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Purification and Characterization of Ferulate and *p*-Coumarate Decarboxylase from *Bacillus pumilus*

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Bacillus pumilus **PS213 isolated from bovine ruminal fluid was able to transform ferulic acid and *p*-coumaric acid to 4-vinylguaiacol and 4-vinylphenol, respectively, by nonoxidative decarboxylation. The enzyme responsible for this activity has been purified and characterized. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of crude extract from a culture induced by ferulic acid or *p*-coumaric acid shows three bands that are not present in the crude extract of an uninduced culture, while the purified enzyme shows a single band of 23 kDa; the molecular mass calculated by size exclusion chromatography is 45 kDa. Enzyme activity is optimal at 37°C and pH 5.5 and is not enhanced by any cation. Kinetic studies indicated a K_m of 1.03 mM and a V_{max} of 0.19 mmol · min^{-1}/mg · liter^{-1} for ferulic acid and a K_m of 1.38 mM and a V_{max} of 0.22 mmol · min^{-1}/mg · liter^{-1} for *p*-coumaric acid.**

Lignin degradation is a very complex process involving many enzymatic reactions and many microorganisms; its final result is lignin mineralization. The rumen is one of the environments where the lignin seems to be degraded, as shown by significant weight loss of the lignocellulosic material (1), owing to the presence of bacteria and fungi described as potential utilizers of lignin or lignin compounds. The degradation mechanism of lignin in plant cell walls depends on the following steps: (i) separation of core and noncore lignin from the cell wall polysaccharides, mainly hemicellulose (13); (ii) depolymerization of lignin, which is a polymer of phenylpropanoid units interconnected by several types of carbon-carbon or carbon-oxygen bonds (18); and (iii) degradation of low-molecular-weight aromatic compounds.

We focused our attention on the catabolism of low-molecular-weight aromatic compounds, in particular ferulic acid and *p*-coumaric acid, which are the main phenolic monomers released from graminaceous cell wall (8) and are very important in repressing lignocellulose utilization in the rumen, besides being toxic in vitro to rumen bacteria, protozoa, and fungi (3, 5). These two lignin-related aromatic compounds are extremely abundant and widely distributed in higher plants, and thus they could be of interest as a renewable resource for the production of useful and value-added chemicals such as guaiacol derivatives through biotransformation (9).

The bacterium *Bacillus pumilus* PS213 isolated from the bovine rumen has been shown to convert ferulic acid and *p*-coumaric acid to 4-vinylguaiacol and 4 vinylphenol, respectively, by a nonoxidative decarboxylation, while its ability to degrade the two products further is still unknown. Decarboxylation of both classes of phenolic monomers, substituted cinnamic and benzoic acids, has already been described for several microorganisms (6, 9, 10, 15), including *Bacillus* species (11) such as *B. pumilus* (2). However, no purification or characterization of this enzyme has been reported to date. In this paper we report the presence of a decarboxylase enzyme in the crude extract of an induced *B. pumilus* culture and describe the purification and characterization of this enzyme.

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MATERIALS AND METHODS

Culture conditions. The aerobic bacterium *B. pumilus* PS213, initially isolated from ruminal fluid, was maintained in LB medium containing 15% glycerol at −70°C. For enzyme production, the bacterium was grown at 37°C for 8 h in 1-liter flasks each containing 250 ml of M9CA medium (17) and 125 mg of ferulic acid or *p*-coumaric acid. Media were inoculated with 1% of a starting culture grown overnight in LB medium.

Analysis of substrate degradation. For identification of degradation products of an overnight culture supernatant, we used UV spectroscopy followed by high-pressure liquid chromatography (HPLC) analysis, comparing the spectra with those related to the standard compounds. HPLC analysis was performed on a Star 9010 solvent delivery system equipped with a Star 9050 detector connected to a computing integrator (model 4400; Varian Associates Inc., Walnut Creek, Calif.), with a Supelcosil LC8 column (Supelco Inc., Bellefonte, Pa.). Samples were dissolved in water, and methanol-H_2O-acetic acid (60:39.9:0.1) was used as the mobile phase.

Preparation of cell extract. The culture was centrifuged at 8,000 rpm for 10 min in a GSA rotor (Sorvall, DuPont, Inc., Wilmington, Del.), the supernatant was removed, and the pellet was washed with 40 mM Tris-HCl (pH 7.6). The pellet was resuspended in 5 ml of lysis buffer per g of wet cells. Lysis buffer was composed of 50 mM Tris-HCl (pH 7.6), 10% sucrose, 1 mM dithiothreitol, 1 mM EDTA, 0.1% Triton X-100, and 600 μg of lysozyme per ml, according to Penalva and Salas (16), and freshly prepared before addition. The cell suspension was incubated on ice for 1 h or until it clarified. Protamine sulfate (50 μg/mg of protein) was added to the suspension to precipitate nucleic acids by forming an aggregate. Samples were centrifuged in an Eppendorf centrifuge (Eppendorf, Hamburg, Germany) at maximum speed for 10 min, and the supernatant was recovered. Cell extract was stored at −20°C in 0.1 M sodium phosphate buffer (pH 6.0) containing 0.5 mM phenylmethylsulfonyl fluoride as a proteinase inhibitor.

