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Nocardia sp. vanillic acid decarboxylase

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Abstract

Vanillic acid is an abundant renewable chemical useful for the biocatalytic synthesis of aromatics. The bacterium, *Nocardia* sp. NRRL 5646 efficiently catalyzes the decarboxylation of vanillic acid to the phenolic product, guaiacol. For purification and characterization of vanillic acid decarboxylase (VAD), a simple, rapid enzyme assay was devised to couple the vanillic acid decarboxylase reaction with horseradish peroxidase/H_2O_2. Guaiacol formed in the decarboxylation reaction is oxidized by peroxidase to tetraguaiacol, a chromophoric substance, which is measurable at 470 nm. Soluble, VAD activity was inducible in cells grown with vanillic acid. VAD was purified 185-fold by a combination of DEAE cellulose, phenylsepharose and sephacryl S-200 chromatographies. SDS-PAGE gave an estimated molecular mass of 46 ± 0.8 kDa while active native VAD was 92 kDa by gel chromatography. VAD required no cofactors, displayed optima at pH 7.0 and 30 °C, and a K_m of 0.8 mM for vanillic acid. The N-terminal amino acid sequence was determined to be AEYTLPDLDYDYSALEPHIS. 4-Hydroxybenzoic acid, vanillic acid, and syringic acid were excellent substrates, while VAD showed no activity for isovanillic acid, ferulic acid, *p*-coumaric acid, 2-hydroxybenzoic acid and 3-hydroxybenzoic acid. Purified VAD required a phenolic functional group *para* to the carboxylic acid moiety in the best substrates. The vanillic acid decarboxylation reaction is non-oxidative in nature. Conduct of the decarboxylation process in deuterium oxide results in incorporation of one deuterium atom *para* to the phenolic hydroxyl group of guaiacol. Vanillic acid decarboxylation likely involves enzymatic tautomerization to a vinylogous β-keto acid that spontaneously decarboxylates to guaiacol.

Keywords: Vanillic acid; *Nocardia*; Vanillic acid decarboxylase; Mechanism; Novel purification

1. Introduction

Vanillic acid is the major product [1] obtained by β-oxidative cleavage of the side chain of ferulic acid by species of *Rhodotorula* [2], *Bacillus* [3], *Pseudomonas* [4], and *Streptomyces* [5,6]. As such, vanillic acid is a biorenewable chemical feedstock available for the biocatalytic synthesis of other aromatic compounds such as vanillin and vanillyl alcohol by reductive metabolism, protocatechuic acid by *O*-demethylation, guaiacol by non-oxidative decarboxylation [7], and methoxyhydroquinone by oxidative decarboxylation [8–14]. *Nocardia* sp. NRRL 5646 produced two major products from vanillic acid biotransformation; guaiacol in 69% yield, and vanillyl alcohol in 11% yield. These products were produced by decarboxylation and reduction of the carboxylic acid, respectively [15].

Vanillic acid decarboxylation is apparently a non-oxidative enzyme reaction resembling the process by which ferulic acid decarboxylase achieved the decarboxylation of ferulic acid [1,16]. Such enzymes require no cofactors, are relatively stable, and are amenable to extracellular biocatalytic applications for the synthesis of valuable aromatic compounds. The present study was undertaken to evaluate the biocatalytic mechanism of *Nocardia* VAD, and to evaluate the nature of the enzyme itself. We established that the *Nocardia* decarboxylation reaction likely involved enzymatic tautomerization to a vinylogous β-keto acid that spontaneously decarboxylates to give guaiacol. We also report the purification and characterization of VAD from cell free extracts of *Nocardia* by use of a novel and rapid enzyme assay based upon the coupling of the VAD reaction with a peroxidase/H_2O_2 oxidation of guaiacol product. Finally, we compare *Nocardia* VAD to other phenolic acid decarboxylases (PADs) that are of interest because of their roles in generating flavor and off-flavors in foods and beverages [2,15,17–22].

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2. Materials and methods

2.1. Materials

Vanillic acid (4-hydroxy-3-methoxybenzoic acid) (**1**), guaiacol (2-methoxyphenol) (**3**), protocatechuic acid, syringic acid, *trans*-ferulic acid (4-hydroxy-3-methoxy-cinnamic acid 99%), *p*-coumaric acid, isovaleric acid, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, 2-hydroxybenzoic acid and *p*-aminobenzoic acid were purchased from Aldrich Chemical Company (Milwaukee, WI). Bovine serum albumin, protein markers and hydrophobic interaction chromatography gel (phenyl-sepharose CL-6B) were purchased from Sigma Chemical Co. (St. Louis, MO). Protein assay dye reagent was from Pierce (Rockford, IL). DEAE-cellulose (DE-53) was from Whatman Ltd. (Maidstone, Kent, England). HPLC grade methanol was obtained from EM Science (Gibbstown, NJ). Other chemicals and reagents were of analytical grade from Fisher Scientific (Pittsburgh, PA).

