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Purification and Characterization of a Microbial Dehydrogenase

A Vanillin:NAD(P)$^+$ Oxidoreductase

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Abstract

Pseudomonas fluorescens (strain BTP9) was found to have at least two NAD(P)-dependent vanillin dehydrogenases: one is induced by vanillin, and the other is constitutive. The constitutive enzyme was purified by ammonium sulfate fractionation, gel-filtration, and Q-Sepharose chromatography. The subunit *Mr* value was 55,000, determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The native M_r value estimated by gel-filtration chromatography gave a value of 210,000. The enzyme made use of NAD^+ less effectively than $NADP^+$. Benzaldehyde, 4-hydroxybenzaldehyde, hexanal, and acetaldehyde were not oxidized at detectable rates in the presence of NAD^+ or $NADP^+$. The ultraviolet absorption spectrum indicated that there is no cofactor or prosthetic group bound. The vanillin oxidation reaction was essentially irreversible. The pH optimum was 9.5 and the p*I* of the enzyme was 4.9. Enzyme activity was not affected when assayed in the presence of salts, except $FeCl_2$. The enzyme was inhibited by the thiol-blocking reagents 4-chloromercuribenzoate and *N*-ethylmaleimide. NAD^+ and $NADP^+$ protected the enzyme against such a type of inhibition along with vanillin to a lesser extent. The enzyme exhibited esterase activity with 4-nitrophenyl acetate as substrate and was activated by low concentrations of NAD^+ or $NADP^+$. We compared the properties of the enzyme with those of some well-characterized microbial benzaldehyde dehydrogenases.

Index Entries: Vanillin; enzyme purification; vanillin dehydrogenase; *Pseudomonas fluorescens*; benzaldehyde dehydrogenase.

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Introduction

Vanillin (4-hydroxy-3-methoxy benzaldehyde) is one of the most important aromatic flavor compounds in the production of food flavorings and fragrances for perfumes. The first step for its biodegradation is its oxidation to vanillic acid (4-hydroxy-3-methoxy benzoic acid). Vanillic acid is a phenolic compound of high added value compared to vanillin, which is used as an antibacterial agent in dental prophylaxis and a specialty chemical and synthon in chemical synthesis *(1)*.

Oxidation of vanillin to vanillic acid occurs during the lignin degradation by some soil microorganisms such as *Pseudomonas acidovorans (2)*, *Neurospora crassa (3)*, *Corynebacterium* sp. *(4)*, *Streptomyces viridosporus (5)*, *Aspergillus japonicus (6)*, *Bacillus subtilis (7)*, *Pseudomonas fluorescens (8)*, and *Serratia marcescens (9)*. *P. fluorescens* BTP9 can also grow on vanillin as the sole source of carbon and energy and can bioconvert vanillin to vanillic acid *(10,11)*.

Priefert et al. *(12)* more recently reported the identification, cloning, and molecular characterization of the structural gene of vanillin dehydrogenase (*vdh*) in *Pseudomonas* sp. strain HR199. They showed that the deduced amino acid sequence of the gene exhibited 35.3% amino acid identity to benzaldehyde dehydrogenase encoded by TOL plasmid pWW0 of *Pseudomonas putida*. On the other hand, no data are available on the physicochemical properties of the dehydrogenase catalyzing the conversion of vanillin to vanillic acid. In this article, we describe the purification and characterization of vanillin dehydrogenase from *P. fluorescens* strain BTP9. In addition, we compare the properties of the enzyme with those of some well-characterized microbial benzaldehyde dehydrogenases.

Materials and Methods

All chemicals were from Sigma-Chemie (Bornem, Antwerpen, Belgium) except the following: vanillin was from Acros Chimica (Geel, Antwerpen, Belgium); acrylamide, bisacrylamide, sodium dodecyl sulfate (SDS), ammonium persulfate, bromophenol blue, TEMED, and Coomassie blue were from Bio-Rad (Nazareth EKE, Gent, Belgium); Sephadex G-25, Sephacryl S-200, Q-Sepharose Fast Flow, and isoelectrofocusing gels were from Pharmacia Biotech (Roosendaal, The Netherlands); acetaldehyde, phenyl methane sulfonyl fluoride, and 60F-254-silica gels plates (Thin-layer chromatography [TLC])) were from Merck (Overijse, Leuven, Belgium); and nitro blue tetrazolium chloride and EDTA were from UCB (Drogenbos, Bruxelles, Belgium).

Organism

Strain BTP9 was isolated from the rhizosphere of tomato plants and was identified as *P. fluorescens* (API 20NE assay).