Protein determination. The total protein concentration was determined with a protein assay kit (Bio-Rad, Hercules, Calif.) with bovine serum albumin as the standard (4).

Enzyme assay. Ferulate and *p*-coumarate decarboxylase activity was assayed by monitoring the appearance of new peaks at 258 nm (4-vinylguaiacol) and 256 nm (4-vinylphenol), respectively. The assay mixture consisted of 50 mM sodium phosphate buffer (pH 6.0) and 0.5 g of ferulic acid or *p*-coumaric acid per liter. Reactions were started by adding cell extract (5% [vol/vol]) and incubating the mixture at 37°C for 1 h; 1 U of activity was defined as the degradation of 1 μmol of ferulic acid per min.

Protein purification. The enzyme purification was performed at 4°C. The cell extract from 2 liters of bacterial culture was fractionated by the addition of $(NH_4)_2SO_4$ up to 40, 60, 80, and 100% saturation at 0°C and centrifuged at 52,000 × *g* for 20 min. The pellet was resuspended in 3 ml of 20 mM Bis-Tris-HCl buffer (pH 6.0) and dialyzed against the same buffer. Fractions containing decarboxylase activity were pooled and applied to a Q Sepharose Fast Flow ion-exchange chromatography column (10 by 50 mm; Pharmacia LKB Biotechnol-

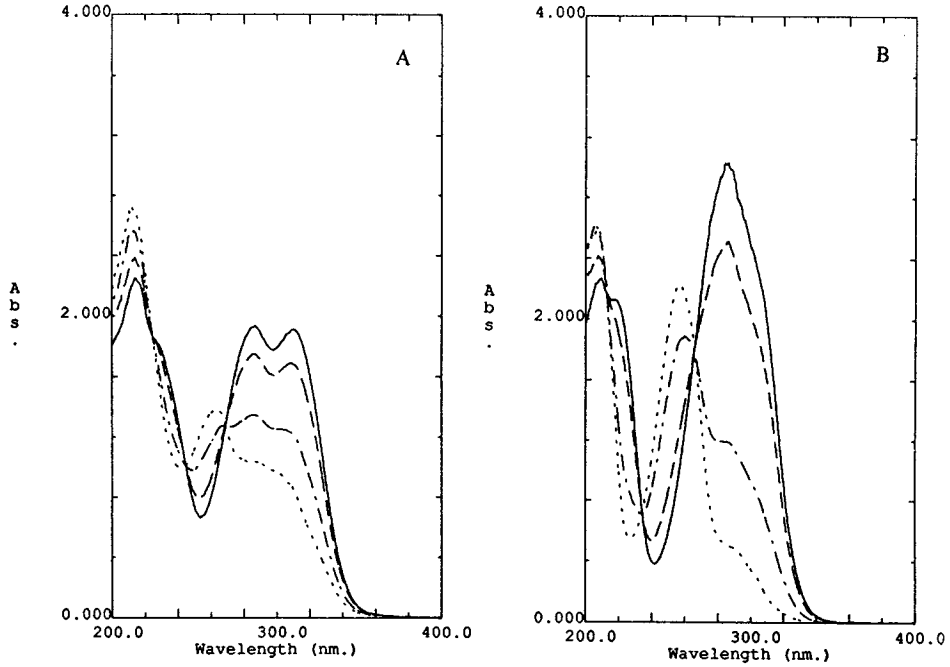

FIG. 1. Decrease of ferulic acid (A) and *p*-coumaric acid (B) levels in the enzyme assay mixtures containing crude cell extract from *B. pumilus* grown in M9CA (— — —), M9CA with *p*-coumaric acid (— · — · —), M9CA with ferulic acid (·····), and lysis buffer without cell extract (——) after 6 h of incubation.

