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Arecaceae sub *Palmae*
Asteraceae sub *Compositae*
Brassicaceae sub *Cruciferae*
Clusiaceae sub *Guttiferae*
Fabaceae sub *Leguminosae* (including Mimosoideae = *Mimosaceae*, Caesalpinioideae = *Caesalpiniaceae* and Papilionoideae = *Papilionaceae* = Fabaceae sensu stricto)
Hypericaceae sub *Guttiferae*
Lamiaceae sub *Labiatae*
Oenotheraceae sub *Onagraceae*
Poaceae sub *Gramineae*

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Journal of Medicinal Plant Research **Planta medica**

Partial Purification and Properties of (S)-Norlaudanosoline Synthase from Eschscholtzia tenuifolia Cell Cultures

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Eschscholtzia tenuifolia; Papaveraceae; Cell Cultures; Alkaloid Biosynthesis; (S)-Norlaudanosoline Synthase.

Abstract

A new enzyme, (S)-norlaudanosoline synthase, which catalyses the synthesis of (S)-norlaudanosoline from dopamine and 3,4-dihydroxyphenylacetaldehyde was isolated from the soluble protein extract of *Eschscholtzia tenuifolia* cell suspension cultures and purified approximately 40-fold. The apparent molecular weight of the enzyme is 15 500 Dalton. The pH optimum is 7.8, temperature optimum 40° C, apparent K_M values for dopamine and dihydroxyphenylacetaldehyde are 1.5 mM and 0.7 mM respectively. The synthase shows high substrate specificity in that only the phenylacetaldehydes are transformed but not the phenylpyruvates. No apparent cofactor requirement could be demonstrated. By means of isoelectric focusing and disc-gel electrophoresis evidence was obtained for the existence of four norlaudanosoline synthase isoenzymes, none of which catalyses the reaction of dopamine with 3,4-dihydroxyphenylpyruvate. These enzymes are responsible for the synthesis of (S)-norlaudanosoline, the key intermediate in the formation of isoquinoline alkaloids occurring in the plant kingdom.

Introduction

The largest group of alkaloids in the plant kingdom is the one based on the tetrahydroisoquinoline nucleus [1]. This nucleus occurs in a vast array of structure types, all derived from L-tyrosine. The nature of the intermediates in the initial reaction of isoquinoline biosynthesis from tyrosine has, however, not been fully established, neither at the *in vivo* nor at the *in vitro* level. According to the scheme outlined in 1910 by WINTERSTEIN and TRIER [2], the isoquinoline nucleus arises in nature by condensation of dopamine and 3,4-dihydroxyphenylacetaldehyde (route I, Fig. 1). As the first isoquinoline intermediate in the pathway, norlaudanosoline was postulated as early as 1917 [3]. More than 40 years later, this compound was indeed recognized by radioactive substrate feeding experiments as an early precursor of papaver-alkaloids [4, 5].

However, above scheme was modified [6] in as far as it was postulated that DOPA is converted into the pyruvic acid, rather than the aldehyde, and that condensation with dopamine provides norlaudanosoline-1-carboxylic acid which subsequently undergoes decarboxylation as shown in Fig. 1 (route II). The intermediary nature of norlaudanosoline-1-carboxylic acid was recently claimed to be supported by *in vivo* [7–9] and *in vitro* [10, 11] experiments.

In order to distinguish between route I or II (Fig. 1) it was clearly necessary to study the early steps of isoquinoline biosynthesis at the cell-free level. Experiments, using crude enzyme preparations from cell cultures of 10 different genera from 3 plant families containing benzylisoquinoline alkaloids led to the discovery of a novel enzyme which condenses dopamine with 3,4-dihydroxypenylacetaldehyde to yield (−)1-α-H-(S)-norlaudanosoline [12]. The enzyme catalysing this reaction was named (S)-norlaudanosoline synthase. No reaction was observed using cell-free extracts of the above plant material with 3,4-dihydroxyphenylpyruvate as carbonylic substrate. Thus it was demonstrated that route I of Fig. 1 is operative in isoquinoline alkaloid producing plants. The first natural intermediate in this pathway is therefore (S)-norlaudanosoline rather than norlaudanosoline-1-carboxylic acid. In this report we present the partial purification and properties of (S)-norlaudanosoline synthase, a central enzyme of the isoquinoline pathway.

