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Unusual 4-hydroxybenzaldehyde synthase activity from tissue cultures of the vanilla orchid *Vanilla planifolia*

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Abstract

Tissue cultures of the vanilla orchid, *Vanilla planifolia,* produce the flavor compound vanillin (4-hydroxy-3-methoxybenzaldehyde) and vanillin precursors such as 4-hydroxybenzaldehyde. A constitutively expressed enzyme activity catalyzing chain shortening of a hydroxycinnamic acid, believed to be the first reaction specific for formation of vanilla flavor compounds, was identified in these cultures. The enzyme converts 4-coumaric acid non-oxidatively to 4-hydroxybenzaldehyde in the presence of a thiol reagent but with no co-factor requirement. Several forms of this 4-hydroxybenzaldehyde synthase (4HBS) were resolved and partially purified by a combination of hydrophobic interaction, ion exchange and gel filtration chromatography. These forms appear to be interconvertible. The unusual properties of the 4HBS, and its appearance in different protein fractions, raise questions as to its physiological role in vanillin biosynthesis in vivo.

Keywords: *Vanilla planifolia*; Orchidaceae; Non-oxidative chain-shortening; Benzaldehydes

1. Introduction

Vanilla is one of the world's most popular flavors, with an estimated annual worldwide consumption of over 2000 tons. Natural vanilla flavor is obtained from beans of the vanilla orchid, *Vanilla planifolia*. The major flavor compound is vanillin (4-hydroxy-3-methoxybenzaldehyde) (**1**), although over 250 different compounds have been isolated from vanilla beans, including 4-hydroxybenzaldehyde (**2**), 4-hydroxybenzoic acid (**3**) and 4-hydroxy-3-methoxybenzoic acid (vanillic acid) (Guarino and Brown, 1985; Dignum et al., 2001). The vanilla beans are harvested up to 8 months post-pollination; at this stage, the green beans are flavorless but contain large quantities of glucosides of the various flavor compounds. The characteristic flavor develops on "curing" of the beans, a process that can last for as much as 6 months and which is associated with increases in hydrolytic enzymes such as glycosidases, esterases, proteases and lipases, and oxidative enzymes such as polyphenol oxidases and peroxidases (Dignum et al., 2001). During curing the various glycosides are hydrolyzed and also undergo oxidation.

There is extensive literature documenting the formation in plants of benzoic acids and aldehydes from hydroxycinnamic acids derived from phenylalanine via the phenylpropanoid pathway of secondary metabolism (Funk and Brodelius, 1990a,b, 1992; Yazaki et al., 1991; Schnitzler et al., 1992; Löscher and Heide, 1994; Yalpani et al., 1993; Verberne et al., 1999). However, considering the wide usage of vanilla flavor, and the importance of benzoic acids in the biosynthesis of molecules as diverse as the plant defense signal molecule salicylic acid (Verberne et al., 1999), the napthoquinone pigment shikonin (Löscher and Heide, 1994), cocaine (Bjorklund and Leete, 1992), xanthones with anti-HIV activity (El-Mawla et al., 2001) and the anticancer drug taxol (Chu et al., 1994), there is still considerable uncertainty as to the nature of the biochemical reactions

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leading to shortening of the side chain of hydroxycinnamic acids to yield substituted benzoic acids.

In plants, there may be at least five different routes to benzoic acids. One, which has been proposed to occur during vanillin biosynthesis in *V. planifolia* (Zenk, 1965) and shikonin biosynthesis in *Lithospermum erythrorhizon* (Löscher and Heide, 1994), involves conversion of hydroxycinnamic acids to their coenzyme A esters, with subsequent chain-shortening by a process analogous to NAD-dependent β-oxidation of fatty acids, leading to a benzoic acid, as shown in Fig. 1. The operation of this pathway is supported by radiolabeling studies (El-Basyouni et al., 1964), and enzymatic activities have been verified in crude cell extracts (Löscher and Heide, 1994). However, the enzymes themselves have not been purified. A second route involves a non-oxidative pathway for conversion of 4-coumaric acid (**4**) to 4-hydroxybenzaldehyde, with no co-factor requirement. This type of reaction, outlined in Fig. 1, has been proposed to occur during formation of 4-hydroxybenzoic acid in potato tubers (French et al., 1976) and elicited carrot cell cultures (Schnitzler et al., 1992), and in an alternative pathway to shikonin in *L. erythrorhizon* (Yazaki et al., 1991). In these cases, 4-hydroxybenzoic acid is formed from 4-hydroxybenzaldehyde by a separate NAD-dependent dehydrogenase. The chain-shortening enzyme itself was reported to be very unstable (French et al., 1976). A very different pathway to vanillin in cell cultures of *V. planifolia* was proposed involving methylation of the 4-position of hydroxycinnamic acids, followed by glucosylation, side-chain shortening and 4-demethylation (Funk and Brodelius, 1990a,b). Direct enzymatic evidence for this pathway is yet to be obtained. The benzoic acid precursors of xanthones in *Centaurium erythraea* appear to originate directly from the shikimate pathway rather than via cinnamic acid (El-Mawla et al., 2001). In the bacterium *Pseudomonas fluorescens*, vanillin is produced non-oxidatively from feruloyl CoA by an enzyme of the enoyl-SCoA hydratase/isomerase family (Gasson et al., 1998; Mitra et al., 1999). Finally, recent studies have indicated that some benzoic acid derivatives, such a salicylic acid (2-hydroxybenzoic acid) and 2,3-dihydroxybenzoic acid, may be synthesized in plants from the shikimate pathway via isochorismic acid, a pathway that is also found in bacteria and which does not involve phenylalanine as an intermediate (Wildermuth et al., 2001; Muljono et al., 2002).

