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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Cloning, sequencing, and overexpression in *Escherichia coli* of the *Enterobacter* sp. Px6-4 gene for ferulic acid decarboxylase

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Abstract Ferulic acid decarboxylase (FADase) can catalyze the transformation of ferulic acid into 4-vinyl guaiacol via decarboxylation in microorganisms. In this study, a gene encoding FADase was first isolated from the bacterium *Enterobacter* sp. Px6-4 using degenerate primers and a genome walking technique. The putative encoding gene (*fad*) of FADase consists of 507-bp nucleotides, coding a polypeptide of 168 amino acid residues. In addition, a putative gene encoding the transcriptional regulator was identified from the upstream of the *fad* gene. The deduced peptide sequence of the FADase from *Enterobacter* sp. Px6-4 showed a 51.2–53.3% sequence identity to decarboxylases from other bacteria. The gene *fad* was successfully expressed in *Escherichia coli* BL21, and the recombinant FADase was purified as a protein of ca. 23 kDa with an optimal activity at pH 4.0 and 28 °C. The purified FADase could convert ferulic acid to 4-vinyl guaiacol effectively, and its hydrolytic activity could be inhibited by Cu^{2+} (99%) and Hg^{2+} (99.5%). A phylogenetic analysis of the FADase protein from bacteria revealed several different clades. Our result provided a basis for further studies of the ferulic acid transformation pathway and for enhanced production of vanillin in the future.

Keywords *Enterobacter* sp. Px6-4 · Ferulic acid decarboxylase · Cloning · Expression · Enzyme analysis

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Introduction

Ferulic acid is an extremely abundant cinnamic acid derivative found in the cell wall of woods, grasses, and corn hulls (Rosazza et al. 1995; Ishii 1997; Oosterveld et al. 2000). It is covalently linked to various carbohydrates as a glycosidic conjugate (Smith and Hartley 1983; Muller-Harvey and Hartley 1986; Saulnier and Thibault 1999). It has been suggested that ferulic acid is released during ruminal degradation of plant cell walls (Akin 1988). Moreover, ferulic acid and other aromatic acids can be released from these polymeric structures by feruloyl esterases produced from fungi and other microorganisms (Benoit et al. 2008). Feruloyl esterases are a subclass of the carboxylic acid esterases that hydrolyze the ester bond between hydroxycinnamic acids and sugars present in plant cell walls, and they have been isolated from a wide range of microorganisms (e.g., Brezillon et al. 1996; Donaghy and McKay 1997; Brunati et al. 2004; Mathew and Abraham 2004). Free ferulic acids are metabolized by different microorganisms into 4-vinyl derivatives and then reduced into 4-ethyl derivatives (Priefert et al. 2001;

Torres and Rosazza 2001; Mathew and Abraham 2006). These volatile phenols are potential contributors to the aroma of wine, perfumes, pharmaceuticals, and other fermented foods and beverages (Ramachandra and Ravishankar 2000; Priefert et al. 2001).

Recently, six major pathways have been reported to be involved in the initial ferulic acid conversion, namely: (1) non-oxidative decarboxylation, (2) side chain reduction, (3) coenzyme-A-independent deacetylation, (4) coenzyme-A-dependent deacetylation, (5) demethylation, and (6) oxidative coupling (Priefert et al. 2001; Mathew and Abraham 2006). In non-oxidative decarboxylation, catalyzed by decarboxylase, ferulic acid is converted to 4-vinyl guaiacol (4-hydroxy-3-methoxystyrene) (Donaghy and McKay 1997). 4-Vinyl guaiacol is up to 30 times more costly than ferulic acid. Thus, this bioconversion is a high-value-added process (Priefert et al. 2001). The decarboxylation of ferulic acids to 4-vinyl guaiacol has already been reported for many microorganisms (e.g., Huang et al. 1993; Cavin et al. 1997a, b, 1998; Prim et al. 2003; Gury et al. 2004), and the catalyzing enzyme has been purified from *Pseudomonas fluorescens* (Huang et al. 1994), *Lactobacillus plantarum* (Cavin et al. 1997a), and *Bacillus pumilus* (Degrassi et al. 1995). However, the genes encoding these functions have not been fully investigated (Cavin et al. 1997b, 1998; Prim et al. 2003; Gury et al. 2004).