Culture Maintenance and Fermentation

Bacteria were kept at 4°C on a solid medium containing 2% proteose peptone, 0.15% $MgSO_4 \cdot 7H_2O$, 0.15% $K_3PO_4 \cdot 3H_2O$, 1% glycerol, and 1.5% agar-agar. To obtain large amounts of bacteria, *P. fluorescens* BTP9 was grown in a nutrient broth (1% $Na_2HPO_4 \cdot 12H_2O$, 0.1% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% NaCl, 0.005% $CaCl_2 \cdot 2H_2O$ and 0.6% yeast extract) in a 15-L fermentor (Biolafitte, St Germain en Laye, France), a 20-L fermentor (Biolafitte, St Germain en Laye, France) or a 400-L fermentor (Amos, Angleur, Liege, Belgium).

An inoculum was prepared in a conical flask (100 mL for the 15- and 20-L fermentors, 2 L for the 400-L fermentor) containing 25 mL (for the first two fermentors) or 625 mL (for the third fermentor) of nutrient broth inoculated with one bacterial colony and shaken (190 rpm) at 30°C for 24 h. The entire culture was used to inoculate the fermentors, which contained 10 and 250 L of nutrient broth, respectively. The fermentors were operated for 16 h at 30°C with an aeration rate of 10 L/min (for the first two fermentors) and 250 L/min (for the third fermentor) using a stirring rate of 300 and 200 rpm, respectively.

For the 15- and 20-L fermentors, the bacteria were harvested by centrifugation for 30 min at 4470*g*. For the 400-L fermentor, a preliminary centrifugation at 12,000*g* (SA7 centrifuge equipped with an Optek system 110/AF10 and a time switch TA2RS; Westfalia, Oelde, Germany) was used. The liquid cell mud obtained at the end of this centrifugation was further centrifuged at the conditions used for cultures in the 15- and 20-L fermentors. Pelleted cells were suspended in 3 L of 100 m*M* potassium phosphate buffer/1 m*M* dithiothreitol (DTT), pH 6.0, and centrifuged again. The cells were stored at –20°C.

Preparation of Cell-Free Extracts

Frozen pelleted cells were resuspended in 3 vol of 100 m*M* potassium phosphate buffer/1 m*M* DTT, pH 6.0, and made 1 m*M* in phenyl methane sulfonyl fluoride. They were disrupted by two passages through a French press (FA-073; American Instrument, Silver Spring, MD) at a pressure of 40 MPa. The homogenate was centrifuged at 20,200*g* for 15 min at 4°C. The supernatant was frozen at –20°C or used, without any delay, as the starting material for the purification of vanillin dehydrogenase.

Purification of Constitutive Vanillin Dehydrogenase

Ammonium Sulfate Fractionation

Fifty milliliters of cell-free extract (12 g of cells wet wt) was brought to 1.3 *M* $(NH_4)_2SO_4$. After gentle stirring for 30 min, the solution was centrifuged for15 min at 20,200*g* and the resulting pellet removed. The supernatant was brought to 3.0 *M* $(NH_4)_2SO_4$ and centrifuged for 15 min at 20,200*g*.

The resulting pellet was resuspended in 5 mL of 20 m*M* potassium phosphate buffer, 2 m*M* DTT, and 0.33 m*M* vanillin, pH 6.0 (buffer A). The protein solution was loaded onto a Sephadex G-25 medium gel filtration column (47 × 26 mm) preequilibrated in buffer A. The first 24 mL was harvested and concentrated by vacuum dialysis on a 5-kDa cutoff membrane to a volume of 15 mL.

Gel filtration on Sephacryl S-200

The desalted fraction (15 mL; 705 mg of protein) was divided into three aliquots. Each aliquot was loaded onto a Sephacryl S-200 gel filtration column (600×16 mm) pre equilibrated with buffer A. The proteins were eluted at a flow rate of 0.2 mL/min and 6-mL fractions were collected. Those fractions containing the vanillin dehydrogenase activity were pooled, and the solution was concentrated by vacuum dialysis as already described to a volume of 5 mL.

Chromatography on Q-Sepharose

The sample was loaded onto a Q-Sepharose column (94 x 26 mm) pre equilibrated with 20 m*M* piperazine, 2 m*M* DTT, and 0.33 m*M* vanillin, pH 6.0 (buffer B). The proteins were eluted from the column using a fast protein liquid chromatography system by the following gradient system at a flow rate of 2 mL/min: 0.45 min, buffer B; 45–90 min, gradient of 0–0.5 *M* NaCl in buffer B; 90–150 min, gradient of 0.5–1.0 *M* NaCl in buffer B. Fractions of 2 mL were collected, and those containing the vanillin dehydrogenase activity (eluted at 0.4 *M* NaCl) were pooled and concentrated by vacuum dialysis to a volume of 3 mL. The purified enzyme was stored at –20°C.