Tissue cultures of *V. planifolia* have been established that accumulate 4-coumaric acid, 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol, 3,4-dihydroxybenzaldehyde, 4-hydroxy-3-methoxybenzyl alcohol, and vanillin (Havkin-Frenkel et al., 1996). The pattern and levels of these metabolites are consistent with, but do not prove, the involvement of 4-hydroxybenzaldehyde as an intermediate in vanillin biosynthesis. We here report the identification from these cultures of 4-hydroxybenzaldehyde synthases (4HBS)

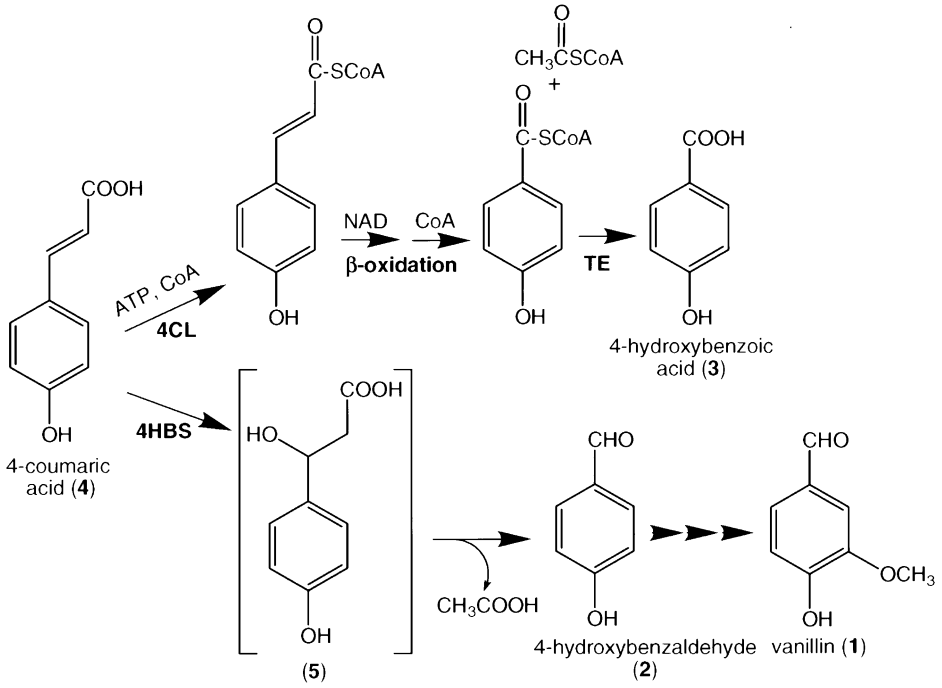

Fig. 1. Oxidative and non-oxidative pathways for the chain-shortening of cinnamic acid derivatives in plants. The upper pathway shows the oxidative pathway to 4-hydroxybenzoic acid via the coenzyme A ester. The lower pathway shows the non-oxidative pathway to 4-hydroxybenzaldehyde via an unstable 4-hydroxyphenyl-β-hydroxypropionic acid intermediate (**5**). 4CL, 4-coumarate:coenzyme A ligase; TE, thioesterase; 4HBS, 4-hydroxybenzaldehyde synthase.

that produce 4-hydroxybenzaldehyde from 4-coumaric acid in the presence of a thiol reagent, but with no co-factor requirement.

2. Results

2.1. 4HBS activity in crude extracts

4HBS activity could be demonstrated when crude extracts of *V. planifolia* cell cultures were incubated with 4-coumaric acid and dithiothreitol (DTT) at pH 8.0. HPLC analysis indicated time-dependent production of a new compound that was identified as 4-hydroxybenzaldehyde by comparison of its chromatographic mobility, UV absorption spectrum and mass spectrum with those of an authentic standard, as shown in Fig. 2. Production of 4-hydroxybenzaldehyde in the extracts was also accompanied by appearance of acetic acid, as determined by conversion to methyl acetate and determination by GC/MS (data not shown), consistent with the non-oxidative reaction shown in Fig. 1.

Production of 4-hydroxybenzaldehyde from 4-coumaric acid also occurred spontaneously at a low rate under the assay conditions used; this conversion, which was dependent on the presence of DTT, is subtracted from the enzyme-catalyzed rate in all the results reported below. The pH optimum for the reaction was 8.0. Chain shortening of 4-coumarate required the presence of a thiol reagent, and DTT, dithioerythritol and coenzyme A were all equally effective, whereas 2-mercaptoethanol, glutathione or cysteine were much less effective (Table 1). At 10 mM 4-coumarate, the reaction rate increased linearly with concentrations of DTT up to 20 mM (data not shown). The reaction with 4-coumarate and DTT was almost completely inhibited in the presence of 1 mM ascorbate (Table 1). The thiol-dependent 4HBS activity remained relatively constant during growth of the cultures, with an average value of around 88 nmol 4-hydroxybenzaldehyde produced min^{-1} mg $protein^{-1}$ (approximately 1.5 nkat mg^{-1}) over a 40 day period.