2.2. Analytical methods and instrumentation

Analytical and preparative thin layer chromatography (TLC) was carried out on 0.25 and 1 mm layers of silica gel GF_{254} (Sigma) precoated on polyester plates and glass plates (20 cm × 20 cm), respectively. The developing solvent was a mixture of dichloromethane:acetonitrile:formic acid (75:25:1, v/v/v). Developed TLC plates were directly visualized under 254 and 366 nm UV light for fluorescence quenching or fluorescence. Under these conditions, vanillic acid and guaiacol gave R_f values of 0.5 and 0.8, respectively.

High performance liquid chromatography (HPLC) was performed with a Shimadzu LC-10AD liquid chromatograph equipped with dual pumps and a photodiode array UV–vis detector (SPD-M6A). Products were separated from substrate over a hypersil C_{18} column (250 mm × 4.6 mm; 5 μm, Alltech, Deerfield, OH) protected with a guard column of the same composition (10 mm × 4.6 mm, 5 μm, Alltech). The mobile phase was a mixture of methanol:water:formic acid (70:30:1.2, v/v/v) and columns were eluted at a flow rate of 0.8 mL/min. Under these conditions, vanillic acid and guaiacol gave retention volumes of 8.2 and 14.1 mL, respectively. The HPLC method enabled detection of a minimum of 0.1 nmol of both guaiacol and vanillic acid, and an average standard deviation of 5%.

One-dimensional proton (^{1}H) nuclear magnetic resonance (NMR) spectra were obtained with a Bruker AMX-600 spectrometer (for ^{1}H operating 600 MHz, Karlsruhe, Germany), equipped with an IBM Aspect 2000 processor with VNMR version 4.1. Impurities in deuterated NMR solvents were used as internal standards. Chemical shift values are reported in parts per million (ppm) and coupling constants (*J* values) are given in Hz. Abbreviations for NMR are s, singlet; dd, double doublet; m, multiplet. FABMS spectra were performed using a Fisons VG-ZAB-HF reversed geometry mass spectrometer.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed [23] with 3% stacking and 12% separating gels containing 0.1% SDS. The protein standards used for estimation of subunit molecular masses were bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), bovine pancrease trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa) and bovine milk α-lactoglobulin (14.2 kDa). Protein was measured by the Bradford protein microassay [24] with bovine serum albumin as the standard.

2.3. Incubations

Nocardia species NRRL 5646 was grown and maintained on slants of Sabouraud-dextrose agar or sporulation agar [25] and stored in screw cap-sealed tubes in a refrigerator at 4 °C, prior to use. A two-stage incubation procedure [15] used a stage I culture, grown in 25 mL of sterile medium held in stainless steel-capped, 125 mL DeLong culture flasks. The medium contained 2% glucose, 0.5% yeast extract, 0.5% soybean meal, 0.5% NaCl and 0.5% K_2HPO_4 in double distilled water, and was adjusted to pH 7.0 with 6N HCl before being autoclaved at 121 °C for 15 min. Cultures were incubated with shaking at 250 rpm at 28 °C either on G25 or Innova 5000 Gyrotory three tier Shakers (New Brunswick Scientific Co., Edison, NJ). A 10% inoculum derived from 72 h-old first stage cultures was used to initiate second stage cultures, either as before in 125 mL DeLong flasks, or in 200 mL of medium held in 1 L stainless steel capped DeLong flasks, which were incubated as above for 24 h. For VAD induction, 160 mg of vanillic acid (in 1 mL of dimethyl sulfoxide) was added to each 1 L, stage-two culture flask and incubations were continued for 48 h before cultures were harvested by centrifugation at 10,000 × *g* at 4 °C for 20 min (Sorvall RC 26 Plus centrifuge with a SLA-600TC rotor, Kendro Laboratory Products, Newton, CT, USA). Induced cell pellets were suspended and washed in cold, pH 7.0, 50 mM potassium phosphate buffer (Buffer A) and finally filtered through cheesecloth to remove soybean meal solids. Centrifugation of this filtrate at 10,000 × *g* for 10 min at 4 °C gave cell weights of 20–24 g/L.