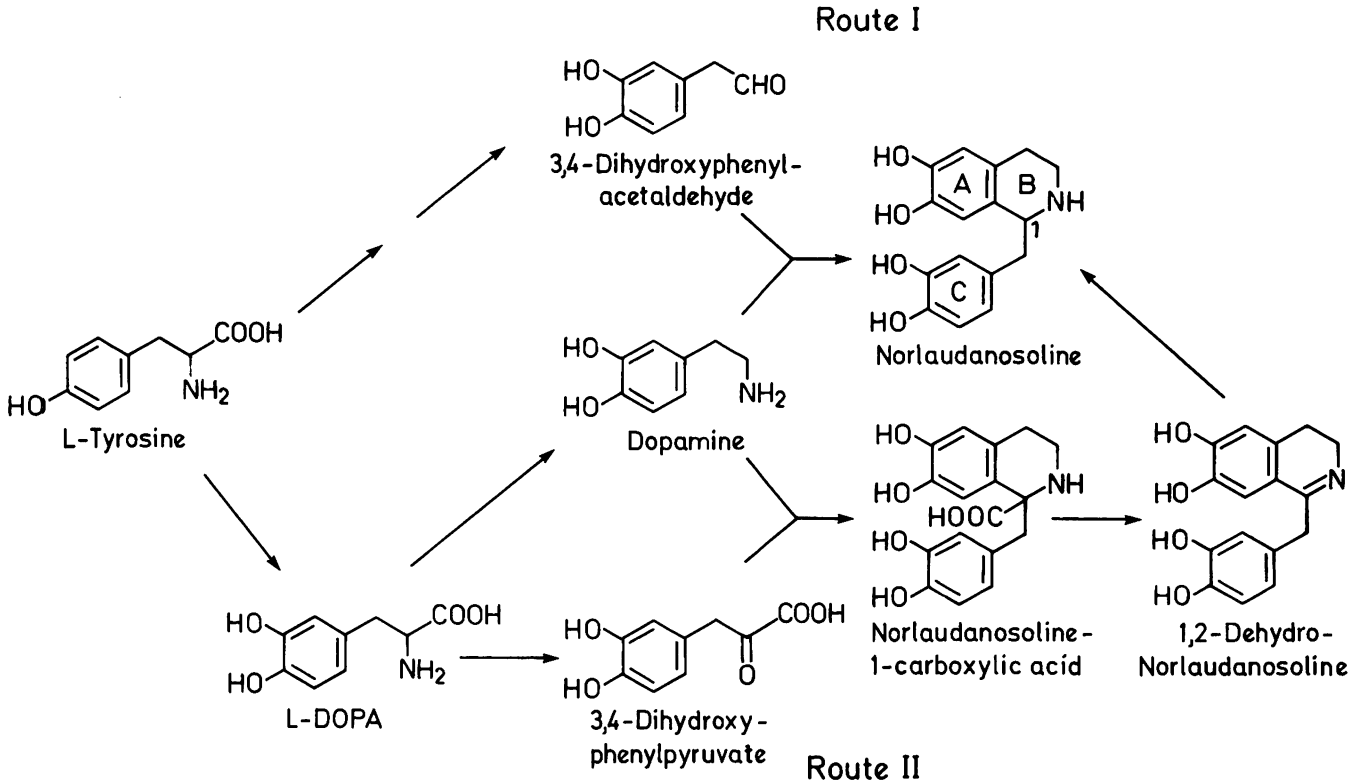


Fig. 1. Two putative biosynthetic routes to the benzylisoquinoline skeleton.

Material and Methods

Plant material

For this investigation a cell culture line of *Eschscholtzia tenuifolia* was used which has been established and is growing in this laboratory since 1975. The cells are cultivated on Linsmaier and Skoog (LS)medium [13] in 1 litre Erlenmeyer flasks which contain 250 ml of liquid medium and shaken at 23° C and 750 lux on a gyratory shaker at 100 rpm. Maximal enzyme yield was observed after 8 to 10 days at which time cells were filtered, frozen in liquid nitrogen, and stored at −20° C.

Substrates

As already described [12] dopamine (ring 2,6-^{3}H) was used as a substrate. This compound was synthetized in the following way: ring-2,6-^{3}H-L-tyrosine (Amersham) 0.25 µmol (0.93 mCi) was dissolved in 1 M KPO_4^{2-}-buffer pH 5.5 and tyrosine decarboxylase (Sigma, 5 mg) was added. The reaction took place in a total volume of 150 µl. Reaction time was 20 minutes at 37° C. The tyramine (ring 2,6-^{3}H), formed during this reaction period by action of the decarboxylase, was oxidized to dopamine without further purification: 0.5 mg tyrosinase (Sigma) in 100 µl KPO_4^{2-}-buffer (1M, pH 6.1) and 100 µl Na^+-ascorbate solution (1M) were added to this reaction mixture to yield a total volume of 350 µl. The reaction mixture was gassed with oxygen. Reaction time was 7 minutes at 37° C. Optimal reaction time for this enzyme had to be determined prior to the preparative synthesis involving the ^{3}H-labelled substrate. Dopamine thus formed was purified by paper chromatography (Whatman No. 1, solvent system: n-butanol : acetic acid : water = 4:1:5, upper phase). The labelled dopamine was eluted with water, the overall yield of ring-2,6^{3}H-dopamine was 51.5 %, spec. act.: 3.7 mCi/µmol.

3,4-dihydroxyphenylpyruvate was the kind gift of Prof. Steglich (Bonn). 4-hydroxyphenylpyruvate was obtained from Sigma. 3,4-dihydroxyphenylacetaldehyde and 4-hydroxyacetaldehyde were prepared from adrenaline and synephrine respectively by the procedure of [15] as modified by [16]. In highly purified form both aldehydes were surprisingly stable at −20° C.

Norlaudanosoline-synthase assay

Standard assay conditions were as in [12] except that final concentration of 0.4 mM ^{3}H-dopamine (1.4×40^4 cpm) was used in the assay. Varying amounts of enzyme were used in a final volume of 0.25 ml. Sodium ascorbate had to be added to the incubation mixture in order to assure protection from oxidation of the substrates. Final concentration of 64 mM ascorbate in the assay has been found to be optimal for the reaction rate. After incubation for 40 minutes at 30° C, 0.3 ml dextran coated charcoal (30 mg) suspension was added, the sample was agitated for 1 minute and subsequently centrifuged (9980 rpm) for 5 minutes (Eppendorf system). 0.2 ml of the aqueous phase was transferred to the scintillation vial, mixed with 5 ml Quickszint scintillation fluid (Koch-Light-Zinsser) and counted for radioactivity (Bertold system). Controls with either heat denatured enzyme or without carbonylic substrates were always included in the tests. The amount of ^{3}H release by non-enzymatic transformation of the substrate under these conditions was subtracted from the sample containing active enzyme [12]. The ^{3}H-release measured by these conditions (charcoal absorption) was chequed by a sublimation technique which proved that all the nonabsorbed ^{3}H did appear in form of ^{3}HOH in the incubation mixture. ^{3}H-dopamine solution on standing released some ^{3}H in form of water. If this phenomenon was observed ^{3}H-dopamine had to be rechromatographed and was used after purification.

Enzyme purification

Step 1. 700 g tissue of frozen *E. tenuifolia* cells were crushed in 1.5 litres of phosphate buffer pH 7 containing 20 mM EtSH and stirred for 20 minutes in an ice bath. The suspension was pressed through cheese cloth and the filtrate was centrifuged at 48000 xg at 4° C.

Step 2. A fractionated ammonium sulphate precipitation (0–50 %, 50–70 %) was carried out with the supernatant, the centrifuged precipitate (10 min at 48000 xg) taken up in a minimum amount of phosphate buffer (pH 7.5, 20 mM EtSH), and dialysed for 18 hours at 4° C against 20 mM KPO_4^{2-}-buffer pH 7.5 (20 mM EtSH). The slightly turbid solution was centrifuged again as above.