The chain shortening enzyme activity was quite specific for 4-coumaric acid. Cinnamic, caffeic, sinapic and

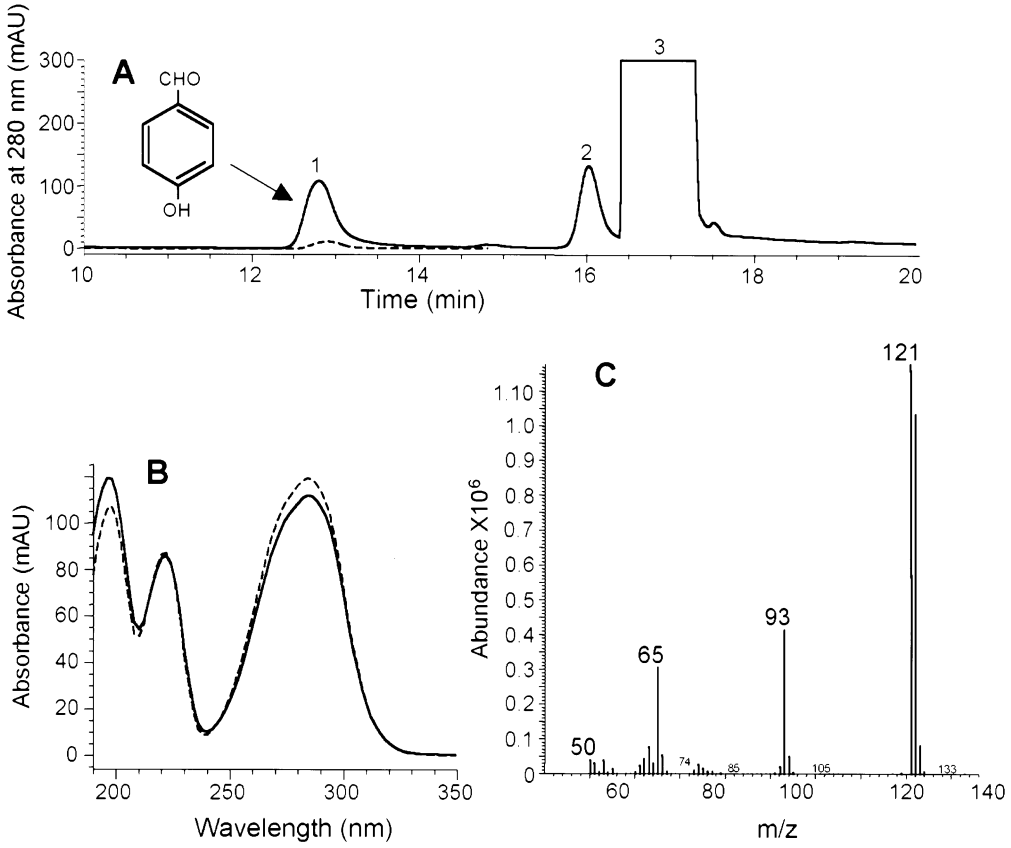

Fig. 2. Characterization of the reaction product of 4HBS by HPLC, UV and MS. (A) HPLC separation of substrates and products of the 4HBS reaction. Peak 1 is 4-hydroxybenzaldehyde (product, produced at low amounts in controls stopped at zero time, dashed line). Peak 2 is *cis*-4-coumaric acid (minor contaminant of the substrate). Peak 3 is *trans*-4-coumaric acid (substrate). (B) UV absorption spectra of the product of the 4-HBS reaction (peak 1 above) and authentic 4-hydroxybenzaldehyde (dotted line). (C) Mass spectrum of peak 1. Note the molecular ion of m/z 121 diagnostic of 4-hydroxybenzaldehyde $[M\text{-}1]^+$.

Table 1
Effects of thiol reagents and co-factors on 4HBS activity in crude extracts from *V. planifolia* cell cultures

Additions to assay	4HBS activity (nkat/mg protein)	Relative activity (%)
Dithiothreitol (10 mM)	1.47±0.08	100
Dithioerythritol (10 mM)	1.48±0.03	101
Glutathione (reduced) (10 mM)	0.12±0.01	8
Glutathione (oxidized) (10 mM)	0.0	0
Cysteine (10 mM)	0.28±0.01	19
Coenzyme A (1 mM)	0.46±0.06	31
Coenzyme A (10 mM)	1.48±0.19	101
Dithiothreitol (10 mM) + ascorbate (1 mM)	0.01±0.01	1
Dithiothreitol (10 mM) + MgATP (1 mM)	2.19±0.16	149
Dithiothreitol (10 mM) + MgATP (10 mM)	3.15±0.37	214
Coenzyme A (1 mM) + MgATP (10 mM)	0.34±0.02	23
Coenzyme A (10 mM) + MgATP (10 mM)	1.26±0.08	86

The standard assay contained 10 mM 4-coumaric acid and 26 μg protein in 0.1 M Tris–HCl, pH 8.0, total volume 0.5 ml. Results are the mean and standard deviation from 3–5 replicate assays.

o-coumaric acids were not substrates, and low activity (approximately 2% of the activity with 4-coumaric acid) was obtained with ferulic acid, which was converted to 3-methoxy-4-hydroxybenzaldehyde. These results are consistent with earlier proposals for the vanillin biosynthetic pathway in beans of *V. planifolia*, in which coumaric acid may be the major precursor and ferulic acid a minor precursor (reviewed in Dignum et al., 2001).

The oxidative pathway proposed for chain shortening of cinnamic acids involves generation of a coenzyme A thioester. To test whether this type of activity was present in the *V. planifolia* cultures, crude and purified extracts were incubated with ATP, Mg^{++} and coenzyme A (Table 1). Significant 4-hydroxybenzaldehyde production occurred when 4-coumarate was incubated with 10 mM CoA and 10 mM MgATP, but it was nevertheless less than production with CoA alone, suggesting that the reaction was not occurring through production of coumaroyl CoA. No 4-hydroxybenzoic acid, the product of oxidative chain shortening, could be detected. Furthermore, incubation of crude *V. planifolia* extracts with 4-coumarate, ATP, coenzyme A and NAD did not result in production of 4-hydroxybenzoic acid (data not shown). Thus, at least in vitro, non-oxidative chain shortening appears to be the major route to vanillin precursors in *V. planifolia* cell cultures.