Nocardia resting cells were prepared in a similar manner by being filtered first through cheese cloth to remove soybean meal solids and cells were concentrated by centrifugation at 12,000 × *g* at 4 °C for 20 min. Cell pellets were suspended and washed in cold pH 7.0, 0.1 M phosphate buffer and centrifuged again.

2.4. Resting cell decarboxylations of vanillic acid to guaiacol in H_2O and ^2H_2O

Deuterium incorporation experiments used 600 mg (wet weight) of *Nocardia* resting cells in 125 mL DeLong flasks containing 25 mL of one of the following buffers: (a) pH 7.0, 0.1 M phosphate buffer containing 0.5% glucose prepared with H_2O; (b) with 50% ^2H_2O and (c) with 100% ^2H_2O. Each reaction mixture was furnished with 1 mg/mL of vanillic acid, and incubated on the G25 shaker (250 rpm) at 28 °C for 24 h. The cultures were separately harvested, acidified to pH 2.0 with 6N HCl and pelleted by centrifugation at 12,000 × *g*, 4 °C for 20 min. The resulting supernates were extracted with 25 mL of ethyl acetate three times each. After being dried over anhydrous Na_2SO_4, the ethyl acetate extracts were evaporated to dryness. The dried extracts were dissolved in a minimum volume of methanol, and purified by preparative TLC. A UV quenching band corresponding to guaiacol identified by co-TLC with the authentic compound was scraped from the plate and extracted with 25 mL of methanol three times. Methanol extracts were evaporated to dryness for ^{1}H NMR and mass analysis.

2.5. Vanillic acid decarboxylase (VAD) assay

VAD activity was determined using a coupled enzyme assay procedure in which the product of VAD, guaiacol, was further oxidized to a readily measured chromophore, tetraguaiacol ($\varepsilon = 26.6\ cm^{-1}\ mM^{-1}$ at 470 nm) by the action of horseradish peroxidase/H_2O_2 (Fig. 1). Crude (100 μg) or purified protein (2–10 μg) samples were incubated with shaking at 200 rpm at 30 °C in 500 μL of pH 7.0, 50 mM phosphate buffer containing 4 mM vanillic acid. After 30 min, H_2O_2 (500 μM) and horseradish peroxidase (10^{-7} M) was added to a final volume of 1 mL, and samples were incubated for 5 min before absorbances were measured at 470 nm. A linear relationship of absorbance to concentration was observed over the range 25–500 μM guaiacol. Using this assay, one unit of VAD activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of guaiacol from vanillic acid per minute under defined conditions.

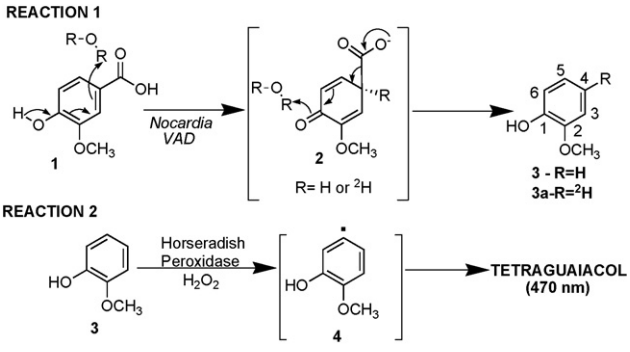

Fig. 1. The vanillic acid decarboxylase assay showing enzymatic conversion of vanillic acid (**1**) to guaiacol (**2**) and peroxidase/H_2O_2 oxidation of **2** to the chromophore, tetraguaiacol.

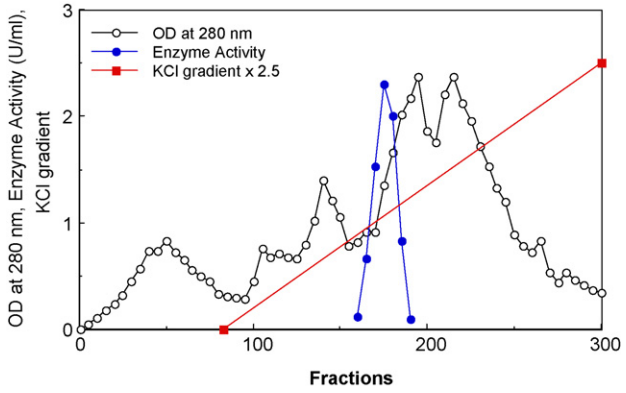

Fig. 2. Elution profile of vanillic acid decarboxylase using DE-53 column chromatography.