Electrophoresis

The purification of vanillin dehydrogenase was monitored by SDS-polyacrylamide gel electrophoresis (PAGE) system developed by Bollag and Edelstein *(13)*, which consisted of 6.0% (w/v) polyacrylamide as the stacking gel and 10.0% (w/v) polyacrylamide as the separating gel.

Nondenaturing polyacrylamide gels were prepared as just described, except that they contained 1 m*M* DTT, the separating gel was only 7% polyacrylamide, and SDS was omitted from all solutions. The samples were loaded directly onto the gel without any treatment. Nondenaturing gels were either stained for protein using Coomassie blue or activitystained by incubating the gel in the dark from 4 to 12 h, at 29°C, in a 0.2 *M* Tris/HCl buffer (pH 8.0) containing 0.1% (w/v) vanillin, 0.02% (w/v) nitroblue tetrazolium chloride, 0.02% (w/v) phenazine methosulfate, and 0.02% (w/v) $NADP^+$ (or NAD^+).

The p*I* of the enzyme was determined on Phastgel homogeneous polyacrylamide gels containing Pharmalyte ampholytes (pH gradient from 3.5 to 9.5).

Determination of M_r Values

The apparent subunit M_r of the constitutive vanillin dehydrogenase was determined by SDS-PAGE. The native M_r value was determined in duplicate by gel filtration. The calibration proteins were catalase (240 kDa), alkaline phosphatase (140 kDa), bovine serum albumin (BSA) (66 kDa), and lactate dehydrogenase (36 kDa).

Enzyme Assay

The activity of vanillin dehydrogenase was measured at 37°C in 1.2-mL reaction mixtures containing 100 m*M* glycin buffer, 1 m*M* DTT, 2.75 m*M* $NAD(P)^+$ and enzyme (usually 100 µL), at pH 9.5. The reaction was initiated by the addition of vanillin to a final concentration of 5.5 m*M*. The reduction of $NAD(P)^+$ could not be monitored at 340 nm owing to the relatively low activity of the vanillin dehydrogenase and the differential absorption of the substrates and products; absorption coefficients of vanillin and NAD(P)H are similar: 5300 M^{-1} cm^{-1} for vanillin and 6300 M^{-1} cm^{-1} for NAD(P)H. Consequently, for each enzyme assay, the amount of vanillic acid was measured by high-performance liquid chromatography (HPLC) in five reaction mixtures of the same initial composition, after five different incubation times (from 0 to 2 h). The reaction was stopped by heating the reaction mixtures for 5 min in a water bath at 80°C.

The samples were centrifuged for 10 min at 25,850*g*, and vanillic acid was measured in the supernatant by liquid chromatography, using an HPLC apparatus (32x; Kontron, Milano, Italy) equipped with an autosampler. The compounds were separated by reverse-phase chromatography with two glass columns (100-mm length and 4.6-mm id), connected in series and packed with a reverse stationary phase (Chromspher C8; Chrompack, Antwerpen, Belgium). The eluent was a water/methanol/85% phosphoric acid mixture in a 800:200:2 ratio. The flow rate was 0.6 mL/min; vanillic acid was detected at 254 nm. Standards of vanillic acid were used for calibration. One unit of enzyme activity was defined as 1 µmol of product formed per minute, in the assay conditions; specific activities are given as U/mg of protein. Protein concentrations were determined by the procedure of Lowry *(14)*; BSA was used as the standard.

Enzyme Detection

For detection of vanillin dehydrogenase activity, 500 µL aliquots of purification fractions were combined with 100 µL of a 33 m*M* $NADP^+$ (or NAD^+) aqueous solution. The reaction was initiated by adding 50 µL of a 66 m*M* vanillin aqueous solution. The resulting reaction mixtures were left overnight at 37°C. The reaction was stopped by heating the reaction mixtures for 5 min at 80°C (water bath). The course of vanillin transformation was followed by TLC on 60F-254-silica gel plates (0.25 mm thick) devel-

oped in chloroform/methanol (19:1[v/v]). The spots of vanillic acid and residual vanillin were located on the plates under ultraviolet light at 254 nm.

Measurement of Esterase Activity

Esterase activity of the constitutive vanillin dehydrogenase was measured at 37°C in 1.2-mL reaction mixtures containing 100 m*M* potassium phosphate, 1 m*M* DTT buffer (pH 8.5), various $NAD(P)^+$ concentrations (2.75, 5.50, and 8.25 m*M*) and enzyme (80 µg). The reaction was initiated by adding 4-nitrophenol acetate (final concentration of 5.5 m*M*) dissolved in acetone. The hydrolysis of 4-nitrophenol acetate was monitored spectrophotometrically at 400 nm. The spontaneous hydrolysis rate of 4-nitrophenol acetate—measured with a control reaction mixture containing the heat-inactivated enzyme—was substracted from the observed rate. The molar extinction coefficient of 4-nitrophenol at 400 nm was assumed to be 14,690 M^{-1} cm^{-1}, at pH 8.5 *(15)*.