2.2. Tissue-specificity of 4HBS activity

4HBS activity was observed in stems, roots, leaves, pods and embryo cultures of *V. planifolia*, with highest specific activity in pods (Fig. 3A and B). 4-HBS activity could not be induced further in the cell cultures by treatment with yeast elicitor (Hertz, 2000) or transfer to media containing various concentrations of gibberellic or abscisic acids (data not shown). The effect of developmental age of the pods on 4HBS activity is shown in Fig. 3B. Maximum activity occurred approximately 7–8 months post-pollination; 4-hydroxybenzaldehyde appeared in the pods three months post-pollination, with maximum levels after 10–11 months (Fig. 3C).

2.3. Purification and properties of 4HBS

4HBS was purified from crude tissue culture extracts by a four step procedure involving ammonium sulfate fractionation, hydrophobic interaction chromatography, ion exchange chromatography and size exclusion chromatography, as described in Experimental. Fractionation of the 40% ammonium sulfate supernatant on phenyl-sepharose Cl 6B resulted in the bulk of the 4HBS activity eluting in a broad double peak at around 1.0–0.8 M ammonium sulfate (Fig. 4A). When this material was re-fractionated on a Mono Q FPLC ion exchange column, approximately 50% of the 4HBS activity did not bind and was eluted in the initial wash, with the majority of the remaining activity eluting as a biphasic peak soon after the start of the NaCl gradient (Fig. 4B). The unbound material (4HBSI) was further fractionated by gel filtration on Superdex 200HR. The bulk of the activity (IB) appeared as a rather broad peak that eluted between the marker proteins egg albumin (Mr 44,000) and equine myoglobin (Mr 17, 000) (Fig. 4C; positions of markers shown in Fig. 4D), with a smaller peak (IA) of higher Mr. An almost identical elution pattern was observed when the 4HBS activity that was retained on Mono Q (4HBSII) was further fractionated on Superdex 200HR (Fig. 4D). This suggests that the two forms of 4HBS resolved on ion exchange chromatography may be closely related. That these forms are interconvertible is shown by the fact that re-chromatography of fraction IIB on Mono Q resulted in an almost identical activity profile to that of Fig. 4B, with major peaks at the elution volumes of the original fractions I and II (data not shown). However, re-chromatography of fraction I resulted in a single peak that was not retained by the column.

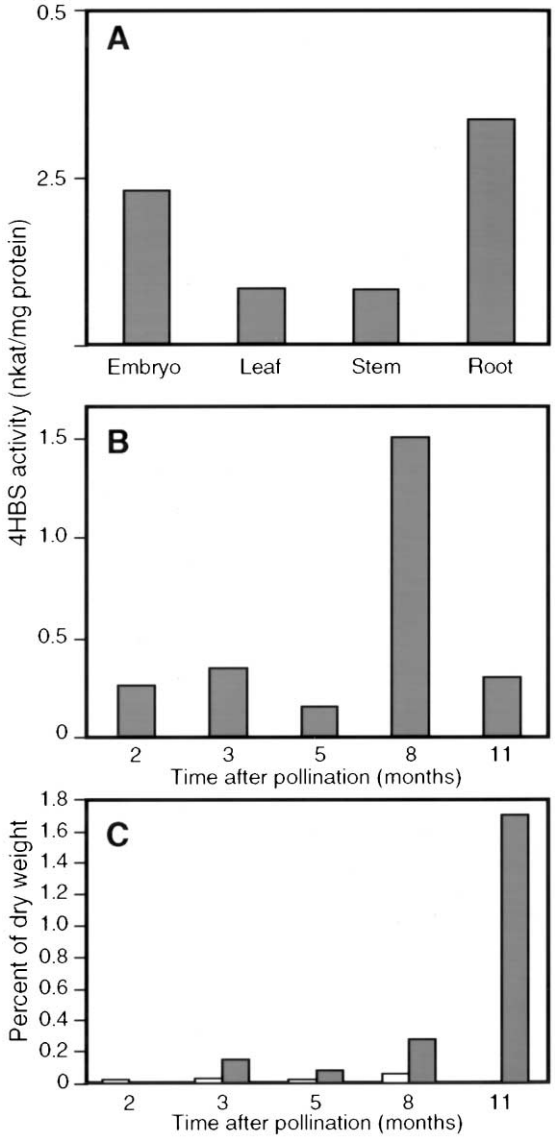

Fig. 3. 4HBS activity in various tissues of *V. planifolia*. (A) Enzyme activity in leaf, stem and root tissue of mature *V. planifolia* plants, and in embryo cultures. (B) Developmental changes in 4HBS activity in *V. planifolia* pods. (C) Developmental changes in levels of 4-coumaric acid (open bars) and 4-hydroxybenzaldehyde (shaded bars) in *V. planifolia* pods.

The molecular weights corresponding to the peak of 4HBS activities in fractions IIA and IIB were determined by comparison of the peak elution volumes to those of a number of standard molecular weight markers run through the same Superdex column (Fig. 4E). From this analysis, the holoenzyme molecular weights corresponding to the peaks of IIA and IIB were 79 and 28 kDa respectively. It should be noted that the width of peak IIB was 2.5–3 times greater than that of the molecular weight standards with which the column was calibrated, suggesting further heterogeneity of activity within this fraction.

The major 4HBS activity peaks were further analyzed by SDS-PAGE. First, the *V. planifolia* cell extract was resolved by ammonium sulfate fractionation, hydrophobic interaction chromatography and gel-filtration on Superdex 200 HR. Peak B was then further resolved by chromatography on Mono Q, and fractions analyzed for 4HBS activity and constituent proteins. Fig. 5A shows a silver-stained gel of Mono Q fractions from this preparation. Peaks with maximum 4HBS activity are marked with an asterisk. Fig. 5B compares the protein profiles of two highly purified 4HBS fractions. Extracts were fractionated by hydrophobic interaction chromatography and ion-exchange on Mono Q. Peak I from Mono Q was re-fractionated on Mono Q (smaller fraction size, same elution profile), and then further fractionated on Superdex. The second peak (fractions 16–19) was collected and termed 4HBSA (Fig. 5B, lanes 2 and 3). Peak II from the Mono Q fractionation of the same extract was further fractionated on Superdex, and fractions 16–19 collected. This fraction was termed 4HBSB (Fig. 5B, lanes 5 and 6). It can be seen that all fractions with high 4HBS activity contain a protein of Mr 28 kDa and several proteins of between 31 and 45 kDa.