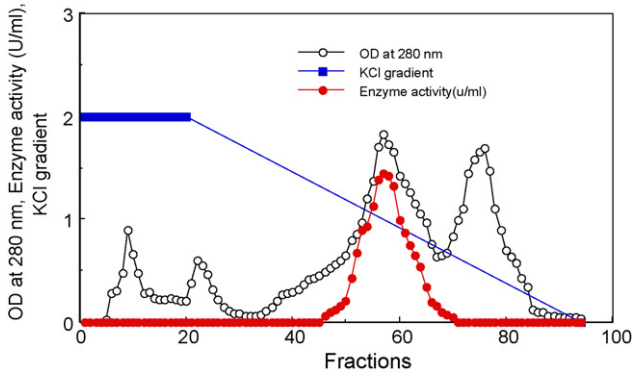

Fig. 3. Elution profile of vanillic acid decarboxylase by hydrophobic interaction chromatography.

2.6. VAD purification

All purification steps were carried out at 4 °C. A cell pellet (40 g) was suspended in 120 mL cold Buffer A containing 0.5 mM PMSF, 1 mM β-mercaptoethanol and 10% (v/v) glycerol and sonicated with a Branson Sonifier Cell Disrupter 350 (Branson Sonic Power Company, Danbury, CT) for a total of 4 min with 30 s cooling intervals between two 15 s pulses. The broken cell suspension was centrifuged at 10,000 × *g* for 30 min and the resulting supernatant was centrifuged again at 100,000 × *g* (Beckman LB-70 Ultracentrifuge, Palo Alto, CA) for 40 min. The 100,000 × *g* supernatant was the crude cell extract used in subsequent steps.

The crude extract was brought to 30% ammonium sulfate saturation and stirred for 30 min before the cloudy suspension was centrifuged at 20,000 × *g* for 20 min. This supernatant was brought to 65% ammonium sulfate saturation, stirred for 30 min, and centrifuged at 20,000 × *g* for 20 min. The resulting pellet was dissolved in 10 mL of pH 7.0, 20 mM phosphate buffer and dialyzed against 2 L of the same buffer containing 1 mM β-mercaptoethanol and 10% (v/v) glycerol (Buffer B). The dialyzed sample (20 mL, 235 mg protein) was loaded onto a 3 cm × 20 cm DEAE-Cellulose column (DE-53) that had been previously equilibrated with pH 7.0, 20 mM phosphate buffer containing 1 mM β-mercaptoethanol. The column was washed with 150 mL of equilibration buffer at a flow rate of 30 mL/h. The enzyme was eluted (Fig. 2) by a linear gradient of 0–0.5 M KCl in the same buffer (450 mL) while 2 mL fractions were collected and assayed for VAD activity. The active fractions were pooled and concentrated by ultrafiltration and reconstituted with 4 mL of pH 7.0, 300 mM phosphate buffer containing 2 M KCl for hydrophobic interaction chromatography (HIC).

HIC was performed on a 1.5 cm × 20 cm column of phenyl sepharose CL-4B (Sigma), which was equilibrated with 2 M KCl in pH 7.0, 300 mM phosphate buffer. The column was first washed with 40 mL of equilibration buffer to remove unbound proteins. Bound proteins were eluted with a linear 150 mL gradient ranging from equilibration buffer to pH 7.0, 20 mM phosphate buffer at a flow rate of 30 mL/h while 2 mL fractions were collected (Fig. 3). Active fractions (54–62, 18 mL) were pooled and concentrated by ultrafiltration with an Amicon stirred cell unit containing a PM10 (10,000 kDa cutoff) membrane. The concentrated enzyme solution (1 mL, 3.2 mg protein) was loaded onto a 1 cm × 100 cm sephacryl S-200 column equilibrated and eluted with pH 7.0, 20 mM phosphate buffer while 0.5 mL fractions were collected. Fractions containing VAD activity were combined for subsequent analysis.

2.7. VAD substrate specificity, kinetics, and N-terminal amino acid sequence

Purified VAD from the S-200 column step was used in all of the following studies. Phenolic acids (Table 3) were incubated with purified enzyme and analyzed by HPLC for the formation of products. Incubation mixtures each contained 53.6 units of pure enzyme and 10 μmol of phenolic acid substrate in 0.5 mL buffer. Reactions were stopped by adding 0.5 mL of methanol. After microfiltration, 10 μL samples were injected for HPLC analysis.