Step 3. The dialysed protein solution was added to a DEAE-cellulose column (39.4 ml, Ø = 2.5 cm, l = 8 cm) equilibrated with 20 mM KPO_4^{2-}-buffer, pH 7.5 (20 mM EtSH). The protein solution was absorbed, washed with equilibration buffer and a gradient from 0–200 mM KCl (400 ml) was applied. 80 Fractions of 5 ml were collected and the enzyme activity was found in fractions 4–15.

Step 4. The enzyme containing fractions of step 3 were pooled and applied to a CM-cellulose column (vol. 15 ml, Ø = 1.5 cm, l = 8 cm) equilibrated with 20 mM KPO_4^{2-}-buffer pH 6.0 (20 mM EtSH). After absorption of the protein, the column was washed with equilibration buffer and eluted with a gradient from 0–250 mM KCl (280 ml). 70 Fractions of 4 ml were collected and the enzyme activity was found in fractions 16–21.

Step 5. The enzyme containing fractions of the step above were concentrated to 6 ml and put onto a Sephadex-G-100 column (150 ml, Ø = 1.5 cm, l = 85 cm), equilibrated with 50 mM KPO_4^{2-}-buffer pH 7.0 (20 mM EtSH). 100 Fractions of 2 ml were collected and the enzyme activity was found in fractions 43–54. The 11 fractions containing the enzyme were pooled, concentrated, and used for subsequent experiments.

Protein was determined by a modified Lowry-test [17] or in more highly purified samples with optical methods [18].

Molecular weight determination

The molecular weight of the purified (S)-norlaudanosoline synthase was carried out by gel filtration on a calibrated Sephadex-G-100 superfine column. Although only the Stokes radius of a protein can be determined by this method, it is often used for the determination of the molecular weight on the premise of a globular shape of the protein tested. The column (150 ml, Ø = 1.5 cm, l = 85 cm), equilibrated with 25 mM KPO_4^{2-}-buffer (20 mM EtSH) pH 7.5, was eluted with 15 ml/h in 100 fractions of 2 ml. The column was calibrated with proteins of known molecular weight: cytochrom C (12500), chymotrypsinogen A (25000), ovalbumin (45000), and bovine serumalbumin (67000). Ferritin (450000) was used for the determination of the void volume of the column. The standards were monitored by the absorbance at 280 nm. The results are given as Stokes-radii.

Polyacrylamide gel electrophoresis, isoelectric focusing

In order to search for the existence of isoenzymes a disc gel electrophoresis was performed. The system used was a 10 % polyacrylamide gel [19]. The electrophoresis was carried out at 4° C and 2 mA per gel using imidazol (0.68 mM) and D,L-asparagine (9.6 mM) pH 7 as electrode buffer. Protein of step 2 was used for this electrophoresis. Gels were either stained with amido black or sliced and the enzyme activity eluted in 200 µl of step 1 buffer for 24 hrs at 4° C. The enzyme activity was measured with a 50 µl aliquot in the standard assay.

Isoelectric focusing (LKB system) was used according to [20]. The protein of step 2 dialysed against 10 % glycine was used for this assay. The column was run for 36 hrs. The final parameters were 300 V, 0.4 mA, 0.12 watt. The column was fractionated into 60 fractions of 2 ml, with a flow rate of 120 ml/h. 50 µl of the fractions were used for the standard assay. Dialysis of the fractions did not increase the catalytic activity.

Both assay systems were accomplished using 3,4-dihydroxyphenylacetaldehyde or 3,4-dihydroxyphenylpyruvate as substrates.

Results

The basis for the successful application of the tritest principle was the fact that one of the tritium atoms of ring-2,6-^{3}H-dopamine was removed during the enzymatically catalysed condensation with phenylacetaldehydes [12]. It had been known previously [21] that incubation of dopamine and appropriate aldehydes under so-called physiological conditions leads to the non-enzymatic formation of benzylisoquinoline derivatives. Both of these reactions, the nonenzymatic and the enzymatic condensation of the substrates, do occur under standard conditions. Fig. 2 shows the kinetic of norlaudanosoline formation under standard conditions with of without enzyme catalysis. As can be seen clearly, there is a high rate of nonenzymatic formation of norlaudanosoline which has to be subtracted from the gross conversion observed in the standard incubation mixture in the presence of the synthase enzyme. From this figure it also can be deduced that proper enzymatic values can only be obtained in relatively short periods of incubation time. During later phases of the reaction the uncatalyysed condensation of the reactants is larger than in the

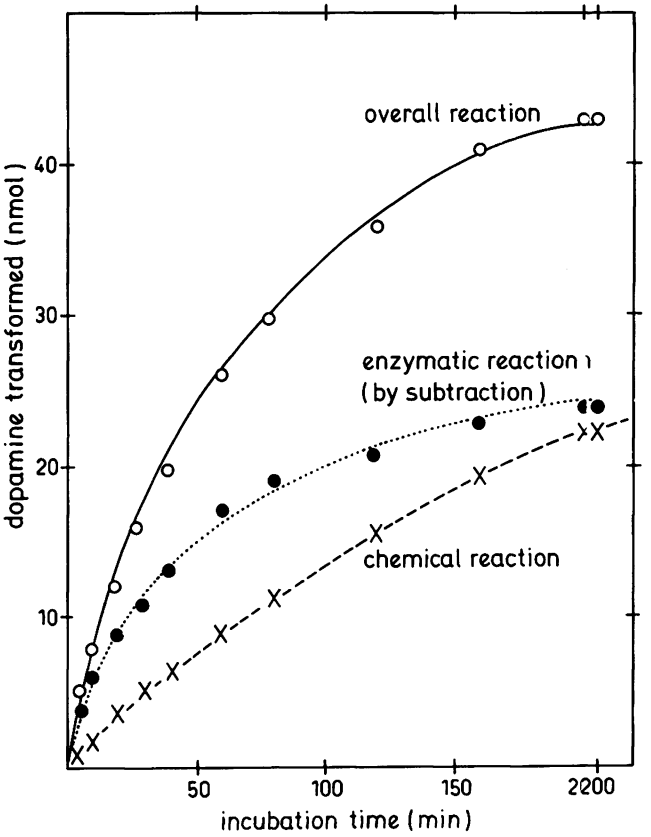


Fig. 2. Kinetics of the reaction of dopamine with 3,4-dihyddroxyphenylacetaldehyde in the standard incubation mixture. The graph designated "chemical reaction" (x – x) shows the time course of the uncatalysed Pictet-Spengler reaction. The graph naamed "overall reaction" (O – O) shows the dopamine transformatition in the presence of enzyme (2.1 µg; 8.74 pkat). The kinetic of thhe net enzymatic reaction (● – ●) was obtained by subtraction.

presence of the enzyme. Incubations were therefore carried out only for 15 minutes and several controls were run parallelly [12].