Kinetic studies with 4HBS purified through the ammonium sulfate and hydrophobic interaction chromatography steps revealed the unusual finding that the reaction rate did not saturate with concentrations of 4-coumaric acid up to as high as 100 mM (Fig. 6), and therefore a K_m value could not be calculated. This phenomenon has been observed previously for 4HBS activity from *L. erythrorhizon* (Yazaki et al., 1991). The kinetics also suggest some degree of positive cooperativity, consistent with a multimeric enzyme system (Ricard et al., 1974). Fig. 6 also documents the rate of spontaneous conversion of 4-coumaric acid to 4-hydroxybenzaldehyde in the presence of 10 mM DTT.

3. Discussion

Previous studies on the enzymes leading to 4-hydroxybenzoate derivatives in plants have led to proposals for at least three different mechanisms for the chain-shortening reaction (Funk and Brodelius, 1990a,b; Yazaki et al., 1991; Löscher and Heide, 1994). The present data provide a more detailed biochemical characterization of one type of plant chain-shortening enzyme system, and confirm a non-oxidative mechanism most likely involving a hydrolyase activity that proceeds by hydration of the side chain 2,3 double bond of 4-coumaric acid with subsequent cleavage of the side chain to yield acetate and 4-hydroxybenzaldehyde. This reaction, which has been previously proposed as the first step in formation of 4-hydroxybenzoic acid in *L. erythrorhizon* and carrot cell cultures (Yazaki et al., 1991;