Kinetic parameters (V_{max} and K_m) were obtained for VAD using the peroxidase/H_2O_2 coupled assay described above. Reactions were conducted under initial velocity conditions and vanillic acid concentrations were varied from 0.1 to 5 mM. Kinetic constants were determined by using Lineweaver-Burk and the Ez-fit program [26]. Optimal incubation temperature and pH were estimated in assay mixtures containing 0.2 U of purified enzyme. Incubation temperatures were varied from 15 to 50 °C with vanillic acid as substrate. The influence of pH on reaction rates was determined in 100 mM acetate (pH 4 and 5), 100 mM phosphate buffer (pH 6.0, 6.5, 7.0, 7.5 and 8.0) and Tris·HCl (pH 8–9).

Purified VAD (10 μg) was analyzed by SDS-PAGE and transblotted onto a polyvinylidine difluoride membrane (Problott, Applied Biosystems, Foster City, CA). N-terminal amino acid analysis was done by automated microsequencing by Edman degradation on a 475A sequencer (Applied Biosystems, Foster City, CA) at the Protein Structure Facility, College of Medicine, University of Iowa, Iowa City, IA.

3. Results

3.1. Resting cell incorporations of deuterium into guaiacol during vanillic acid decarboxylation

To evaluate the decarboxylation mechanism, resting cell cultures were incubated in H_2O- and ^2H_2O-containing buffers

Table 1
^1H NMR and mass spectral analyses of guaiacol isolated from resting cell *Nocardia* sp. decarboxylations of vanillic acid conducted in H_2O- or ^2H_2O-containing buffers

^2H_2O % (v/v) in buffer	Guaiacol relative intensity at *m/z*				^1H NMR relative intensity H-3, 4, 6 protons at δ 6.87
	124 $[M]^+$	125 $[M+1]^+$	109 $[M-CH_3]^+$	110 $[M+1-CH_3]^+$	
0	100	12	100	9	3.0
50	100	23	100	16	2.8
100	0	60	0	86	2.2

containing vanillic acid. The guaiacol product from each reaction was isolated by solvent extraction, and purified by preparative TLC to give samples that were analyzed by mass spectrometry and NMR spectroscopy (Table 1).

The product (**3**) recovered from the standard H_2O-containing reaction gave mass ion peaks at *m/z* 124 ($[M]^+$, 100%), 125 ($[M+1]^+$, 12%), 109 ($[M-CH_3]^+$, 100%), and 110 ($[M+1\text{-}CH_3]^+$, 9%). The ^{1}H NMR spectrum contained signals at 6.92 ppm (m, 1H, Ar-5-*H*), 6.87 ppm (m, 3H, Ar-3, 4, 6-*H*), 5.62 ppm (s, 1H, Ar-O*H*), and 3.90 ppm (s, 3H, Ar-OCH_3). These spectral data were identical to those for authentic guaiacol. The product (**3a**) from the 100% ^2H_2O-containing buffer reaction furnished major mass spectral ions at *m/z* 125 (60%), and 110 (86%), while samples of **3a** from the 50% ^2H_2O-containing buffer reaction showed mass spectral ions at *m/z* 124 (100%), 125 (23%), 109 (100%) and 110 (16%). The results were clearest with the 100% ^2H_2O sample showing the incorporation of one deuterium atom into the decarboxylated product, **3a**. As expected, **3a** from the 50% ^2H_2O reaction contained an apparent mixture of deuterated and non-deuterated water.

Analysis of the ^{1}H NMR spectra of **3a** obtained from 50% to 100% ^2H_2O containing media showed all signals found in unlabeled guaiacol (**3**). However, the signal for 5-H at 6.93 ppm that existed as a multiplet in **3** resonated as a doublet of doublets with *J* values of 7.6 and 1.2 Hz. This simplification of the signal for H-5 can be accounted for by incorporation of a deuterium atom at the 4-position of guaiacol, *para* to the phenolic functional group, and on the position previously occupied by the carboxylic acid functional group. In addition, as summarized in Table 1, the relative intensities of signals clustered at 6.87 ppm for H-3, 4 and 6 for unlabeled **3**, 50% ^2H_2O **3a** and 100% ^2H_2O **3a** were 3H, 2.8H and 2.2H, respectively. These NMR results show increasing incorporation of deuterium in **3a** from 50% ^2H_2O to 100% ^2H_2O reactions.