Inhibition by Thiol-Blocking Reagents

The constitutive vanillin dehydrogenase (0.4 mg of enzyme/mL) was incubated at 0°C for 1 h in the presence of various concentrations (from 0 to 0.4 m*M*) of p-hydroxymercuribenzoate (PHMB) or *N*-ethylmaleimide (NEM). The enzyme activities were then measured in the assay conditions defined before. Protection of the inhibition by cofactors or substrate was estimated by the addition of $NAD(P)^+$, or vanillin prior to the treatment with PHMB. The constitutive vanillin dehydrogenase was diluted in 100 m*M* potassium phosphate buffer, pH 6.0, containing 0.1 m*M* PHMB and 0.5 m*M* NAD^+, $NADP^+$ or vanillin. A control was carried out with only enzyme and PHMB (absence of protection). Enzyme activities were measured as described previously. The inhibition rates (percentage) for each thiol-blocking reagent concentration were calculated as followed:

$$\text{IR (\%)} = 100 \times \left[1 - \frac{\text{dehydrogenase activity (with thiol-blocking reagent)}}{\text{dehydrogenase activity (without thiol-blocking reagent)}}\right]$$

Measurement of Enzyme Activity with Benzaldehyde, 4-Hydroxybenzaldehyde, Acetaldehyde, and Hexanal

The oxidation reaction of the constitutive vanillin dehydrogenase was measured in reaction mixtures (720 µl) containing 100 m*M* glycin, 1 m*M* DTT, 2.75 m*M* $NAD(P)^+$, and enzyme (usually 20 µL) at pH 9.5. The reaction was initiated by the addition of benzaldehyde, 4-hydroxybenzaldehyde, acetaldehyde, or hexanal (final concentration of 0.1 m*M*). The reduction of $NAD(P)^+$ was monitored at 340 nm, 25°C. The absorption coefficient of NAD(P)H at 340 nm is 6.30×10^3 M^{-1} cm^{-1} *(16)*. Enzyme activity with benzaldehyde was also measured by HPLC as described earlier, to compare the enzyme activity with vanillin with those with the other aldehydes.

Stoichiometry of Dehydrogenation

The stoichiometry of the reaction between vanillin and NAD(P)$^+$ catalyzed by the constitutive vanillin dehydrogenase was determined in the following manner. Two reaction mixtures were prepared in the assay conditions defined before. After 20 min of incubation at 37°C, the reactions were stopped by heating at 80°C for 5 min. Vanillic acid concentrations were measured by HPLC, and NAD(P)H concentrations were determined spectrophotometrically (at 340 nm) after acidification at pH 4.0 (at this pH value, the molar absorption coefficients at 340 nm for vanillin and NAD(P)H were assumed to be approx 0 and 6.3×10^3 M^{-1} cm^{-1}, respectively).

Results

Evidence of Two Vanillin Dehydrogenase Activities

The vanillate-producing activity was determined by growing *P. fluorescens* BTP9 in a medium containing 0.6% yeast extract (as source of carbon) in the absence or presence of vanillin (0.01%). Samples were removed at 2-h intervals after 11–25 h of incubation and after 35 h, and cell-free extracts were prepared (in this experiment, the pH of the extraction buffers and the reaction mixtures was 8.0 and 7.0, respectively). The vanillic acid–producing activity was measured with NAD$^+$ and NADP$^+$.

With NAD$^+$, the vanillic acid–producing activity reached a maximum (1.9 mU/mg of protein) after 15–19 h of incubation (beginning of stationary phase) with 0.01% vanillin as the inducer (Fig. 1). After 19 h of incubation, induced activity decreased up to 50% of its maximum value (activity measured after 35 h), probably owing to the degradation of vanillin by cells. When no vanillin was added, the specific activity with NAD$^+$ was threefold lower (Fig. 2). With NADP$^+$ as cofactor, the enzyme activity was essentially constitutive.

One can conclude that *P. fluorescens* BTP9 produces at least two kinds of vanillin dehydrogenase: the first was NAD$^+$ dependent and is induced by vanillin, whereas the second has a mainly NADP$^+$ linked activity and is constitutive.

Purification of Constitutive Vanillin Dehydrogenase

We decided to purify and characterize the constitutive dehydrogenase rather than the enzyme that is induced by vanillin because the former enzyme can be easily separated from the latter by using a nutrient broth without vanillin for the production of the dehydrogenase.

