclaurine. In both cases the phenolase reaction inevitably supplies the *meta*-hydroxyl group in the aromatic rings A and C of the benzylisoquinoline alkaloids. The phenolase described here had a surprisingly low pH optimum (pH 6.0), compared to all other enzymes of the reticuline pathway [6] which have

their optima at pH 7.5 or above. This suggests that the enzyme could be compartimentalized. Furthermore, plant phenolases have been located in chloroplasts and mitochondria [27, 28] and can also be associated with thylakoid membranes [29]. Electron microscopic investigations have shown [30] that in *Berberis* cell cultures up to 7% of the cytoplasmic space is filled with proplastids. This question has to be investigated independently. On the basis of our present knowledge we conclude that the phenolase described in this paper has an important metabolic function in alkaloid metabolism, in that this enzyme catalyses the non-stereospecific 3'-hydroxylation of *N*-methylcoclaurine to yield 3'-hydroxy-*N*-methylcoclaurine, the penultimate precursor to reticuline.

EXPERIMENTAL

Materials. Cell cultures of *Berberis stolonifera* were grown as previously described [23] and frozen in liquid N_2. (*R,S*)- and (*S*)-*N*-Methylcoclaurine as well as its 3'-hydroxylated congener were synthesized by Dr R. Stadler [5]. (*R,S*)-[N-$^{14}CH_3$]-*N*-Methylcoclaurine was synthesized by incubating [$^{14}CH_3$]-SAM, (*R,S*)-coclaurine, *N*-methyltransferase [31], and subsequently isolating the radio-labelled *N*-methylated product. [3,5-$^3H_2$]-L-Tyrosine and [$^{14}CH_3$]-SAM were purchased from Amersham. (*R*)- and (*S*)-6-*O*-[C^3H_3]-Coclaurine were synthesized by incubating the respective norcoclaurine enantiomers in the presence of (C^3H_3)-SAM and commercial catechol-*O*-methyltransferase (Sigma). The (*S*)-counterpart was formed at double the rate of the (*R*)-enantiomer with the desired methylation reaction occurring exclusively at the C-6 position. However, in the case of the (*R*)-enantiomer, some unspecific *O*-methylating activity could be observed leading also to (*R*)-isococlaurine and (*R*)-norarmepavine. The basic products were extracted with EtOAc at pH 9.5 and subsequently purified by TLC [CH_2Cl_2–MeOH–NH_3 (25%) = 90:9:1].

Enzyme assay. The standard assay mixt. (300 μl) contained the following: 50 μmol K-Pi buffer pH 6.0, 30 μmol Na-ascorbate, 10 nmol [3,5-$^3H_2$]tyrosine (15 000 cpm) and enzyme. The mixt. was incubated for 30 or 60 min at 30° and terminated by the addition of 300 μl dextran-coated charcoal suspension (10 g activated charcoal, 250 mg dextran in 100 ml H_2O), agitated (1 min) and centrifuged (all Eppendorf systems). The supernatant (300 μl aliquot) was added to 5 ml of scintillation fluid (Rotiszint, Roth, Karlsruhe) and radioactivity determined. In the case of *N*-methylcoclaurine incubations, the incubation mixt. (50 μl) was applied directly to silica gel TLC sheets (0.25 mm Polygram Sil G/UV_{254}, Macherey and Nagel) and developed in Me_2CO–$CHCl_3$–$NHEt_2$ (5:4:1) (*N*-methylcoclaurine: R_f 0.62; 3'-hydroxy-*N*-methylcoclaurine: R_f 0.17).

Enzyme purification. All procedures were carried out at 0–4°. To 250 g of 7-day-old deep frozen cells were added 25 g polyvinylpyrrolidone (Sigma) and 500 ml 100 mM K-Pi buffer at pH 7.5. The slurry was stirred until a homogeneous brei was obtained, which was then pressed through cheesecloth and centrifuged for 10 min at 10 000 *g*. The supernatant was fractionated between 30 and 60% saturation with $(NH_4)_2SO_4$ and the suspension centrifuged. The resulting pellet was taken up in 30 ml 10 mM KPO_4 buffer at pH 7.5. The protein solution was freed from alkaloids and desalted by passage through a Sephadex G-25 column (3 × 65 cm), pre-equilibrated with 10 mM K-Pi buffer adjusted to pH 7.5. The protein-containing eluate in a 60 ml soln was subsequently passed through a hydroxylapatite column (3 × 21 cm) at a flow rate of 25 ml hr^{-1}. The eluate containing the enzyme was directly loaded onto a DEAE column (2.5 × 29 cm) pre-equilibrated with 10 mM K-Pi buffer at pH 7.5 buffer. The DEAE column was washed with 215 ml of the same equilibration buffer and then eluted with the same buffer supplemented with 0.75 M KCl. The eluate (26 ml) was desalted by dialysis for 2 hr against 5 l 10 mM K-Pi buffer (pH 7.5) and applied (1 ml min^{-1}) onto a Q Sepharose Fast Flow column (1 × 10 cm), pre-equilibrated with 10 mM K-Pi buffer (pH 7.5). The protein was eluted with a linear KCl gradient (0–400 mM KCl in standard buffer) with the phenolase appearing between 0.2–0.4 mM KCl. The enzyme-containing fractions (58–70), which were light brown in colour, were pooled and dialysed against the standard buffer. A portion of the dialysate (12 ml) was loaded onto a Mono Q column (0.5 × 5 cm, Pharmacia) at a flow rate of 1 ml min^{-1}, equilibrated in standard buffer. Elution was accomplished with a linear KCl gradient (0–200 mM) in standard buffer. The active fractions were concd to a vol. of 350 μl (1 mg protein) with polyethyleneglycol (PEG 20 000, Serva). Isoelectric focusing (flat gel, ampholytes pH 4.0–6.5) was applied and the gel prefocused at 400 V overnight, and then for 3 hr at 2000 V. For protein detection, the gel was blotted with a cellulose membrane. One part of the membrane was used to stain for protein (Ponceau S) while the other portion was allowed to react with tyramine which was subsequently transformed to dopamine. This technique enabled visualization of the phenolase as a coloured band within 2 min. The major band (IEP 4.25) was removed from the gel, eluted with 500 μl H_2O, electrodialysed, and concd to a vol. of 200 μl. The enzyme was found to be homogeneous by SDS-PAGE [32]. Protein was determined according to ref. [33], relative protein values as described in ref. [34].

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