ogy, Uppsala, Sweden); the column was washed with 15 ml of 300 mM NaCl–20 mM Bis-Tris-HCl (pH 6.0), and a 300 to 800 mM NaCl gradient was used to elute the enzymatic activity (50 ml of each NaCl concentration). The proteins were eluted at a flow rate of 0.5 ml/min. Fractions of 2 ml were collected, and the protein content and decarboxylase activity were assayed. Fractions 13 to 19, containing enzymatic activity, were pooled, dialyzed, and concentrated by ultrafiltration in Centriprep 3 (Amicon Inc., Beverly, Mass.). The protein was loaded again on the same column and eluted as described above with a 300 to 500 mM NaCl gradient. Fractions 18 to 21 were pooled and concentrated in Centricon 3 (Amicon) to a final volume of 2 ml. The sample was loaded on a Sephacryl HR200 size exclusion chromatography column (10 by 1,000 mm; Pharmacia LKB Biotechnology) equilibrated with 20 mM sodium phosphate buffer (pH 6.0) containing 150 mM NaCl. The protein was eluted at a flow rate of 0.1 ml/min, and 2-ml fractions were collected. The active fractions 26 to 32 were collected and loaded again on the Q Sepharose Fast Flow column. The elution was performed as described above with a 300 to 400 mM NaCl gradient. The last step of purification was performed by size exclusion chromatography on Sephacryl HR200 under the conditions describe above, with Gel Filtration Standard molecular weight marker proteins (Bio-Rad Laboratories).

Effect of pH and temperature. Optimal pH and temperature were determined in the pH 3.0 to 9.5 range (50 mM sodium acetate [pH 3.0 to 5.5], sodium phosphate [pH 6.0 to 7.0], Tris-HCl [pH 7.5 to 9.5]) and the 4 to 80°C temperature range. Enzyme stability was determined by incubating the purified enzyme, without substrate, at different pHs and temperatures. For pH stability determination, samples were incubated in buffers from pH 3.0 to pH 9.0 and at 37°C for 30 min. For determination of thermal stability, various temperatures (4 to 80°C) were used at pH 5.5 for 30 min. The remaining decarboxylase activity was assayed under standard conditions described above.

Effect of O_2 on enzyme activity. Freshly prepared crude cell extract was completely degassed under vacuum and then stored on ice for 4 h in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, Mich.) containing a 10% CO_2–10% H_2–80% N_2 atmosphere. An enzyme assay was performed as described above, but the assay mixture containing 1 μg of resazurin sodium per ml was stored in an anaerobic chamber until the color faded. The reaction was started by addition of 10 μl of crude extract to 90 μl of assay mixture. Samples were boiled in a water bath to stop the reaction before the substrate consumption was measured with a spectrophotometer.

Other assays. The effects of various metallic ions and reagents at 2 mM on the purified enzyme were tested. The purified enzyme was dialyzed against distilled water. Residual activity was measured as described above. K_m and V_{max} values were determined from Lineweaver-Burk plots at ferulic acid and *p*-coumaric acid concentrations varying from 10 to 200 μM.

PAGE analysis. The presence of the enzyme in the induced culture was checked by comparing the protein pattern of the crude extracts from induced and uninduced cultures grown in the same medium with and without ferulic acid or *p*-coumaric acid. Proteins were resolved by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 5% stacking gel and 12% resolving gel) by the method of Sambrook et al. (17). Molecular weights were determined with the low-molecular-weight standards (Bio-Rad). Protein bands were stained with Coomassie blue R-250 after electrophoresis. SDS-PAGE performed at 15°C in a 15% gel by the method described above was used to ascertain the degree of protein purity. The molecular masses of the enzyme polypeptides were determined by comparison with the mobilities of standard proteins of known molecular mass.

Analytical isoelectric focusing of the purified enzyme was performed with an Ampholine PAGplate precast polyacrylamide gel (Pharmacia), with pH values ranging from 3 to 10, and the broad-pI calibration kit (Pharmacia) as pI marker.

The molecular mass and the pI of the enzyme were evaluated, with the help of an UltroScan XL Laser Densitometer (Pharmacia), by comparing the migration distance of the enzyme with that of the molecular mass and pI markers in SDS-PAGE and isoelectric focusing, respectively.

HPLC analysis of protein. The purified protein was analyzed by reverse-phase HPLC to control the degree of purification obtained by low-pressure liquid chromatography. The HPLC system used was the same as described above, and the column used was a Supelcosil LC304 measuring 5 cm by 46 mm (inner diameter) with a pore size of 300 nm. The eluted protein was detected at 226 nm, the flow rate was 0.6 ml/min, eluent A was water–0.1% trifluoroacetic acid, and eluent B was acetonitrile–0.1% trifluoroacetic acid. A 10-μl sample from size exclusion chromatography was loaded onto the column and eluted with a gradient of 95% A and 5% B to 10% A and 90% B in 30 min.