Table 1 summarizes the results of a typical purification of the constitutive vanillin dehydrogenase. The enzyme was purified 33-fold with a 28% yield. It was homogeneous, as judged by denaturating and nondenaturating PAGE and by isoelectrophoresis (Fig. 3). The enzyme was purified several times by this procedure to obtain sufficient amounts for its characterization. DTT (1 m*M*) was always required to stabilize the enzyme activity.

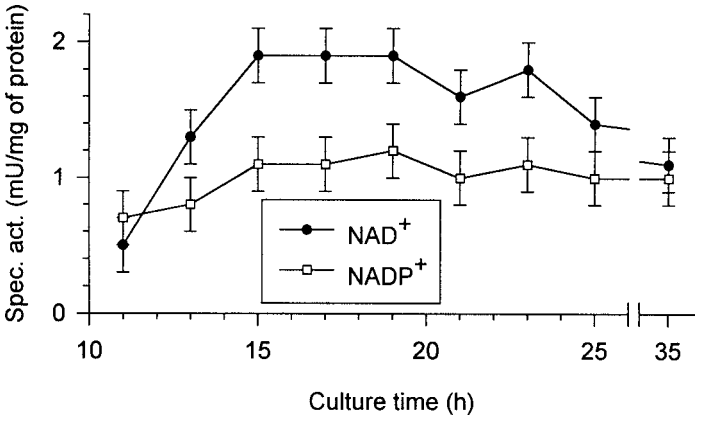

Fig. 1. Specific activity as function of time (measured with NAD^+ and $NADP^+$) of cell-free extracts obtained from culture of *P. fluorescens* BTP9 grown in presence of vanillin (0.01%).

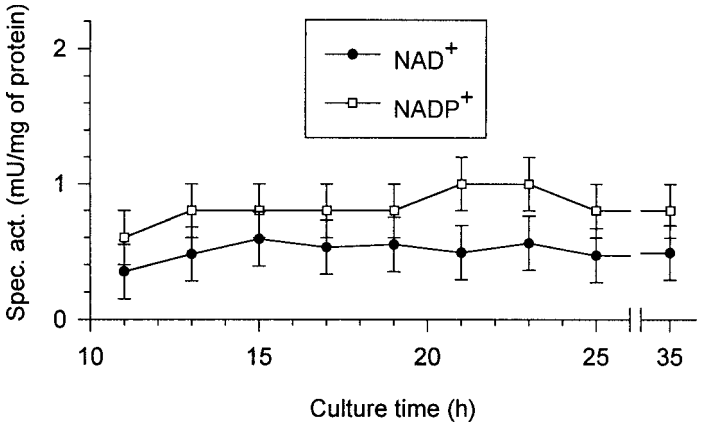

Fig. 2. Specific activity as function of time (measured with NAD^+ and $NADP^+$) of cell-free extracts obtained from culture of *P. fluorescens* BTP9 grown in absence of vanillin.

Table 1
Purification of Constitutive Vanillin Dehydrogenase from *P. fluorescens* BTP9[a].

	Volume (mL)	Total activity (mU)	Total protein (mg)	Specific activity (mU/mg protein)	Yield (%)	Purification (fold)
Cell-free extract	50.0	6710	1400	4.8	100	1
$(NH_4)_2SO_4$ fractionation	15.0	6350	705	9.0	95	2
Sephacryl S-200	5.0	3350	62	54.0	50	11
Q-Sepharose	3.0	1900	12	158.0	28	33

[a] For full experimental details see the text.