The enzyme under investigation was isolated from *E. tenuifolia* suspension cells by extracting frozen cell powder with phosphate buffer. Partial purification was achieved by ammonium sulphate fractionation (50–70 % saturation). DEAE-cellulose- and CM-cellulose, as well as gel chromatography on Sephadex G-100. This procedure yielded a purification of approximately 40-fold with a recovery of only 3.3 %.

Table I

Purification of (s)-Norlaudanosoline Synthase from 700 g (wet wt) Suspension Cells

Step	Protein mg	Total Activity (nkat)	Specific Activity (pkat/mg)	Yield (%)	Purification (-Fold)
Crude Extract	1000	53	52	100	0
Ammonium Sulfate Factionation/ Dialysis	330	49.5	150	92	2.8
DEAE-Cellulose	70	42	600	78	11.3
CM-Cellulose	5.7	6.3	1110	12	20.8
Gelchromatography Sephadex G-100	0.4	1.7	2080	3.3	39

The data for a typical purification procedure are summarized in Table I. The protein solution at this stage did not contain any other enzymes of the isoquinoline biosynthetic pathway thus far tested. A typical elution profile of a gel chromatrography experiment on Sephadex G-100 using a step 2 enzyme is shown in Fig. 3. As can be noted there is a relatively sharp peak between fractions 43 and 54 which contains the catalytic activity. Disc gel electrophoresis of a step 2 enzyme under the conditions given in material and methods gave a surprising result. As shown in Fig. 4 definite catalytic activity was found in 4 areas of the gel. This was the first indication for the occurrence of isoenzymes. Further evidence for the existence of 4 distinct proteins catalysing the formation of norlaudanosoline was obtained by isoelectric focusing. As shown in Fig. 5, a step 2 protein again separated into 4 distinct enzyme activities. Independent experiments led to the assumption of multiple enzyme forms at the isoelectric points pH 4.6; pH 5.3; pH 6.7; pH 7.6. There is good evidence that the protein which has been enriched as given in Table I is that one with the IEP of pH 7.5 which also might explain the relatively low yield (3.3 %) of the purification procedure. It is noteworthy that these 4 forms of norlaudanosoline synthase can also be clearly distinguished during chromatography on hydroxy-apatite columns (data not shown). In *E. tenuifolia* there are undoubtedly 4 forms of norlaudanosoline synthase which all condense dopamine with 3,4-dihydroxyphenylacetaldehyde, none of which works with the phenylpyruvates.

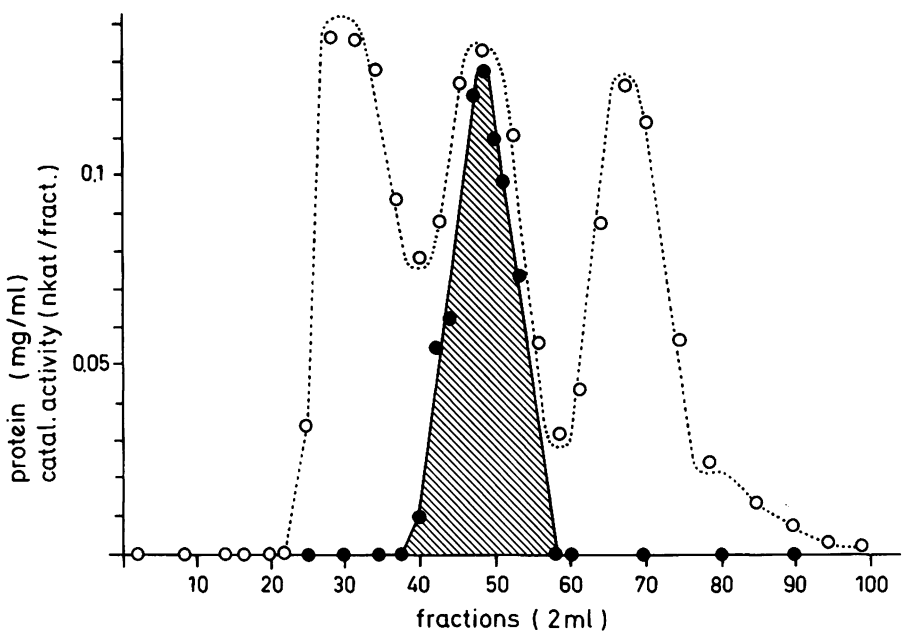


Fig. 3. Elution profile of (S)-norlaudanosoline synthase activity from a Sephadex G-100 gel filtration column. The applied protein solution (9.6 mg protein, 5.45 nkat) had been prepurified by ammonium sulphate fractionation, dialysis and DEAE cellulose column chromatography. The gel column was equilibrated and eluted with 50 mM phosphate buffer at pH 7.0. Enzyme activity was measured as mentioned in the text.