In our recent report, a bacterium *Enterobacter* sp. Px6-4 was isolated from vanilla roots, and this bacterium could utilize ferulic acid as the sole carbon source to produce vanillin. This bacterium was found capable of transforming ferulic acid into 4-vinyl guaiacol via non-oxidative decarboxylation (Li et al. 2008). Here, we describe the identification of the *Enterobacter* sp. Px6-4 ferulic acid decarboxylase gene (*fad*) and report its expression in *E. coli*. The biochemical properties of FADase were characterized and the evolutionary relationships of this FADase with decarboxylases from other bacteria were discussed.

Materials and methods

Chemicals

Ferulic acid (*trans*-, 99%) and 4-vinyl guaiacol (99%) were purchased from Sigma-Aldrich (America). All the solvents used for high-performance liquid chromatography (HPLC) were of HPLC grade. All of the other chemicals were of analytic grade.

Strains, vector, and growth condition

The bacterium strain *Enterobacter* sp. Px6-4 was isolated from vanilla roots (Li et al. 2008) and deposited in the China General Microbiological Culture Collection Center (CGMCC 1999). *E. coli* DH5α and *E. coli* BL21 (DE3) were used for the transformation and propagation of plasmids harboring the desired DNA fragments. Bacteria were grown in Luria–Bertani medium (tryptone, 10 g/l; yeast extract, 5 g/l; NaCl, 10 g/l) at 37 °C.

Vectors pMD18-T (Takara, Japan) and pET28a (Novagen, Germany) were used for TA-cloning and gene expression, respectively.

Cloning of the gene coding ferulic acid decarboxylase from *Enterobacter* sp. Px6-4

The genomic DNA of *Enterobacter* sp. Px6-4 was extracted according to a previously described method (Li et al. 2008). A pair of degenerate primers, PS and Px2, was designed according to the reported decarboxylases (GenBank nos. AF017117, AJ276891, AJ27863, AJ492219, U63827, and X84815) from different bacteria. These primers were used for amplifying the conserved nucleotide sequence of *fad* from *Enterobacter* sp. Px6-4. PCR amplification was performed according to our previously described protocol (Li et al. 2008). The PCR product was purified and ligated into the pMD18-T vector, and the recombinant vector was transformed into *E. coli* DH5α. Colonies containing the desired fragment were randomly picked and sequenced using the ABI 3730XL DNA Analyzer (Applied Biosysterms, USA).

To obtain the full-length nucleotide sequence of *fad* from *Enterobacter* sp. Px6-4, three pairs of nested PCR primers (Table 1) were designed according to the conserved sequence. The 5′ and 3′ terminal fragments of the gene

Table 1 PCR primers used in this study

Primers	Sequence (5′–3′)
PS	GAA(AT)AAC(CT)GGA(ACT)TGGAATAC(CT)G
Px2	ACCCAC(CT)TTA(ACT)GGGAAGAA
P1T31	TCTATAAAATCTCCTGGACCGA
P1T32	GCACCGACGTGAGCCTGATT
P1T33	CCTGTTCCACGGCACGATCT
P1T51	GGTTCACAATCAGGCTCACG
P1T52	CGATGTACGCCTGCTGGTCT
P1T53	GTACGCCTGCTGGTCTTTCA
P2T31	GACATTTTCCACCATTGATGCC
P2T32	CAAACGCAGCTGGAACAAGACG
P2T33	CGCTGCACCTCATCGTTAAGC
P2T51	TTGCCGTTTTCTCGTCCTTCA
P2T52	CGAAAAATCAGACGCTGGAAGAC
P2T53	TCACCCTGATGGCGATGGAT
PX1e[a]	AGG*GAATTC*ATGAACACCTTCGACAAACA
PX2e[a]	GGC*AAGCTT*GCGCTTATTTTAAATTATCAGG