Preparation of protein for sequence determination. A preparative SDS-PAGE was performed, the gel was slightly stained with Coomassie blue R-250, and the band responsible for the activity was cut out and eluted from the gel with LKB 2014 Extraphor Electrophoretic Concentrator (LKB Electrophoresis Division, Bromma, Sweden) as specified in the LKB Laboratory Manual. Protein was dialyzed, concentrated in a Centricon 3 microconcentrator, and loaded onto a gel (12 by 14 cm; 0.75 mm thick) containing a 10 to 15% gradient of polyacrylamide. After electrophoresis, the gel was soaked in transfer buffer (48 mM Tris, 39 mM glycine [pH 9.2], 10% methanol, 1.3 mM SDS) for 15 min. During this time, a polyvinylidene difluoride membrane was rinsed with 100% methanol and stored in transfer buffer. The gel, sandwiched between a sheet of polyvinylidene difluoride membrane and several sheets of blotting paper soaked in transfer buffer, was assembled into a blotting apparatus (Multiphor II Nova Blot; LKB) and electroeluted for 2 h at a constant current of 0.8 mA/cm^2. The polyvinylidene difluoride membrane was washed in deionized water for 5 min, stained with Coomassie blue R-250 in 50% methanol for 10 min, and then destained in 50% methanol–5% acetic acid at room temperature. The membrane was finally rinsed in deionized water for 5 to 10 min, air dried, and stored at −20°C.

Amino acid sequence analysis. Automated Edman degradation of the protein sample was performed on a pulsed liquid-phase protein sequencer (model 470A; Applied Biosystems, Foster City, Calif.) equipped with an on-line phenylthiohydantoin amino acid analyzer (model 120A; Applied Biosystems). Normal, filter,

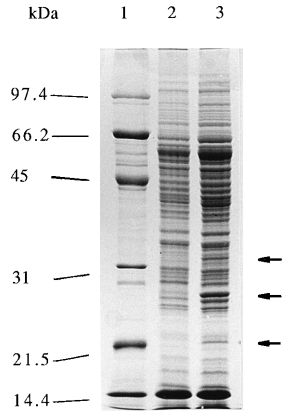

FIG. 2. SDS-PAGE of *B. pumilus* crude cell extract. Lanes: 1, molecular mass standard, consisting of rabbit muscle phosphorylase *b* (97,400 Da) bovine serum albumin (66,200 Da), hen egg white ovalbumin (45,000 Da), bovine carbonic anhydrase (31,000 Da), soybean trypsin inhibitor (21,500 Da), and hen egg white lysozyme (14,400 Da); 2, crude extract from an uninduced culture; 3, crude extract from an induced culture.

and phenylthiohydantoin standard cycles for both reaction and conversion functions were performed as specified by the manufacturer. The sample was loaded onto a fiberglass disk coated with 3 mg of Polybrene (14) and preconditioned by two filter cycles.

RESULTS

Decarboxylase production and activity. *B. pumilus* PS213 produces ferulate and *p*-coumarate decarboxylase under nutrient-limited conditions. This enzyme transforms ferulic acid and *p*-coumaric acid to 4-vinylguaiacol and 4-vinylphenol, respectively, as demonstrated by UV spectroscopy (Fig. 1), via a nonoxidative decarboxylation. In addition, HPLC analysis showed similar retention times for 4-vinylguaiacol and the degradation product of ferulic acid in a *B. pumilus* culture (data not shown). The reaction is catalyzed by a stable ferulic and *p*-coumaric acid decarboxylase present in the crude cell extract from an induced *B. pumilus* culture. The production of this enzyme seems to be induced by the presence of ferulic or *p*-coumaric acid in the culture broth, although the crude extract of an uninduced culture seems to contain a very low enzymatic activity, probably because of a basal constitutive expression (Fig. 1). The enzyme is specific for ferulic acid and *p*-coumaric acid, since it does not decarboxylate other cinnamic acids, benzoic acids, aromatic amino acids, or caffeic acid (data not shown).

Enzyme purification. SDS-PAGE of intracellular total proteins extracted from induced and uninduced 16-h cultures gave similar protein patterns, except for three evident bands present only in the induced sample (Fig. 2). These bands corresponded to approximate molecular masses of 23, 28, and 35 kDa. To confirm the relationship between these proteins and the decarboxylation activity, we proceeded to purify the enzyme. The purification of ferulate and *p*-coumarate decarboxylase (FCD) is summarized in Table 1.

The crude enzyme preparation was precipitated with ammonium sulfate: 81% of the activity was recovered between 40 and 80% saturation, while 41% of the proteins were eliminated. Ion-exchange chromatography of this fraction (Fig. 3A) permitted a further 86.7% elimination of proteins, with 37.5% of the total activity recovered (Table 1). After the first size exclusion chromatography, one major peak of ferulic acid and *p*-coumaric acid decarboxylase became evident, corresponding to an elution volume of 56 to 60 ml. The last two steps of purification confirmed this behavior: the third ion-exchange chromatography further reduced the contaminant proteins by 64% (Fig. 3B), while the following size exclusion chromatography eliminated 88% of remaining proteins, giving almost complete purification (Fig. 3C).