3.2. Enzyme purification

For enzyme purification and characterization, a simple coupled enzyme, spectrophotometric assay method was devised. In Reaction 1 (Fig. 1), VAD decarboxylates vanillic acid to guaiacol. In Reaction 2, guaiacol is oxidized by added horseradish peroxidase/H_2O_2 to produce a deep red complex known as tetraguaiacol, a chromophoric product with A_{470}. Standard curves gave linear absorbances over the range of 25–500 μM guaiacol. VAD reactions were reproducibly linear for up to 1 h. Protein concentrations of greater than 200 μg/mL of crude cell free extract, interfered with tetraguaiacol formation [27–29]. In these cases, VAD reactions could be run as described after which removal of interfering proteins was achieved by treatment of samples with 20 μL of 10% TCA and neutralization with 4N NaOH. The resulting mixtures were suitable for the peroxidase/H_2O_2 analysis. Highly reproducible assays were obtained when as little as 0.02 U of VAD activity was used. The assay offered a novel, rapid, sensitive and easy means of measuring VAD activity during enzyme purification.

Maximum VAD levels in cell extracts gradually increased over time. The specific activity of the cell free extracts of non-induced cells was 0.04 U/mg protein and reached a maximum level to 0.087 U/mg protein in induced cells at 48 h. Thus *Nocardia* sp. contained a basal level of constitutive VAD activity. Cell extracts were routinely prepared from stage II cultures grown for 48 h after induction with 0.8 mg/mL of vanillic acid. Induced cells were harvested by centrifugation, disrupted by sonication and subjected to the sequence of purification steps summarized in Table 2. Ammonium sulfate precipitation removed nearly 60% of the protein from the crude extract while retaining 60.5% of the activity. A 4.2-fold increase in enzyme activity was achieved by DE-53 chromatography, while HIC and sephacryl S-200 column chromatography yielded about six and five-fold increases in activity, respectively. VAD was purified 185-fold in 16.3% overall yield.

3.3. VAD properties

SDS-PAGE of the purified enzyme showed a single band of about 98% purity (Fig. 4), with an apparent molecular mass of 23.0 ± 0.5 kDa. The apparent molecular mass of the native enzyme as measured by gel filtration was 46 (±0.8) kDa, indicating that VAD was active as a homodimeric protein. The UV–vis spectrum of purified enzyme solution showed an absorption maximum at 280 nm indicating the absence of covalently bound prosthetic groups such as FAD, which could display absorption maxima at 354 and 439 nm [30]. Purified decarboxylase had a K_m of 0.8 mM and V_{max} of 170 μmol/(min mg) protein in phosphate buffer (20 mM, pH 7.3) at 30 °C.

Nocardia VAD was stable for 4 weeks at 4 °C, and for 6 months at −70 °C, retaining about 90% of its activity when stored in pH 7.0, 20 mM phosphate buffer, containing 1 mM β-mercaptoethanol and 10% glycerol. Routine incubations of the enzyme in Tris buffer and triethanolamine (20 mM, pH 7.0) produced no appreciable change in activity compared to the same amount of enzyme incubated in phosphate buffer. VAD showed maximum activity at pH 7.0 and 30 °C. The N-terminal amino acid sequence of the VAD subunit was

Table 2
Summary of the purification of *Nocardia* sp. vanillic acid decarboxylase

Steps	Total protein (mg)	Total units (U)	Specific activity (U/mg)	Purification fold	Recovery (%)
Cell free extract	621	180	0.87	–	100
30–65% $(NH_4)_2SO_4$	235	109	1.39	1.6	60.5
DE-53	22.5	43.8	5.85	6.55	24.3
HIC	3.2	35.5	33.3	38.2	19.7
S-200	0.55	29.5	160.8	185	16.4