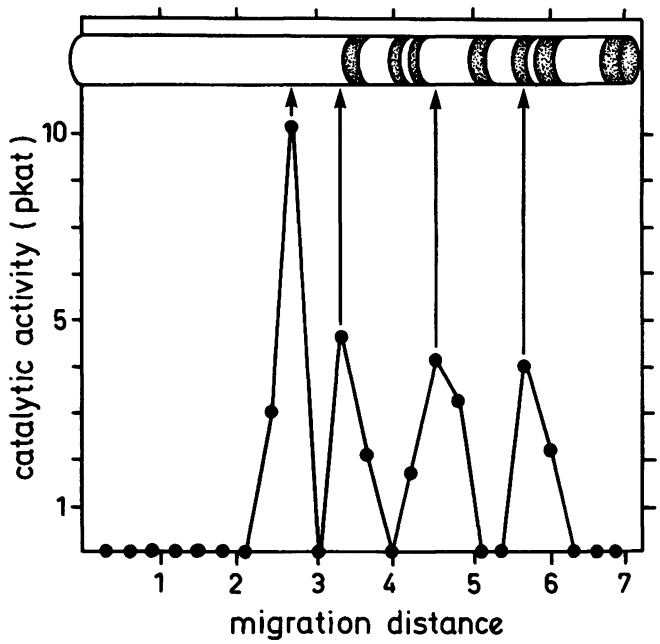


Fig. 4. Migration of catalytic activity of (S)-norlaudanosoline synthase in a 10 % polyacrylamide gel. A volume of 300 µl of a prepurified enzyme solution (step 2) which contained about 3 mg protein (272 pkat catal. activity) was applied to each gel. The figure shows the protein bands and the profile of catalytic activity in the polyacrylamide gels.

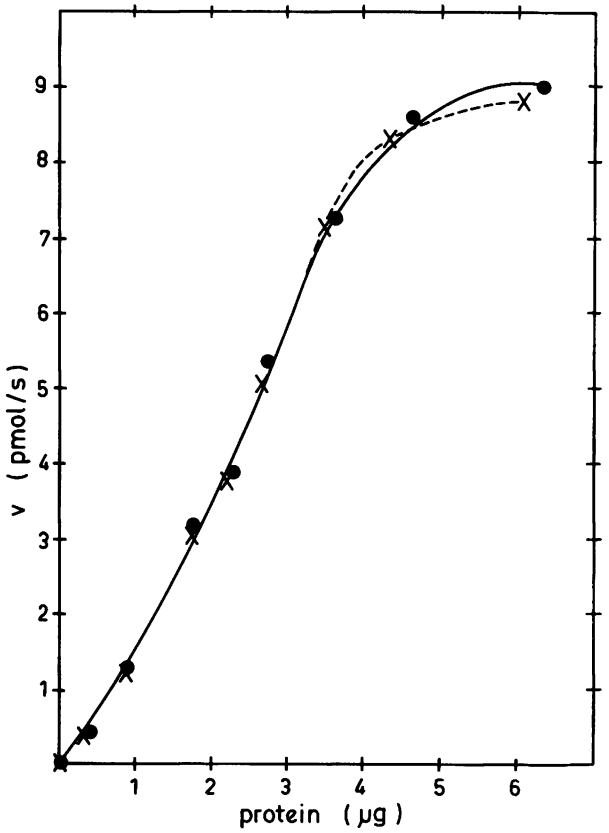


Fig. 6. (S)-norlaudanosoline synthase activity as a function of the protein amount added to the standard incubation mixture. Dopamine transformation was tested with two different cosubstrates: (●) reaction of dopamine with 3,4-dihydroxyphenylacetaldehyde (X) reaction of dopamine with 4-hydroxyphenylacetaldehyde.

Properties of the synthase

The partially purified enzyme was used to determine the protein dependence of the reaction. As shown in Fig. 6 the reaction catalysed by the enzyme was approximately linear up to about 3 µg protein per standard incubation test. The 3,4-dihydroxy- and the 4-

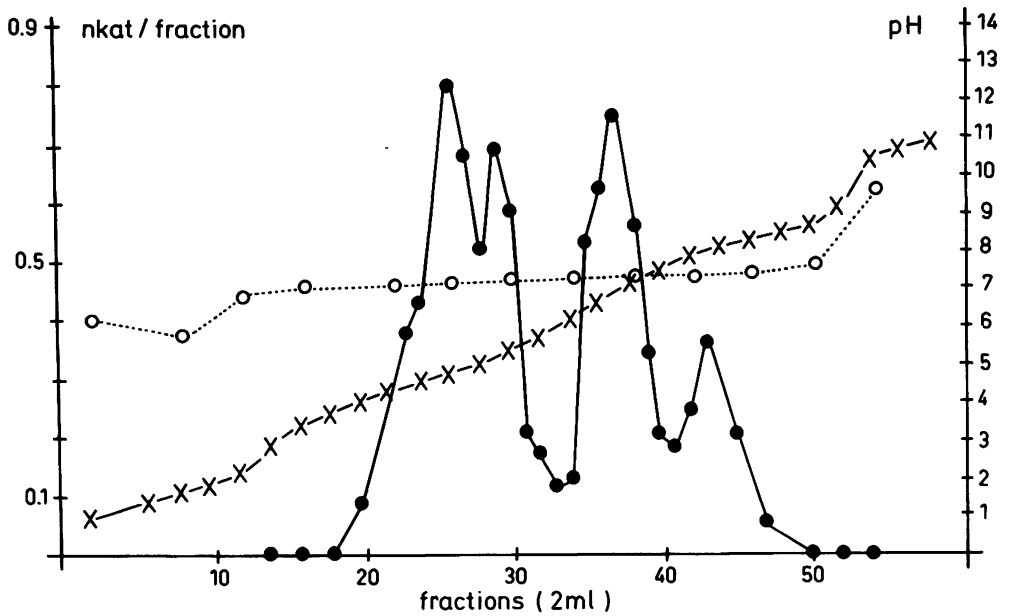


Fig. 5. Partition of catalytic activity in a pH gradient after isoelectric focusing. Isoelectric focusing was performed in a glass column for preparative work containing a sucrose density gradient. After purification by ammonium sulphate fractionation and dialysis against a 10 % glycine solution, 7 ml of an enzyme solution (protein = 44 mg; catal. activity = 15.7 nkat) was applied to the column. After the focusing the column was eluted and the catalytic activity in different fractions determined by the standard assay procedure.

monohydroxyphenylacetaldehydes as substrates were equally well used by the enzyme. At none of the protein concentrations 3,4-dihydroxyphenylpyruvate or 4-hydroxyphenylpyruvate were used as reactants.