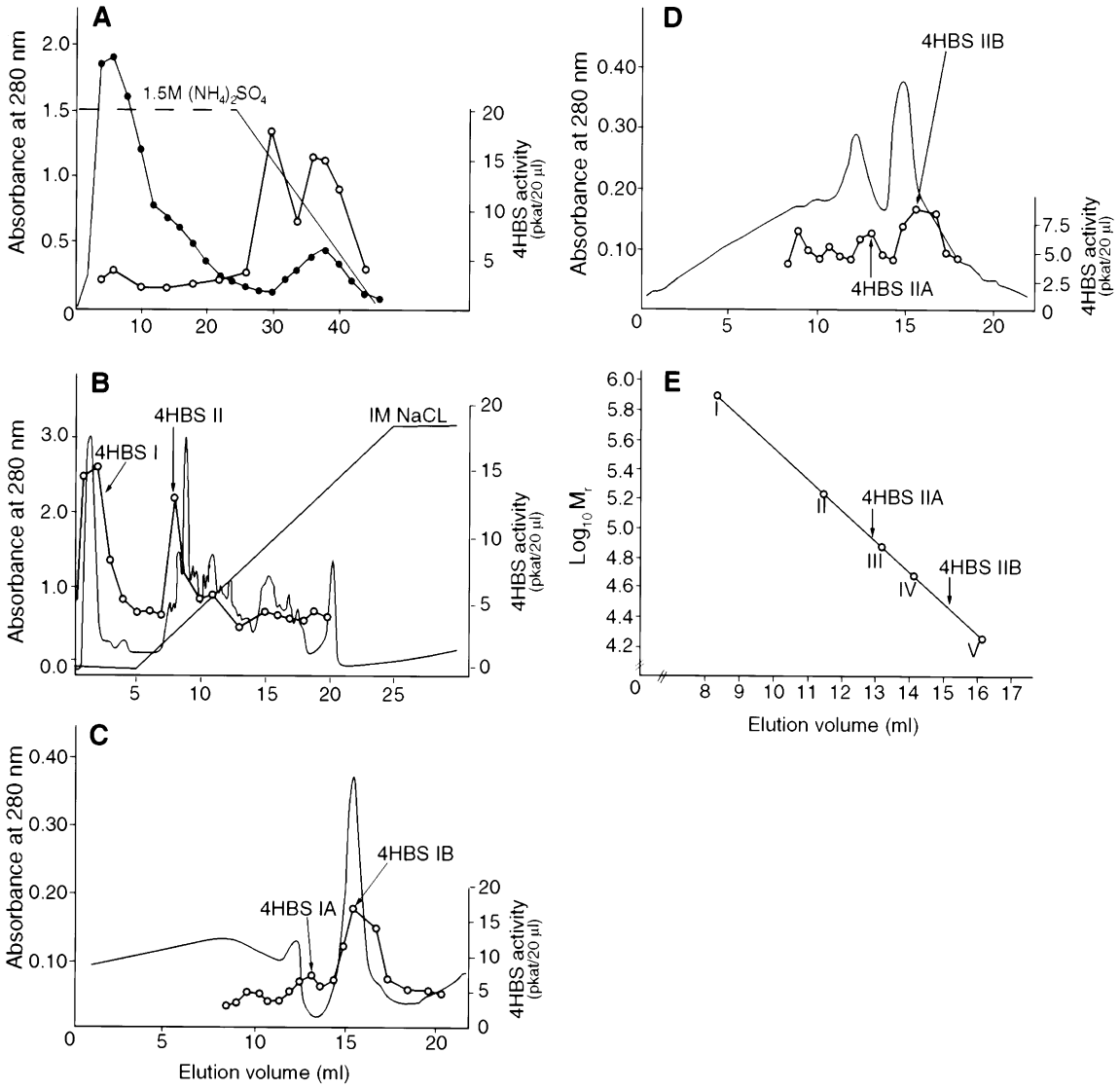

Fig. 4. Purification of 4HBS. (A) Hydrophobic interaction chromatography on Phenyl-Superose Cl 6B. (B) Ion exchange chromatography on Mono Q. (C) Gel filtration of the 4HBS II fraction from Mono Q on Superdex 2000 HR. (D) Gel filtration of the 4HBS I fraction from Mono Q on Superdex 2000 HR. (E) Determination of the Mr of the 4HBS holoenzyme by gel filtration. The marker proteins were: I, thyroglobulin (Mr 670,000), II, bovine γ-globulin (Mr 158,000), III, bovine serum albumin (Mr 70,000), IV, egg albumin (Mr 44,000), V, equine myoglobin, Mr 17,000.

Schnitzler et al., 1992), is presumed to involve the unstable 4-hydroxyphenyl-β-hydroxypropionic acid (**5**) (Fig. 1) as an intermediate, although this compound was not detected in the present work, or in previous studies. Operation of this pathway *in vivo* is indirectly supported by the results of feeding experiments in *V. planifolia* cultures, in which 4-hydroxybenzaldehyde accumulated after feeding 4-coumaric acid (Hertz, 2000).

The exact relationship between the different forms of *V. planifolia* 4HBS resolved in the present study is not clear. The possibility that 4HBS activity comprises a complex of several different protein subunits is suggested by the unusual kinetics of the reaction, the breadths of the peaks of purified enzyme obtained by gel filtration, and the apparent interconvertibility of the major forms of the activity when considered in relation to their similar polypeptide compositions. Alternatively, the data would also support a model in which the spontaneous thiol mediated degradation of 4-coumarate to 4-hydroxybenzoate was non-specifically accelerated by a number of different cellular proteins, which may or may not play physiological roles in vanillin biosynthesis.

One study on 4-hydroxybenzoate synthesis in *L. erythrorhizon* provided convincing evidence for a pathway involving oxidation and cleavage of 4-coumaroyl CoA to 4-hydroxybenzoyl CoA and acetyl CoA in a thiolase type reaction with requirement for NAD (Löscher and Heide, 1994). In this study, non-oxidative formation of 4-hydroxybenzaldehyde was also observed, although at much reduced rates compared to the oxidative conversion.

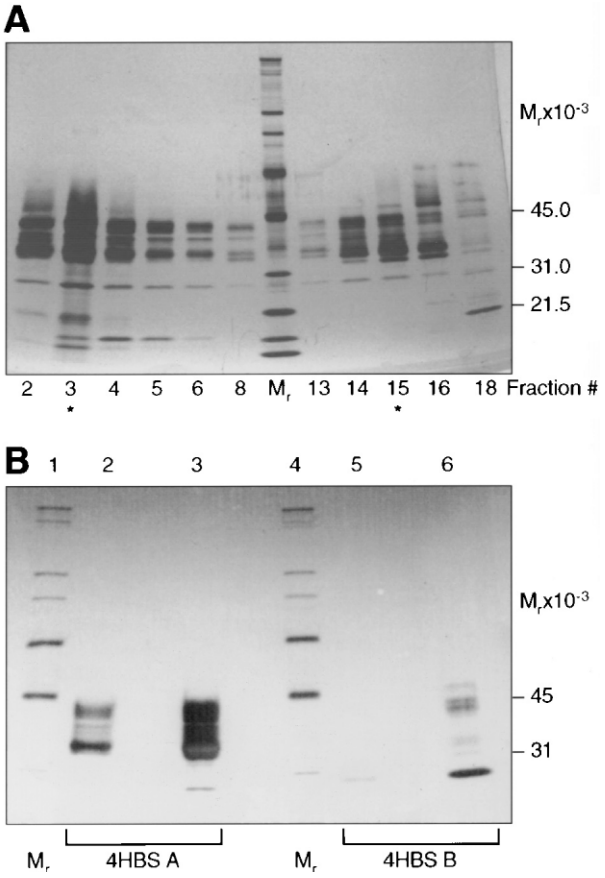

Fig. 5. SDS-PAGE analysis of the purification of 4HBS from *V. planifolia* embryo culture. (A) The activity was fractionated by ammonium sulfate fractionation, hydrophobic interaction chromatography and gel-filtration on Superdex 200 HR. Peak B (see text) was then further resolved by chromatography on Mono Q, and fractions analyzed by SDS-PAGE with silver staining. Fractions marked with an * contained the highest 4HBS activity. (B) Protein profiles of two highly purified 4HBS fractions. Extracts were fractionated by hydrophobic interaction chromatography and ion-exchange on Mono Q, with Peak I being re-fractionated on Mono Q and further fractionated on Superdex to give 4HBSA (lanes 2 and 3). Peak II from the Mono Q fractionation of the same extract was further fractionated on Superdex to give 4HBSB (lanes 5 and 6). Mr markers are included in lanes 1 and 4. Lanes 2 and 6 (500 ng protein), lane 3 (5 μg protein), lane 5 (50 ng protein).