Determination of molecular mass. The molecular mass of the enzyme was estimated by Sephacryl HR200 size exclusion chromatography to be 45 kDa when the elution volume of the enzyme was compared with the elution volume of standard proteins. The purified proteins appeared to be homogeneous, composed of a single polypeptide chain when subjected to SDS-PAGE and Coomassie staining (Fig. 4) followed by UltroScan XL densitometric analysis. The molecular mass estimation of 23 kDa by SDS-PAGE was lower than that estimated by size exclusion chromatography. The overestimated molecular mass obtained by size exclusion chromatography suggests that the protein could be constituted of two subunits with the same molecular mass.

Effect of pH and temperature. The optimal temperature and pH were 37°C and 5.5, respectively (Fig. 5A and B). According to the temperature stability curve, the ferulate and *p*-coumarate decarboxylase rapidly degrades above 37°C (Fig. 5C) and is lost completely above 42°C. The pH stability results show that ferulate and *p*-coumarate decarboxylase is stable in the acidic pH range, exhibiting almost 100% of total activity between pH 5.0 and 6.0 (Fig. 5D).

Effect of O_2 on enzyme activity. Decarboxylation was successfully performed, also under strictly anaerobic conditions, in parallel with the standard reaction, indicating that ferulate and *p*-coumarate decarboxylase is not O_2 dependent and that the rates of disappearance of the two substrates are equivalent with and without oxygen (data not shown).

Other characteristics. The effects of various cations and reagents on enzymatic activity were investigated (Table 2). The activity was completely inhibited by the addition of Cu^{2+}, Ag^{2+}, or SDS and strongly reduced by both Zn^{2+} and Fe^{2+}; it was not enhanced by any cation.

TABLE 1. Purification of *B. pumilus* FCD[a]

Purification step	Total amt of protein (mg)	Total activity (U)	Sp act (U/mg)	Purification factor	Yield (%)
Cell extract	97	117.1	1.2	1	100
$(NH_4)_2SO_4$ precipitation	57.2	94.9	1.66	1.4	81
Q Sepharose Fast Flow (0.3–0.8 M)	35.2	88.5	2.5	2.1	75.5
Q Sepharose Fast Flow (0.3–0.5 M)	7.6	44	5.79	4.8	37.5
Sephacryl HR200	1.25	20.8	16.4	13.6	17.7
Q Sepharose Fast Flow (0.3–0.4 M)	0.45	9.9	22	18.3	8.4
Sephacryl HR200	0.054	3.9	72.2	60.1	3.3

[a] A 2-liter sample of 8-h culture was purified. For details, see Materials and Methods.