The activity of the enzyme was measured at a range of pH 4 to 9 in different buffers as shown in Fig. 7. The synthase from *E. tenuifolia* shows a pH optimum at 7.8 for 3,4-dihydroxyphenylacetaldehyde as a substrate. For 4-hydroxyphenylaldehyde a slightly more acid pH optimum at pH 7.4 was observed. The enzyme exhibits a temperature optimum at 40° C for the dihydroxysubstituted aldehyde while for the monohydroxylated aldehyde the temperature optimum was found to be at 50° C. Under conditions of the standard enzyme test the net enzymatically catalysed condensation of aldehyde with dopamine could be calculated. The ^3H-release by the enzymatically catalysed Pictet-Spengler reaction served as a convenient test. Under standard conditions the appearance of ^3H in the ambient reaction water was linear for a period of up to 15 minutes using 2.1 µg of the 40-fold purified synthase. The effect of the substrate concentrations on the activity of the norlaudanosoline synthase is shown in Fig. 8. Double reciprocal plots of the activity with respect to substrate concentration gave a linear relationship. The apparent K_M-values were 1.5 mM for dopamine and V_{max} 37.4 pmol/s. For 3,4-dihydroxyphenylacetaldehyde as substrate an appa-

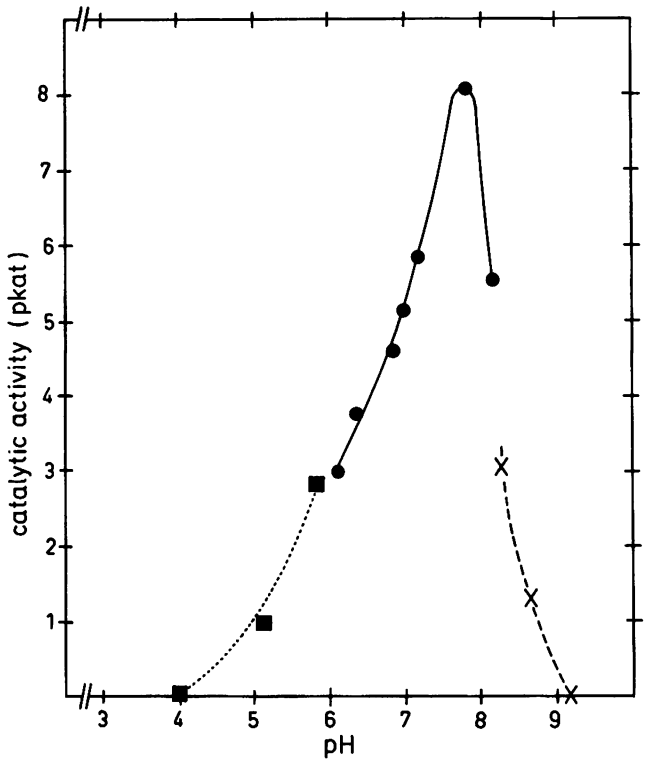
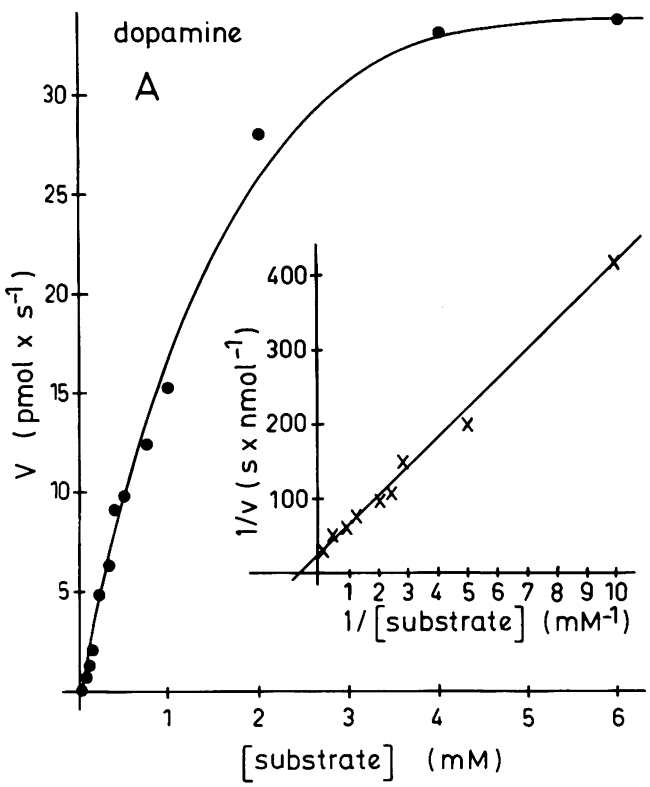


Fig. 7. The effect of pH on the (S)-norlaudanosoline synthase activity. The assay was performed in the presence of (■) 0.24 M citrate/phosphate (●) 0.24 M phosphate buffer and (X) 0.12 M borate buffer.



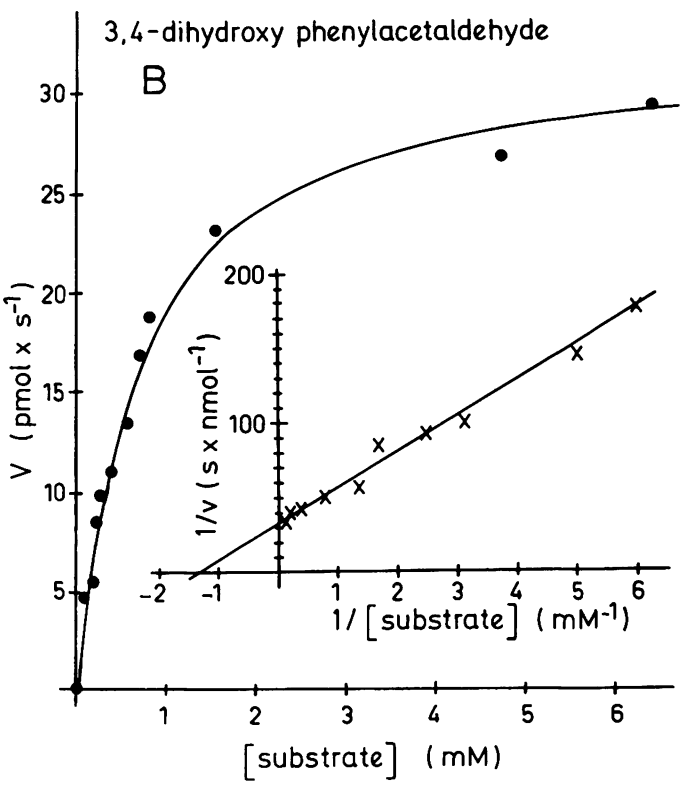


Fig. 8. The effect of different initial concentrations of dopamine (A) and 3,4-dihydroxyphenylacetaldehyde (B) on the reaction velocity of the catalysed reaction. Assay conditions in the standard incubation mixture: pH 7.8; reaction temperature 40° C; protein 2.1 µg; catal. activity 8.74 pkat. The inserts show double reciprocal plots.