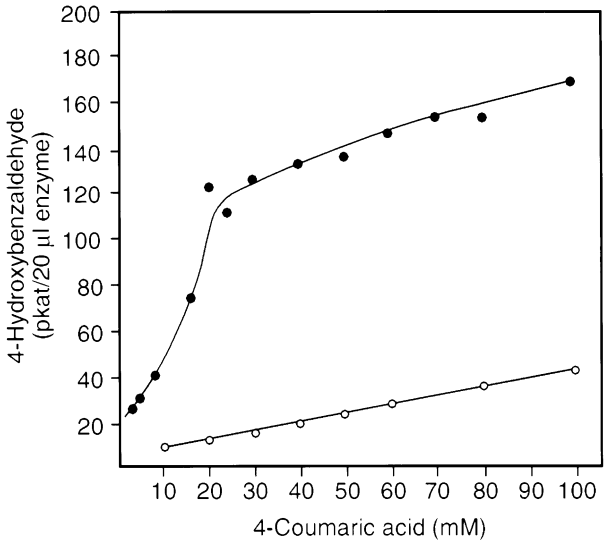

Fig. 6. Production of 4-hydroxybenzaladehyde by 4HBS as a function of 4-coumaric acid concentration (filled circles). Open circles show the spontaneous rate of reaction in the absence of enzyme. The enzyme preparation was partially purified from *V. planifolia* cell cultures by ammonium sulfate fractionation and hydrophobic interaction chromatography.

It was concluded that the non-oxidative pathway could represent either an alternative route to 4-hydroxybenzoic acid, or could be an artifact of the assay (Löscher and Heide, 1994). In view of the multiple enzyme activities and unusual reaction kinetics, our data do not formally rule out this latter possibility Furthermore, the nature of the reductant used for the present type of 4HBS reaction in planta still remains to be determined; it is clearly not DTT, and the physiologically occurring reductant glutathione was much less effective than DTT. It is possible from our data that CoA could be used in vivo, but in a reaction that did not involve formation of the CoA ester. Whatever the in vivo mechanism, the apparent absence of the alternative oxidative pathway in the vanilla tissues suggests that it is worth pursuing the possible biological function of the non-oxidative pathway. This can only be directly determined by genetic or reverse genetic approaches. To this end, one form of the non-oxidative 4HBS activity has now been cloned from *V. planifolia* and shown to catalyze formation of 4-hydroxybenzoate from 4-coumarate when expressed in yeast (G. Kourteva, D. Havkin-Frenkel and R.A. Dixon, unpublished results).

4. Experimental

4.1. Plant materials

To initiate the embryo cultures, green *V. planifolia* beans (4–5 months old) from Indonesia were sterilized, placed in petri dishes, and transferred to fresh solid media every two weeks. The seeds embedded in the beans germinated after 3–6 months. The embryo cultures, which contain differentiated cell aggregates, were maintained in media containing Gamborg's (Gamborg and Eveleigh, 1968) B-5 salts, and 20 g/l sucrose. Vitamins and microelements were added to a final concentration of (mg/l): L-glycine (2.0), myoinositol (50), thiamine-HCl (2.0), nicotinic acid (0.5), D-biotin (0.25), pyridoxine-HCl (0.25), boric acid (1.5), zinc sulfate (1.5), cupric sulfate (0.05). To prevent microbial contamination, 42 mg/l Cefotaxime sodium and 33 mg/l Vancomycin-HCl were added to the media. Solid media also contained 0.8% agar. Liquid cultures were grown

in the light at 50 μE s^{-1} m^{-2} at 25 °C on a gyrotary shaker at 180 rpm.

Cultures grown in liquid media were subcultured at approximately 2 week intervals by sieving the cultures, cutting each aggregate into several pieces, and transferring 4–5 g of fresh cells to 100 ml of new media. Cultures grown in petri dishes were subcultured every four weeks (Havkin-Frenkel et al., 1996).

The cell fresh weight was measured after sieving the cultures, rinsing with deionized water, and drying with a paper towel. The cell dry weight was determined by drying approximately 1 g of fresh cells in a laboratory oven at 65 °C.

4.2. Extraction of phenolic compounds

The procedures used to extract phenolics from the cells and to analyze the extracts by HPLC are based on the methods developed by Havkin-Frenkel et al. (1996). First, 10 ml of 50 mM sodium acetate, pH 5.0, was added to 3 g of fresh cells. The samples were placed in boiling water for 3 min and then homogenized (IKA-Labortechnik Ultra-Turrax T25, Staufen, Germany). After adding almond β-glucosidase (Sigma, G-0395, St. Louis, MO) to a final concentration of 0.2% (w/v), the mixture was incubated at 37 °C for 5 h. Twenty five milliliters of 95% EtOH was then added and the samples kept at 37 °C overnight. The extract was filtered using Fisherbrand P8 filter paper (Fisher Scientific, Pittsburgh, PA) and the EtOH removed by rotary evaporation. The aqueous residue was extracted twice with EtOAc, the pH adjusted to 3.0 with 1 N HCl, and the aqueous fraction extracted a further two times, with the four EtOAc fractions then being combined. The organic fractions were dried through anhydrous ammonium sulfate, evaporated on a heat block at 50 °C under nitrogen, and the residue reconstituted with 3 ml MeOH containing 1.25% glacial acetic acid and filtered using a 0.45 μm syringe filter (Osmonics, Minnetonka, MN).

4.3. Assay of 4HBS

Crude enzyme extracts were prepared by homogenization of *V. planifolia* cell culture material in 100 mM HEPES buffer (1 g wet tissue 2 ml of buffer), pH 8.0, containing 10 mM DTT, at 4 °C. Extracts were freed of low molecular weight compounds by filtration through Sephadex G-25 columns equilibrated in 100 mM Tris–HCl, pH 7.0, containing 10 mM DTT.

Enzyme (10–20 μl) was routinely incubated in a total volume of 200 μl with 100 mM Tris-HCl, pH 8.0, 16 mM 4-coumaric acid and 10 mM DTT at 30 to 35 °C for 5–60 min. Reactions were stopped by addition of ice cold 10% acetic acid in MeOH in 1:1 ratio and the mixtures centrifuged at 10,000 *g* for 10 min. The supernatants were analyzed for production of 4-hydroxybenzaldehyde by HPLC. For studies on requirements for co-factors and thiol reagents, and substrate specificity, the concentration of 4-coumarate in the assay was 10 mM, total volume 500 μl, and incubation time 30 min. The reaction was started by adding coumaric acid or DDT to reduce the background from the spontaneous reaction.