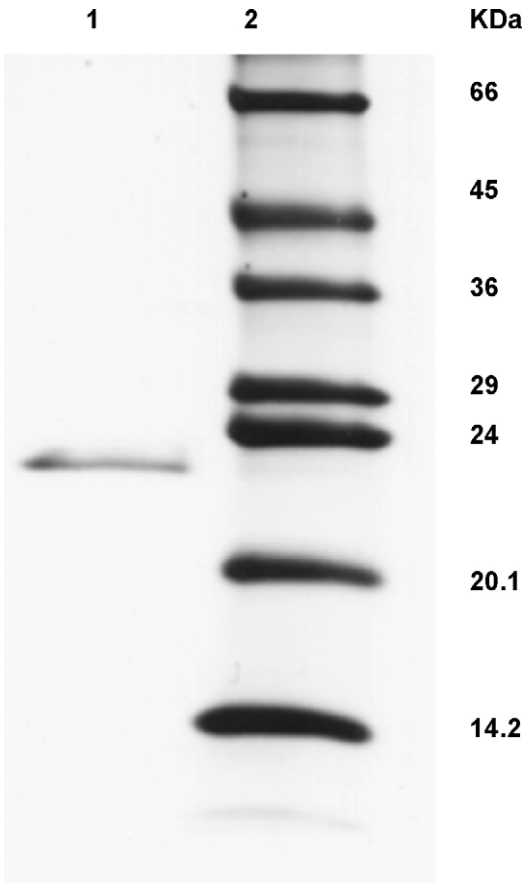

Fig. 4. SDS-PAGE of purified vanillic acid decarboxylase (from S-200 chromatography, 2.5 μg, lane 1) and protein markers (5 μg per band, lane 2) stained with Comassie blue. Markers from top are bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate (36 kDa), carbonic anhydrase (29 kDa), bovine pancrease trypsinogen (24 kDa), soyabin trypsin inhibitor (20.1 kDa) and bovine milk α-lactoglobulin (14.2 kDa).

H_2NAEYTLPDLDYDYSALEPHIS. BLAST [31] and literature comparison of the *Nocardia* sp. enzyme showed homologies with the following enzymes: superoxide dismutase (94%) from *Nocardia asteroids* [32], 4-hydroxybenzoate decarboxylase (55%) from *Thermoplasma acidophilum* [33], phenolic acid decarboxylase (50% and 45%) from *Lactobacillus pentosus* [34], and *Lactobacillus brevis* [35], aromatic acid decarboxylase (45%) from *Pseudomonas aeruginosa* [36], ferulic acid decarboxylase (40%) from *Pseudomonas fluorescens* [37], and *p*-coumaric acid decarboxylase (30%) from *Bacillus pumilus* [38]. Among 10 other aromatic acids, 4-hydroxybenzoic acid, protocatechuic acid and syringic acid gave relative decarboxylation rates (versus vanillic acid) of 90%, 72% and 68% (Table 3). No *o*- or *m*-hydroxy-aromatic carboxylic acids examined were substrates for *Nocardia* VAD. Ferulic acid, *p*-coumaric acid and *p*-aminobenzoic acid were not VAD substrates.

3.4. Effects of metals, coenzymes and inhibitors on enzyme activity

VAD activity was inhibited 90% by 1 mM mercuric chloride, 46% by 1 mM *N*-ethylmaleimide and 63% by 1 mM *p*-chloromercuribenzoic acid indicating the likely involvement of sulfhydryl groups in VAD activity. No effects on VAD activity were observed with 1 mM concentrations of Fe^{+2}, Fe^{+3}, Zn^{+2} salts, or by the additions of FAD, FMN, NAD^+, $NADP^+$, NADH or NADPH, or 1 and 10 mM EDTA.

Table 3
Nocardia vanillic acid decarboxylase activity vs. benzoate and cinnamate derivatives

Substrate	Relative decarboxylation rate[a] (%)
Vanillic acid (control)	100
Isovanillic acid	0
4-OH-benzoic acid	90
3-OH-benzoic acid	0
2-OH-benzoic acid	0
Protocatechuic acid	72
Syringic acid	68
Ferulic acid	0
p-Coumaric acid	0
p-Aminobenzoic acid	0

[a] The specific activity of VAD for decarboxylation of vanillic acid was 160.8 U/mg protein.

4. Discussion

The mechanism of vanillic acid decarboxylation to guaiacol was evaluated by carrying out *Nocardia* sp. resting cell reactions in buffers containing unlabeled water as a control, and in buffers prepared with both 50% and 100% ^2H_2O [2]. If the decarboxylation process involved a quinoid intermediate like (**2**) (Fig. 1), tautomerization of vanillic acid to **2** would involve the addition of a proton (or deuterium) atom from water to the position bearing the carboxylic acid functional group. By this pathway, decarboxylation of the vinylogous β-ketoacid **2** would give guaiacol (**3** or **3a**) containing deuterium at the 4-position (Fig. 1). Mass spectra of guaiacol recovered from the ^2H_2O incubation gave *m*/*z* 125 and no *m*/*z* 124, indicating the incorporation of one deuterium atom in **3a**. The ion at *m*/*z* 110 by loss of CH_3 from **3a** had no *m*/*z* 109 peak confirmed the incorporation of one deuterium into guaiacol.