[a] The restricted sites are in italics

fad were amplified using nested PCR primers (P1T3n and P1T5n) and the DNA Walking Speedup Premix Kit (Seegene, Korea), respectively. To obtain the upstream and downstream sequences of *fad*, genome walking was carried out again using nested PCR primers (P2T3n and P2T5n). These primers were designed based on the sequence from the first genome walking experiment. Genome walking was performed according to the user's manual (Seegene, Korea).

Sequence analysis

The analysis of the DNA sequence was done using the DNAman software package (Version 5.2.2, Lynnon BioSoft, Canada). Homology searching was performed using the BLAST algorithms against various databases in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST). The promoter region was predicted by a Web program (http://fruitfly.org:9005/seq_tools/promoter.htm). The physicochemical parameters of the protein were determined using the Prosite and ProDom databases at ExPASY (http://us.expasy.org). The amino acid sequences of FADases from different bacteria were aligned using the Clustal X software package (Thompson et al. 1997) and a phylogenetic tree was constructed using MEGA version 4.0 (Tamura et al. 2007).

Expression and purification of FADase in *E. coli* BL21

The open reading frame (ORF) sequence of FADase was amplified using primers PX1e and PX2e containing the restricted site *Eco*RI and *Hin*dIII, respectively. These two primers were designed based on *fad* sequence, and the amplified ORF was inserted into vector pET28a. The recombinant vector pET28a/*fad* was transformed into *E. coli* BL21 following the user's protocol (Novagen, Germany). Under the 0.2 mM IPTG at 30 °C incubation overnight, FADase was expressed as a soluble protein in *E. coli* BL21. Cells were harvested by centrifugation at 8,000 *g* for 10 min. Cell pellets were washed and suspended with 50 mM Tris–HCl buffer (pH 7.0) for three times. The cells were broken apart through ultrasonication and the cell-free extract was obtained after the removal of cell debris by centrifugation at 12,000 *g* for 30 min at 4 °C. The crude extracts were stored at −80 °C for purification.

All steps involved in purifying FADase were performed at 4 °C. The supernatant containing the recombinant FADase was loaded onto a chelating sepharose column filled with Ni-NTA agarose (Amersham, Sweden) equilibrated with 50 mM Tris-HCl buffer (pH 7.0) containing 20 mM imidazole and eluted with an imidazole gradient (20 mM to 150 mM). The enzyme-active fractions were collected, dialyzed, and applied to a Resource Q column (Amersham, Sweden) equilibrated with 20 mM Tris-HCl buffer (pH 7.0). The bound protein was eluted with the 20 mM Tris–HCl buffer (pH 7.0) containing 1 M sodium chloride. The FADase-active fractions were pooled and dialyzed against using the 50 mM Tris–HCl buffer (pH 7.0) containing 0.5 M $(NH_4)_2SO_4$. The dialyzed enzyme was applied to a HiLoad 16/60 Superdex column (Amersham, Sweden) equilibrated with 50 mM Tris–HCl buffer (pH 7.0) containing 0.15 M sodium chloride and eluted with the same buffer. The purified FADase was resolved by denaturing SDS-PAGE and stored at −80 °C for later use.

Enzyme analysis

The concentration of the FADase protein was determined by the method of Bradford (1976) using bovine serum album as a standard. The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 4.0) and 2 mM of ferulic acid. The purified FADase was added to start the reaction and the mixture was incubated at 28 °C and sampled at 30s intervals. The amount of ferulic acid and 4-vinyl guaiacol in the reaction mixture was determined using HPLC (Li et al. 2008). One unit of enzymatic activity was defined as the enzyme required for transforming 1 μmol of ferulic acid per minute.