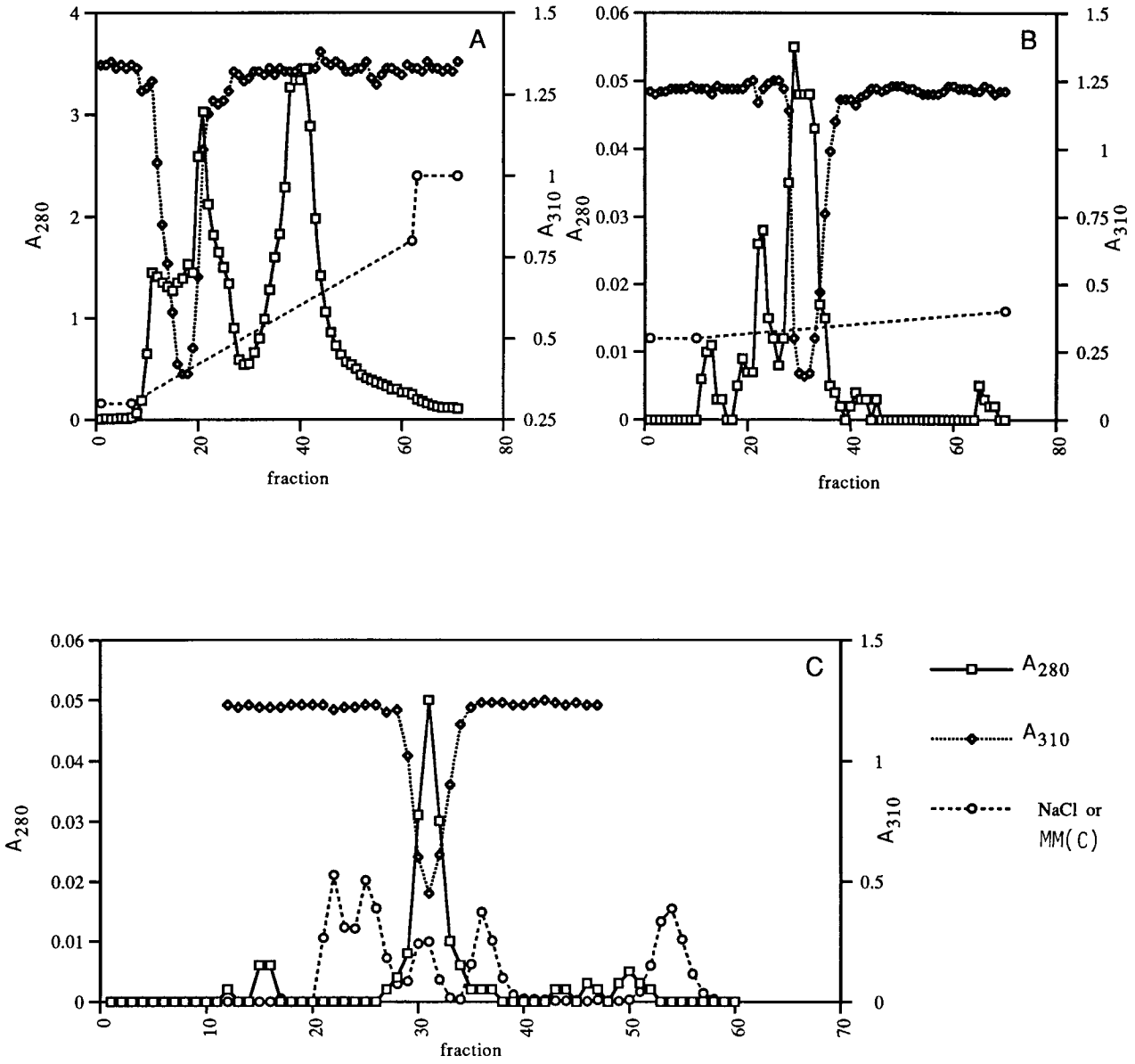

FIG. 3. (A and B) Elution profiles from Q Sepharose Fast Flow ion-exchange chromatography (in 20 mM Bis-Tris-HCl [pH 6.0]) of the *B. pumilus* proteins. The column was washed with 300 mM NaCl, and the protein was eluted with a 300 to 800 mM NaCl gradient (A) and a 300 to 400 mM NaCl gradient (B). (C) Elution profile from Sephacryl HR200 size exclusion chromatography (in 20 mM sodium phosphate [pH 6.0], 150 mM NaCl). The molecular mass marker protein elution profile [MM(C)] is superimposed: bovine thyroglobulin, 670,000 Da; bovine gamma globulin, 158,000 Da; chicken ovalbumin, 44,000 Da; horse myoglobin, 17,000 Da; and vitamin B_{12}, 1,350 Da. Total enzymatic activity is indicated, showing the disappearance of the substrate at 310 nm.

The initial velocity of ferulic and *p*-coumaric acid decarboxylation by ferulate and *p*-coumarate decarboxylase in 20 mM sodium phosphate buffer (pH 6.0) at 37°C was determined over the substrate concentration range of 10 to 200 μM ferulic acid and *p*-coumaric acid. A Lineweaver-Burk plot showed a linear response over this concentration range. The Michaelis constant (K_m) was 1.03 mM for ferulic acid and 1.38 mM for *p*-coumaric acid, and the maximal velocity (V_{max}) was 0.19 $mmol \cdot min^{-1}/mg \cdot liter^{-1}$ for ferulic acid and 0.22 $mmol \cdot min^{-1}/mg \cdot liter^{-1}$ for *p*-coumaric acid.

The isoelectric point of ferulate and *p*-coumarate decarboxylase, determined by means of isoelectric focusing, is 4.6. The isoelectric focusing protein pattern shows a major band at pH 4.6 and two faint bands at slightly higher pH (Fig. 6). Reverse-phase HPLC analysis of the same protein shows a major peak, together with a smaller peak eluted earlier. A late shoulder is also present (Fig. 7).

When subjected to N-terminal sequence analysis, the band of 23 kDa which belongs to the purified ferulate and *p*-coumarate decarboxylase enzyme was revealed to be homogeneous, suggesting the presence of a univocal amino acid sequence. The purified protein (about 500 pmol) was analyzed by automated pulsed liquid-phase Edman degradation. Two analyses of the sample were performed, and the N-terminal sequence was identified as follows:

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1                5                10               15
H2N-Met-Asp-Gln-Phe-Val-Gly-Leu-His-Met-Ile-Tyr-Thr-Tyr-Gln-Asn-Gly-Val.....
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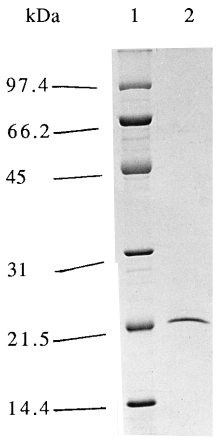

FIG. 4. SDS-PAGE of the peak fraction from the Sephacryl HR200 column. Lanes: 1, molecular mass standard; 2, 15 μg of decarboxylase.