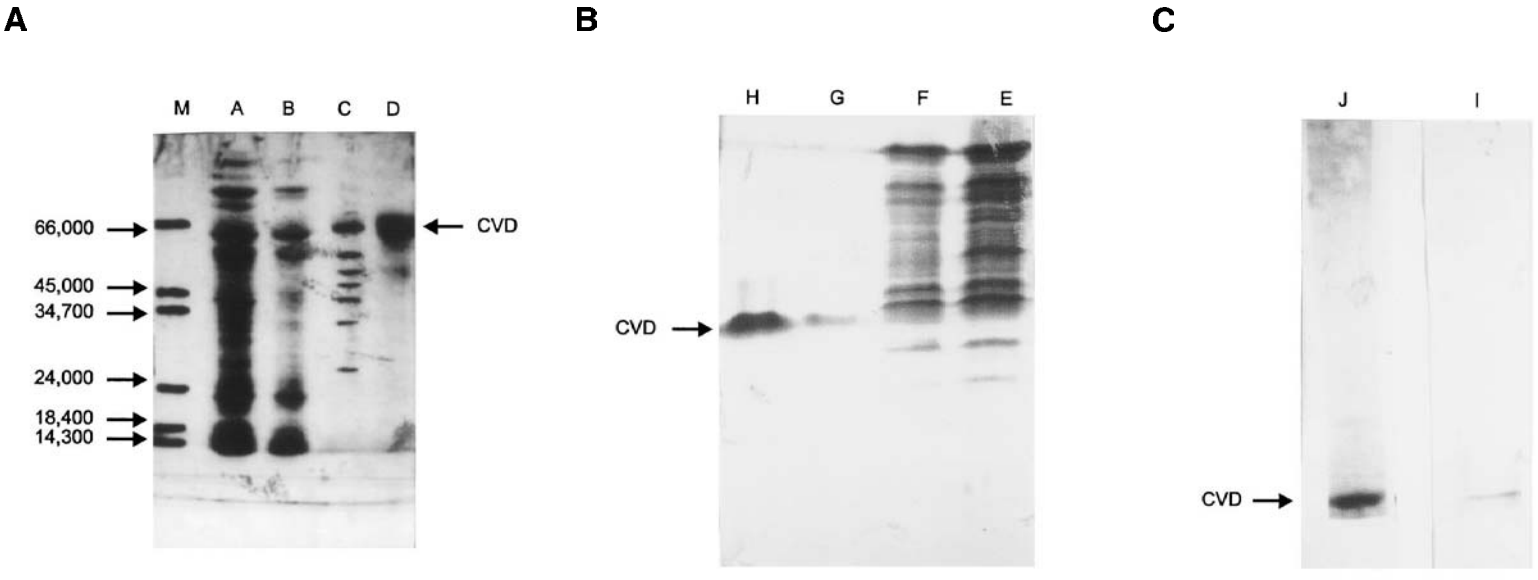

Fig. 3. Electrophoresis of constitutive vanillin dehydrogenase (CVD) from *P. fluorescens* BTP9. (**A**) The purification of the constitutive vanillin dehydrogenase was monitored on a 10.0% polyacrylamide slab gel containing 0.1% SDS. Lane *M*, M_r markers (5 µg of protein); lane A, extract (20 µg of protein); lane B, ammonium sulfate fractionation (15 µg of protein); lane C, Sephacryl S-200 elution pool (7 µg of protein); lane D, Q-Sepharose elution pool (3 µg of protein). (**B**) Nondenaturating 7.0% polyacrylamide slab gel with protein stain. Lane E, extract (25 µg of protein); lane F, ammonium sulfate fractionation (20 µg of protein); lane G, Sephacryl S-200 elution pool (7 µg of protein); lane H, Q-Sepharose elution pool (2 µg of protein). (**C**) Isoelectric focusing gel (pH range: 3.5–9.5). Lane I, Q-Sepharose elution pool (2 µg of protein) with protein stain; lane J, Q-Sepharose elution pool (2 µg of protein) with activity stain. CVD, constitutive vanillin dehydrogenase.

Determination of M_r

The subunit M_r value for the enzyme determined by SDS-PAGE was 55,000. This value is very similar to those reported for the benzaldehyde dehydrogenases previously described: 55,000 for benzaldehyde dehydrogenase II from *A. calcoaceticus (17)*, 56,000 for benzaldehyde dehydrogenase I from *A. calcoaceticus (15)*, 56,300 for benzaldehyde dehydrogenase encoded by the TOL plasmid pWW53 of *P. putida* MT53 *(18)*, 57,000 for benzaldehyde dehydrogenase encoded by the TOL plasmid pWW0 of *P. putida* PaW1 *(19)*. The M_r of the native constitutive vanillin dehydrogenase estimated by gel filtration is 210,000, and, therefore, the number of subunits is assumed to be 4. The aforementioned benzaldehyde dehydrogenases also are tetrameric.

Substrates and Cofactors

The constitutive vanillin dehydrogenase has an activity higher with $NADP^+$ than with NAD^+; the average activity with 2.75 m*M* NAD^+ relative to the activity with 2.75 m*M* $NADP^+$ is approx 30%. Benzaldehyde, 4-hydroxybenzaldehyde, hexanal, and acetaldehyde were not oxidized at detectable rates (rates lower than 0.5% of the enzyme activity with vanillin) with NAD^+ or $NADP^+$. The fact that the enzyme is unable to oxidize benzaldehyde in spite of the structural analogy of benzaldehyde with vanillin is consistent with the inability of benzaldehyde dehydrogenase from *P. putida (20)* and benzaldehyde dehydrogenases I and II from *A. calcoaceticus (16,21,22)* to oxidize vanillin.