A. K_M = 1.53 mM; V_{max} = 37.4 pmol/s
B. K_M = 0.71 mM; V_{max} = 34 pmol/s

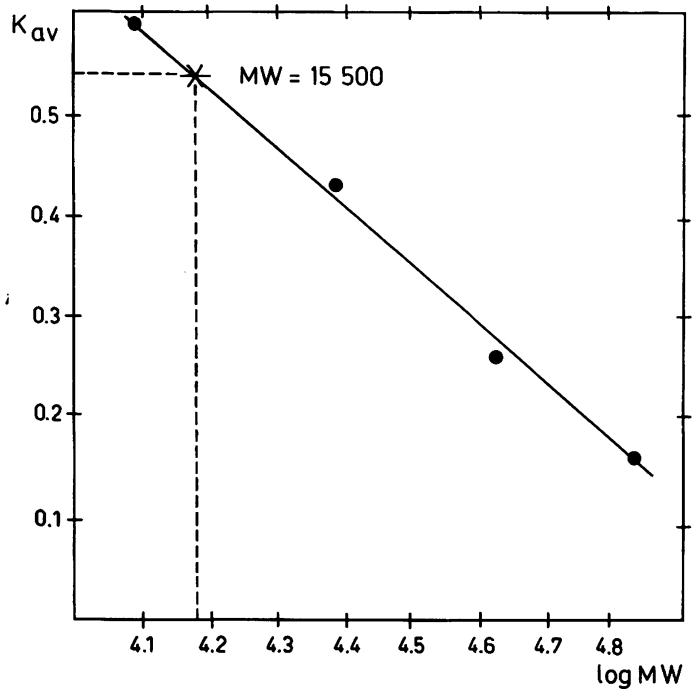


Fig. 9. Estimation of the molecular weight of (S)-norlaudanosoline synthase by gel filtration. For the gel filtration a Sephadex G-100 column equilibrated with 50 mM phosphate buffer at pH 7.0 was used. The column was calibrated with globular proteins of known molecular weight before and after gel filtration of the enzyme. The synthase preparation was 4 ml, containing 4 mg protein (1.85 nkat). Estimated molecular weight assuming globular shape: 15 500 Dalton.

rent K_M of 0.7 mM and a V_{max} of 34 pmol/s were determined under standard conditions. The corresponding values for 4-dihydroxyphenylacetaldehyde were 0.9 mM and V_{max} 27.9 pmol/s. Neither a substrate nor a product inhibition was observed on the catalytic activity of the enzyme. Also phenylacetaldehyde was active as a substrate for this enzyme which proves that for the reaction mechanism a phenolic hydroxy group at the aromatic ring is unnecessary.

Estimation of the Stokes-radius of the norlaudanosoline synthase of *Eschscholtzia* was carried out by measuring the elution volume of a calibrated Sephadex G-100 column using globular proteins of known Stokes-radii as internal standards. The Stokes-radius of the synthase was calculated from a plot of the Stokes-radii of calibration proteins versus the migration velocity in the gel as shown in Fig. 9. The relative migration velocity of the enzyme can be correlated to that of a globular protein of a molecular weight of about 15500 ± 10 %.

The synthase was found to occur in a large number of Papaveraceae, Berberidaceae, and Ranunculaceae cell cultures [12]. Furthermore it was also found in a variety of differentiated plants of the same families which were tested for the occurrence of this enzyme, among these *Papaver somniferum*. However, also in the case of *P. somniferum* only activity with the phenylacetaldehydes was found and not the phenylpyruvates. As already shown, the enzyme activity was completely absent from those species which do not contain isoquinoline alkaloids such as Nicotiana, Catharanthus, or Daucus proving that this enzyme is present and functioning only in specific plants containing the benzylisoquinoline nucleus [12].

Discussion

Recent work in this laboratory [12] has led to the discovery of a new enzyme, named (S)-norlaudanosoline synthase, which catalyses the stereospecific condensation of dopamine with 3,4-dihydroxyphenylacetaldehyde (Fig. 10) or related phenylacetaldehydes. The suggestion by Hahn [6], proposed many years ago, that the carbon atom at position 1 of benzylisoquinoline alkaloids is biogenetically derived from an appropriate α-keto-acid via a Pictet-Spengler-ring closure reaction which has received much recent support, [7–11] is now ruled out in our opinion. (S)-norlaudanosoline is the first intermediate in the benzylisoquinoline pathway. No support for the enzymatic formation of norlaudanosoline-1-carboxylic acid under cell-free conditions was found. This result was made possible by using cell-free extracts of a variety of plant cell suspension cultures which produced substantial amounts of isoquinoline alkaloids [12]. In a preliminary communication this novel enzyme was

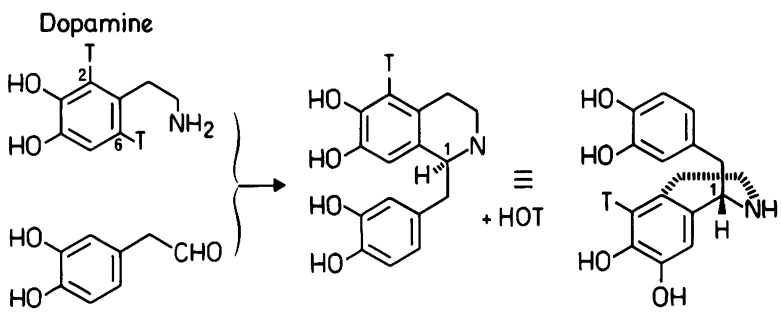


Fig. 10. Scheme of reaction catalysed by (S)-norlaudanosoline synthase indicating also the assay principle.