Analysis of the protein revealed traces of Ala at position 1 and Thr at position 3. The N terminus seemed to be partially blocked, but this did not affect the determination of the sequence, since the two sequences obtained from separate analyses were identical.

DISCUSSION

B. pumilus PS213 isolated from bovine ruminal fluid has been proved to produce ferulate and *p*-coumarate decarboxylase only in the presence of the substrates; therefore, this enzyme is induced by these two cinnamic acids. The role of this aerobic microorganism in the rumen is still unclear, since the rumen is a highly reducing environment, although aerobic microorganisms have already been isolated from ruminal fluid (12) and oxygen is detectable in the bulk liquid phase of the rumen (19). In this work we focused on purification of the ferulate and *p*-coumarate decarboxylase enzyme.

Since this decarboxylation has been described previously and it could be a potential step for the conversion of natural aromatic acids to industrial organic intermediates (9), we purified

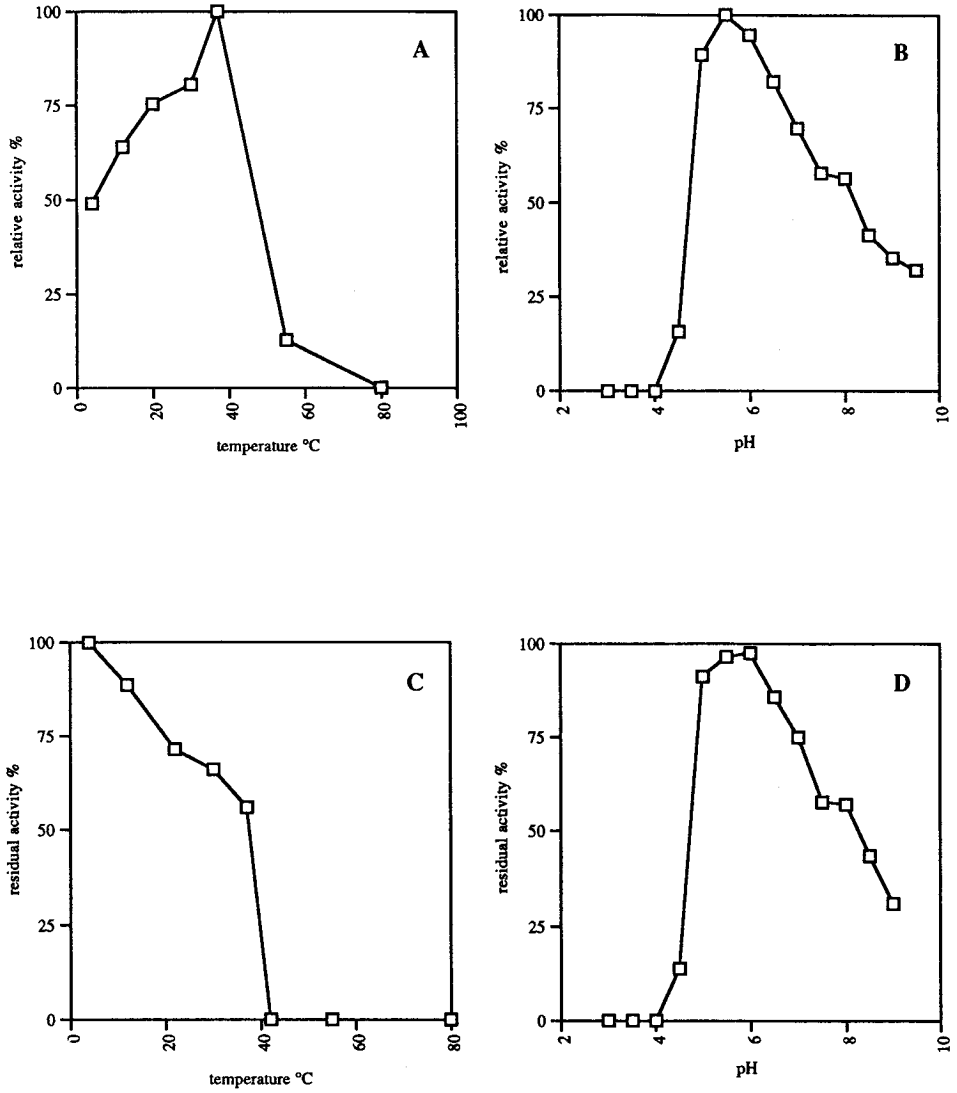

FIG. 5. Temperature optimum (A), pH optimum (B), temperature stability (C), and pH stability (D) of the purified decarboxylase. Enzyme assays for stability were performed after a 30-min exposure at different pHs and temperatures followed by measurement of the relative activity.