On the other hand, the enzyme assay for vanillin dehydrogenase activity, described in Materials and Methods, did not allow accurate measurement of initial velocities with nonsaturating substrate concentrations and, consequently, access to the maximum velocity (V_{max}) and the Michaelis constants (K_m) of the constitutive vanillin dehydrogenase. V_{max} of this enzyme appeared, nevertheless, to be <1 U/mg of protein although it was included between 17 and 110 U/mg of protein for the aforementioned benzaldehyde dehydrogenases and for two TOL plasmid–encoded benzaldehyde dehydrogenases *(18,19,23)*.

Absorption Spectrum

The absorption maxima for the purified enzyme are at 214 and 280 nm. No other maxima were observed, and there was no absorbance above 340 nm, suggesting that the enzyme contained neither a bound cofactor nor a prosthetic group such as cytochrome, flavin, or pyrroloquinoline quinone, which have been found in some other bacterial aldehyde dehydrogenases *(17)*.

Stoichiometry

In the case of the reduction of NAD^+ by vanillin, 0.74 mmol of vanillic acid was formed for 0.71 mmol of reduced NAD. In the case of the reduction

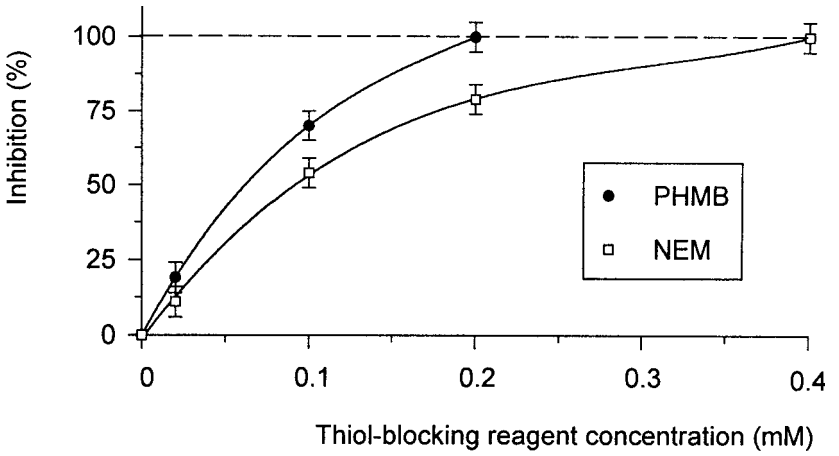

Fig. 4. Influence of thiol-blocking reagent (PHMB or NEM) concentration on enzyme inhibition.

of $NADP^+$, 2.2 mmol of vanillic acid was formed for 2.2 mmol of reduced NADP. It was concluded that the stoichiometry of the reaction between $NAD(P)^+$ and vanillin was 1:1. The enzyme does not apparently catalyze the reduction of vanillic acid by NAD(P)H.

Effects of Temperature and pH

The maximum rate of vanillin oxidation occurred at pH 9.5. This pH optimum is similar to that observed in the case of benzaldehyde dehydrogenases I *(15)* and II *(21)* in *A. calcoaceticus* and benzaldehyde dehydrogenases encoded by the TOL plasmid pWW53 *(18)* and the TOL plasmid pWW0 *(19)* in *P. putida*. The p*I* of the constitutive vanillin dehydrogenase is 4.9. Heat stability of the enzyme was also investigated: the half-life of the enzyme at 50°C was 1.5 min.

Effects of Various Compounds on Stability and Activity of Enzyme

The effect of salts on the activity of the constitutive vanillin dehydrogenase were tested in the assay conditions defined before; NH_4Cl, $MgCl_2$, $CaCl_2$, KCl, or $ZnCl_2$ did not have any effect on the activity of the enzyme but 0.1–1 m*M* $FeCl_2$ significantly decreased (20–50%) the activity, probably owing to the reactivity of Fe^{2+} with a thiol group implied either in the catalysis or in the structure of the active site. The constitutive vanillin dehydrogenase was not affected by the presence of 5 m*M* EDTA.

The constitutive vanillin dehydrogenase exhibits different sensitivities to two thiol-blocking reagents: PHMB and NEM. In the presence of 0.2 m*M* PHMB, the enzyme was totally inactivated but with an identical concentration of NEM, 21% of the enzyme activity was retained (Fig. 4).

Table 2
Protection from inhibition by PHMB of
Constitutive Vanillin Dehydrogenase by Vanillin or $NAD(P)^+$ [a].

Addition	Inhibition (%)
0.1 m*M* PHMB (control)	72
0.1 m*M* PHMB + 0.5 m*M* NAD^+	29
0.1 m*M* PHMB + 0.5 m*M* $NADP^+$	21
0.1 m*M* PHMB + 0.5 m*M* vanillin	40

[a]Inhibition rates were calculated as described above (cf. fig. 4).