partly characterized, introducing also a new and convenient assay for this type of synthase [12]. Use was made of the fact that, during a Pictet-Spengler-type condensation of [ring-2,6-^{3}H] dopamine with an appropriate ketonic intermediate, one hydrogen atom of the phenylethylamine is removed and appears as water in the ambient aqueous incubation mixture [12]. There is an appreciable chemical, non-enzymatically catalysed condensation reaction which has to be subtracted from the gross HOT release in the presence of norlaudanosoline synthase. This assay, when carefully standardized, allowed the purification and characterisation of this enzyme which was the object of the present study. The isolation and partial purification of norlaudanosoline synthase from *Eschscholtzia tenuifolia* was successfully attempted. At the final stage of purification (40-fold) the enzyme was in the presence of S-adenosylmethionine free of other detectable enzyme activities responsible for the formation of methylsubstituted benzylisoquinolines or protoberberines, using norlaudanosoline as substrate. The purified enzyme did not show any apparent dependence on cofactors. The enzyme from *E. tenuifolia* had an exceptionally small molecular weight of about 15500 assuming globular shape of the protein. Evidence was given by disc gel electrophoresis and isoelectric focusing for the existence of four isoenzymes with isoelectric points at pH 4.6; 5.3; 6.7; 7.6. The enzyme purification procedure given in Table I leads to a purification of the enzyme species with the IEP at pH 7.6, which also explains the small yield obtained.

The crude extract, containing all the four isoenzymes, and the partially purified isoenzyme (IEP: pH 7.6) catalysed the condensation exclusively with phenylacetaldehydes but not with corresponding phenylpyruvates. It has been previously determined by CD-spectroscopy that the reaction product catalysed by the unpurified enzyme is (S)-norlaudanosoline [12]. The measurement of the optical property of the product is rather difficult in as far as a masking of the enzyme catalysed product occurs by racemic material produced by the non-stereospecific chemical condensation of the substrates in the incubation mixture [12]. Up to now we have not obtained any experimental evidence that (R)-norlaudanosoline could be formed by the crude enzyme mixtures. This question has to be reinvestigated however, when it will be possible to separate the 4 different isoenzymes in larger quantities so that the stereochemistry can be measured of the enzyme catalysed condensation products of the individual isoenzymes.

It is noteworthy that the K_M-values both for 3,4-dihydroxyphenylacetaldehyde (0.7 mM) and for 4-hydroxyphenylacetaldehyde (0.9 mM) are almost identical. This means that the synthase is also responsible for the formation of demethylcoclaurine. This compound is a precursor of coclaurine which has been shown to be a common precursor for proaporphines of the crotonosin type [22] and the aporphines roemerine and mecambroline [23, 24]. Again only (S)-coclaurine and not the (R)-enantiomer was incorporated. There is, however, evidence for the natural occurrence of (R)-coclaurine [25]. If this compound is enzymatically formed it is expected that there are also norlaudanosoline synthases yielding either pure (R)-enantiomers or (R, S)-racemates of which subsequently the "natural" (S)-form is further metabolized while the "unnatural" (R)-enantiomer accumulates within the plant similar to the situation depicted for the formation of desacetylipecoside [26, 27]. Nevertheless we firmly believe that the predominant pathway catalysed by these synthases leads to the (S)-enantiomers of norlaudanosoline and demethylcoclaurine which are the immediate precursors for the vast majority of the benzylisoquinolines including the morphinans with (R)-configuration [28].

In almost all isoquinoline systems studied so far L-tyrosine has been shown to be incorporated almost equally in both the "upper" (ring-A, B) and the "lower" (ring-C) portions of the molecule (Scheme I). On the other hand DOPA labels only the upper half (ring-A, B) of the isoquinoline which is difficult to envisage if dopamine and 3,4-dihydroxyphenylpyruvate are the precursors, a problem which has masterly been discussed by Holland et al. [29]. The findings presented in this paper demonstrating 3,4-dihydroxyphenylacetaldehyde rather than 3,4-dihydroxyphenylpyruvate as the enzyme-substrate for the condensation reaction with dopamine explain this labelling pattern. The 3,4- or 4-hydroxylated phenylacetaldehydes are produced by a yet to be discovered branch of the tyrosine metabolism not involving DOPA, while DOPA gives rise to dopamine only. In our opinion the DOPA-feeding experiments [cited in 29] clearly rule out the proposed [6–11] role of phenylpyruvate in benzylisoquinoline biosynthesis with the intermediary formation of norlaudanosoline-1-carboxylic acid. Since DOPA is easily transaminated in the plant [e.g. 7] and if the thus formed 3,4-dihydroxypyruvate would truly be a precursor for the "lower" half of the benzylisoquinoline molecule, the C-ring (Scheme I) should contain an appreciable amount of tracer in DOPA-feeding experiments which is not the case. The observed formation of norlaudanosoline-1-carboxylic acid under *in vivo* and *in vitro* conditions [7, 10, 11] can be explained by the ease with which phenylethylamines react with phenylpyruvates non-enzymatically [6]. It is regrettable that neither the stereochemistry of norlaudanosoline-1-carboxylic acid isolated from plants or cell-free preparations has been determined [7, 10, 11] nor the proposed [8] incorporation and trapping experiments with resolved enantiomers of this amino acid have been performed yet. Finally it should be noted that the chemically synthetized norlaudanosoline-1-car-

boxylic acid whose incorporation into several isoquinoline alkaloids has been observed [7, 8, 9, 30] easily undergoes oxidative decarboxylation either in air under slightly basic conditions or by enzymes which occur widely in the plant kingdom such as peroxidases or laccases [31, 32].

On the basis of the results presented in this paper we conclude that the major pathway to the benzylisoquinoline alkaloids involves (S)-norlaudanosoline synthase which condenses dopamine and phenylacetaldehydes in a stereospecific manner, (S)-norlaudanosoline and (S)-coclaurine thus formed being the first intermediate in the pathway.

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