To analyze for acetic acid production the same procedure was done except the reaction was stopped with 2 N HCl in MeOH.

4.4. HPLC and GC/MS analysis

Production of 4-hydroxybenzaldehyde in routine assays used for enzyme purification was determined by HPLC (Agilent HP1100 HPLC with a G1315A diode array detector and G1311A quaternary pump), monitoring at 280 nm, using a C_{18} reversed phase column (Waters Spherisorb 5 μm ODS2 250×4.6 mm), a flow rate of 1 ml/min, and the following gradient: 13% B isocratic for 5 min, followed by an 8 min gradient from 13% B to 20% B, where B = acetonitrile (J.T.Baker, Baker analysed HPLC solvent) and A = 1% phosphoric acid in Milli·Q water. Vanilla culture crude extracts were analyzed on the same instrument, using the same solvents and flow rate, but with the following gradient: 5% B isocratic for 5 min, followed by a gradient to 10% B in 5 min, then to 20% B in 20 min, and finally to 60% B in 15 min.

The peak designated as 4-hydroxybenzaldehyde was further analyzed, without derivatization, by GC/MS using a HP 5890GC/5971MS system. The column and run conditions were HP-Wax (30 m, 0.25 mm ID, 0.25 μm phase ratio), inlet temp 250 °C, 1 ml injection, temp gradient 1 min at 120 °C, 15 °C per min to 200 °C, 10 °C per min to 245 °C, 25 °C per min to 120 °C, at a constant flow at 8 psi.

For analysis of 4HBS substrate and co-factor specificity, HPLC was performed on a Lichrospher 100 column (5 μm, reversed phase C_{18}, 250×4 mm, Merck) with flow rate of 1.5 ml min^{-1} and UV detection. HPLC condiditons were isocratic, with the following solvents: 10% acetonitrile, 25 mM NaOAc, pH 3.0 (4-coumaric, ferulic, sinapic and cinnamic acids as substrates); 20% acetonitrile, 25 mM NaOAc, pH 3.0 (2-coumaric acid acid as substrate); 10% MeOH in acidified water (caffeic acid as substrate).

Metabolite levels in plant tissues were measured with a Waters HPLC equipped with a diode array detector. The column was a Supelco C_{18} DB column of dimensions 250×4.6 mm and a particle size of 5 μm. The mobile phase contained MeOH and water, each of which was acidified with 1.25% acetic acid. The flow rate was 1 ml/min, with a solvent gradient as follows: 0–10 min, 15% MeOH; 10–20 min, 15–20% MeOH; 20–25 min, 20% MeOH; 25–30 min, 20–50% MeOH; 30–42 min, 50% MeOH; 42 min 15% MeOH.

Acetic acid was concentrated, purified and methylated prior to GC/MS analysis as described below. The 4HBS assay was performed as above (Section 4.3). Product from ten reactions was concentrated by running through a sax minicolumn (Supelco) according to the manufacturer's instructions. GC/MS analysis was done using an Agilent GC/MS equipped with flame ionization detector. A capillary column model number HP 19091S- 433, packed with HP-5MS 5% phenyl methyl siloxane was used. Oven temp was 35 °C. Program was: 10 min at 35 °C, followed by an increase at a rate of 10 °C min^{-1} up to 250 °C. Detector temp was 220 °C, flow rate 1.4 l/min, injection volume 5 μl with 25:1 split.

4.5. Purification of 4HBS

All procedures were carried out at 4 °C. The enzyme was extracted from the tissue with 0.1 M HEPES buffer pH 8.0 containing 10 mM DTT. Ammonium sulfate was added to the crude enzyme preparation to 40% saturation, the resulting precipitate was discarded, and the supernatant fractionated by FPLC on Phenyl-Sepharose Cl 6B. The column was equilibrated in 10 mM Tris–HCl pH 7.0 containing 10 mM DTT (buffer A) with 2 M ammonium sulfate. Crude extract in 40% saturated ammonium sulfate was applied to the column, which was then washed with buffer A containing 2 M ammonium sulfate until all unbound proteins were eluted. 4HBS activity was eluted in 0.8 M ammonium sulphate in buffer A. Fractions containing 4HBS activity were pooled, concentrated and desalted using Amicon membrane concentrators with a cut off range of 10,000 Da.

The desalted 4HBS fraction was applied to a Mono Q FPLC ion exchange column equilibrated with 50 mM Tris–HCl pH 7.5 containing 10 mM DTT (buffer B). The 4HBS activity was eluted using a linear gradient of 0–1 M NaCl in buffer B and 1 ml fractions were collected. In a separate experiment in which the 4HBS was run a second time through Mono Q, a 0–0.25 M NaCl gradient at pH 8.0 was used, and 0.25 ml samples were collected. Fractions containing 4HBS activity were pooled, concentrated and desalted as described above.

The 4HBS fraction from Mono Q was further fractionated on a Superdex HR 200 FPLC column equilibrated with 50 mM NaPi buffer pH 7.0 containing 150 mM NaCl and 5 mM DTT. Fractions of 0.6 ml were collected and analyzed for 4HBS activity. The column was calibrated with a range of protein molecular weight standards. Fractions containing 4HBS activity were concentrated and freed of NaCl using Amicon membrane concentrators, and stored frozen at −70 °C in 10 mM Tris–HCl pH 7.0 containing 10 mM DTT.

Fractions containing 4HBS activity from Mono Q and Superdex HR 200 were analyzed on 8–16% SDS-PAGE gels run in Tris–glycine and stained with either Coomassie blue or silver reagent.

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