The protection from inhibition by thiol-blocking reagents of the constitutive vanillin dehydrogenase by vanillin or $NAD(P)^+$ was also investigated. Table 2 shows that for a PHMB concentration of 0.1 m*M*, the inhibition rates in the presence of $NAD(P)^+$ as protective agent ranged between 20 and 30%. The inhibition rate with vanillin as protective agent was 40%.

Therefore, both the substrate and the cofactor manage to partially protect the constitutive vanillin dehydrogenase from inhibition by PHMB. Cofactors appear to be more efficient than vanillin. In the case of benzaldehyde dehydrogenase II from *A. calcoaceticus*, the substrate protects the enzyme better than cofactor *(22)*. In the case of horse liver mitochondrial aldehyde dehydrogenase, inhibition is more important in the presence than the absence of the cofactor *(24)*.

Esterase Activity

The constitutive vanillin dehydrogenase displays an esterase activity with 4-nitrophenol acetate as substrate. The rate was approx 2% of the dehydrogenase activity. The enzyme was activated by NAD^+ and $NADP^+$ (Fig. 5). It was impossible to measure esterase activity at the pH optimum of the dehydrogenase activity (pH 9.5) because of the spontaneous hydrolysis of the substrate; as a result the esterase activity was measured at pH 8.5. Activation of the esterase function by NAD^+ was consistent with the presence of a single, common active site for both the esterase and the dehydrogenase activities *(14)*.

Discussion

The ability of *P. fluorescens* strain BTP9 to bioconvert vanillin into vanillic acid was previously investigated: productivity higher than 3 g/(L·d) and conversion rates near or higher than 80% were reached with immobilized-cell reactors and two-phase reactors *(10,11)*. The enzymes responsible for oxidation of vanillin in *P. fluorescens* strain BTP9 were identified as vanillin dehydrogenases.

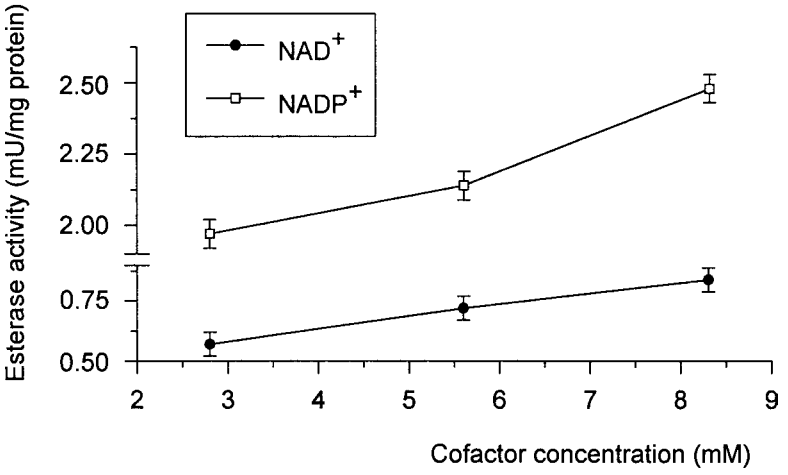

Fig. 5. Esterase activity of constitutive vanillin dehydrogenase activated by NAD^+ and $NADP^+$.

Although those enzymes are implicated in one of the most important natural catabolic pathways (lignolytic pathway), as far as we know they have never been purified before. This could be explained by their low activity compared with that of the benzaldehyde dehydrogenases *(15,18–23)* and by the impossibility of monitoring the reaction progress spectrophotometrically, at 340 nm (enzyme assay usually used for dehydrogenases).

The constitutive vanillin dehydrogenase from *P. fluorescens* BTP9 has several features in common with the aforementioned benzaldehyde dehydrogenases. These enzymes are tetrameric (except the benzaldehyde dehydrogenase encoded by the TOL plasmid pWW0 of *P. putida* PaW1, which would be dimeric *(19)* and have generally similar subunit M_r values (~55 kDa). The sensitivity to thiol-blocking reagents, pH optima (~9.5), esterase activity, and p*I* (~5) are similar. Furthermore, these enzymes, which virtually catalyze irreversible reactions, are not inhibited by EDTA and have no bound cofactor or prosthetic group. The two main differences between the constitutive vanillin dehydrogenase (Vanillin:$NAD(P)^+$ Oxidoreductase according to the enzyme nomenclature) and the benzaldehyde dehydrogenases are the substrates (vanillin for the former and benzaldehyde for the latter) and the activity level (the former enzyme activity is more than 10 or 100 times lower than the latter). Purification and characterization of the induced vanillin dehydrogenase from *P. fluorescens* BTP9 could complement work on the main properties of this new kind of enzyme.

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