TABLE 2. Influence of cations and reagents on *B. pumilus* decarboxylase activity

Cation or reagent	Activity (%)[a]
Control	100
LiCl	88.8
KCl	93.3
NaCl	93.3
$MgCl_2$	95.4
$CaCl_2$	95.4
$FeCl_2$	48.8
$MnCl_2$	97.6
$CoCl_2$	95.4
$CuSO_4$	0
$AgNO_3$	0
$ZnCl_2$	33.3
$Cr_2(SO_4)_3$	85
EDTA	95.4
SDS	0

[a] Activities of the purified enzyme were assayed in the presence of 2 mM effector. The activity assayed in the absence of effector (control) was set to 100%.

the enzyme to homogeneity, as demonstrated by SDS-PAGE, and characterized it from the biochemical point of view.

Separation of the purified protein by isoelectric focusing and HPLC resulted in three bands and three peaks, respectively, suggesting the presence of three isoforms with very similar pIs (4.6) and identical molecular masses. The discrepancy between the molecular masses determined by SDS-PAGE and size exclusion chromatography (23 and 45 kDa, respectively) indicates that the protein could be a homodimer. This is also in agreement with the N-terminal sequence homogeneity of the band excised from the SDS-PAGE gel.

The enzyme loses its activity above 37°C and is stable in the acidic range. Its activity is not affected by most of the cations tested; however, Cu^{2+}, Ag^{2+}, Zn^{2+}, and Fe^{2+} completely or partially inactivate it.

Decarboxylation of ferulic acid and *p*-coumaric acid seems to be quite common in *Bacillus* species (2, 9, 11), and it has already been described in *Fusarium solani* (15), in *Saccharomyces cerevisiae* and *Pseudomonas fluorescens* (9), and in other microorganisms (2, 9). Decarboxylation represents the first step of a degradative pathway, like the one suggested by Nazareth and Mavinkurve for *F. solani* (15). *B. pumilus* cannot utilize ferulic acid or *p*-coumaric acid as the sole carbon source, and other *Bacillus* species are reported to have a different pathway for the metabolism of ferulic acid (7). This implies that this decarboxylase function represents a step of secondary metabolism that could have evolved in response to the toxicity of the two compounds (3). For these reasons, the role and utility of this reaction warrant further investigation.

Isolation and sequencing of the decarboxylase gene from *B. pumilus* are in progress, and the decarboxylase activity from other microorganisms will be investigated in the future.

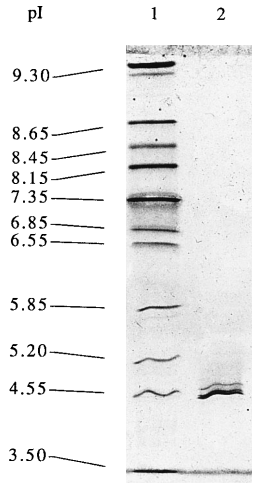

FIG. 6. Analytical isoelectric focusing assay of the peak fraction from Sephacryl HR200 column on a polyacrylamide gel. Lanes: 1, pI markers, consisting of trypsinogen (9.30), lentil lectin (8.65), lentil lectin (8.45), lentil lectin (8.15), myoglobin (7.35), myoglobin (6.85), human carbonic anhydrase B (6.55), bovine carbonic anhydrase (5.85), β-lactoglobulin A (5.2), soybean trypsin inhibitor (4.55), and amyloglucosidase (3.5); 2, 5 μg of decarboxylase enzyme.

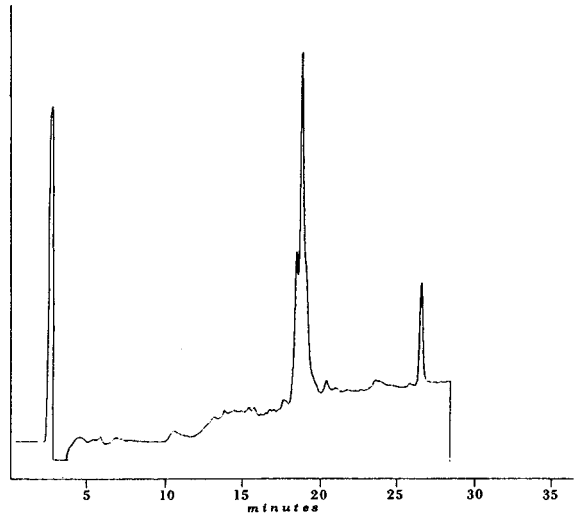

FIG. 7. HPLC analysis of the purified protein eluted at a flow rate of 0.5 ml/min with a water–acetonitrile–0.1% trifluoroacetic acid gradient and detected at 226 nm.

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