The ^{1}H NMR spectrum was used to locate the position of the deuterium atom in **3a**. The ^{1}H NMR spectrum of standard guaiacol shows hydrogens for positions-3, 4, and 6 as an overlapping multiplet at δ 6.87, while the 5-H signal was a multiplet at δ 6.92 due to coupling with protons at positions-3, 4-, and 6. In **3a**, the overlapping multiplet at δ 6.87 integrated for 2.2 protons while the signal for 5-H resonated as a doublet of doublets (J = 7.6/1.2 Hz) [2]. These spectral data indicated the insertion of one deuterium atom at position-4 of guaiacol during the decarboxylation process. These results support a decarboxylation process involving a quinoid intermediate (**2**) as shown in Fig. 1.

The key to enabling the isolation and purification of *Nocardia* sp. VAD resided in our abilities to establish a rapid and sensitive assay for assaying enzymatic activity. VAD was measured by others by trapping $^{14}CO_2$ released from carboxyl-labeled vanillic acid [10]. Decarboxylation of *p*-coumaric acid [39] and 2,3-dihydroxybenzoic acid [40] were measured by monitoring

decreases in UV absorption as substrates were consumed. For VAD, we found that changes in UV/vis absorptions were inadequate for the measurement of substrate utilization or product formation. The assay developed here for detection of guaiacol, the product of VAD decarboxylation of vanillic acid, provided a novel, rapid assay for purification of the enzyme. We confirmed the reliability and initial velocity linearity of at least 1 h of the assay by measuring guaiacol oxidation [27–29] spectrophotometrically, and by HPLC. The simple spectrophotometric assay procedure described here should be generally useful for measuring vanillic acid decarboxylase activity from any source.

Although *Nocardia* VAD is a constitutive enzyme, it was also induced fourfold in cells grown in the presence of vanillic acid. The enzyme was stable in the cold (4 °C). UV–vis spectroscopy showed that VAD contained no apparent flavin component. VAD activity was not affected by the additions of FMN, FAD, NAD^+, $NADP^+$, NADPH, NADH and EDTA, and the reaction did not require oxygen. Thus the enzyme was different than a decarboxylase from *Sporotrichum pulverulentum* [9–12], where decarboxylation was catalyzed by a monooxygenase that required NADP(H). VAD exhibited optima at pH 7.0 and 30 °C, similar to those reported earlier for ferulic acid decarboxylase from *P. fluorescens* [37] and *p*-coumaric acid decarboxylase from *B. pumilus* [38]. The *Pseudomonas* and *Bacillus* enzymes were both homodimeric proteins, similar in mass, while 2,3-dihydroxybenzoic acid decarboxylase from *Aspergillus niger* [40] was a homo tetrameric protein with M_r 120 kDa.

The broad substrate specificity for VAD among hydroxybenzoic acids (Table 3) indicated a requirement for a 4-hydroxy-functional group for decarboxylation to occur. 4-Hydroxycinnamic acid derivatives were not substrates for *Nocardia* VAD, showing a functional difference in VAD versus other known enzymes that decarboxylate ferulic acid or *p*-coumaric acid. The N-terminal sequence for purified VAD was compared to other decarboxylase enzymes by BLAST and literature analysis. VAD showed relatively high homology (94%) with superoxide dismutase from *N. asteroids* [32] and good homology (55%) with 4-hydroxybenzoate decarboxylase from *T. acidophilum* [33], but relatively little with other decarboxylases. A comparison of the complete gene sequence for *Nocardia* sp. NRRL 5646 VAD would be required for further similarity evaluation with other known decarboxylases.

This is the first report of VAD from a species of *Nocardia*. VAD joins an impressive list of other enzymes discovered in *Nocardia* sp. NRRL 5646 that have potential in the synthesis of valuable phenolic products from biorenewables. The abilities of resting cells or pure enzyme to decarboxylate a variety of substrates including vanillic acid to guaiacol, 4-hydroxybenzoic acid to phenol, protocatechuic acid to catechol and syringic acid to 2,6-dimethoxy phenol renders *Nocardia* VAD of interest as a biocatalyst. The use of VAD as a biocatalyst is interesting in that the enzyme requires no cofactors or oxygen in the decarboxylation process. Oxidative and non-oxidative decarboxylases are valuable components of metabolically engineered hybrid pathways for the production of industrially useful chemicals [41]. This work provides the basis for cloning and expression of the *Nocardia* VAD gene to provide abundant supplies of the enzyme, and to enable further structural comparisons to other decarboxylases [17,18,42].

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