Effects of pH, temperature, and metal ions on enzyme activity

The optimum temperature for enzyme activity was investigated at pH 7 by incubating the purified FADase and ferulic acid (2 mM) at 4–70 °C for 20 min. The effect of pH on FADase activity was studied by incubating purified FADase and ferulic acid (2 mM) with the Britton Robinson universal buffer system (Yang et al. 2005) at pH 2–10 at the optimal temperature determined above. The effects of metal ions on enzyme activity were studied by pre-incubated purified FADase with various metal ions dissolved in 50 mM sodium phosphate buffer (pH 4.0) at 28 °C for 20 min. FADase activities were assayed according to the above method.

Results

Cloning and analysis of the *fad* gene from *Enterobacter* sp. Px6-4

A 233-bp fragment was amplified using degenerate primers PS and Px2. Three pairs of nested PCR primers were designed according to the amplified conserved sequence of FADase, and PCR products of 1,252- and 846-bp were amplified from the 5′ and 3′ terminal of the conserved FADase sequence, respectively. In addition, the PCR

products of 872- and 663-bp were obtained from the 5′ and 3′ terminal of the FADase sequence through the second genome walking. The resulting contiguous sequence containing the full-length encoding gene of FADase as well as adjacent genes was assembled from five PCR fragments with the DNAman software package. Based on results from BLAST searches, the resulting assembly contained two complete putative genes, *fad* (encoding for FADase) and *padR* (encoding for transcriptional regulator, PadR), as well as partial sequences of two other genes (short-chain dehydrogenase and aminotransferase) (Fig. 1).

The putative encoding gene of FADase consists of 507-bp nucleotides, a putative promoter from a position at −43 bp to −92 bp upstream of the start codon ATG, and a ribosome binding site (5-AAAGGAG-3) at −8 bp to −14 bp upstream of the start codon. The FADase gene *fad* and its corresponding amino acid residues were shown in Fig. 2. The FADase gene encoded a polypeptide of 168 amino acid residues. The calculated molecular mass and the p*I* of the putative protein was 19.2 kDa and 4.99, respectively. Moreover, a possible rho-independent terminator (inverted repeats) was also found in the downstream region of the termination codon (TAA). A putative gene encoding PadR was identified at −100 bp to −981 bp from the upstream of the *fad* gene (Fig. 3) and this gene encoded a polypeptide of 303 amino acid residues. The calculated molecular mass and the p*I* of the putative PadR protein was 33.9 kDa and 6.09, respectively. Moreover, a partial sequence for a short-chain dehydrogenase was identified at −1,014 bp to −1,746 bp upstream of the *fad* gene, and a partial sequence for an aminotransferase was also identified at 831 bp to 1,680 bp downstream of the *fad* gene (Fig. 1). The complete nucleotide sequence containing *fad* and adjacent genes has been submitted to GenBank (accession number EU853825).

Comparison of FADase with other ferulate (phenolic acid) decarboxylases

The primary sequences of FADase and other decarboxylases from different bacteria were aligned using the DNAman software package (Fig. 4). The deduced peptide sequence of FADase from *Enterobacter* sp. Px6-4 showed 51.9%, 52.8%, 52.4%, 51.2%, 52.5%, and 53.3% sequence identity, respectively, to the decarboxylases from *Bacillus amyloliquefaciens* (ABS75504), *Bacillus coagulans* (EAY44624), *Bacillus licheniformis* (AAU24866), *B. pumilus* (CAA59273), *Bacillus subtilis* (AAC46254), and *L. plantarum* (CAD65735). However, the decarboxylase of *B. amyloliquefaciens* (ABS75504) showed a high degree of amino acid (aa) identity (81.5–91.9%) with those from other *Bacillus* spp. and 71% sequence identity with that from *L. plantarum*. The sequence alignment of FADase from *Enterobacter* sp. Px6-4 with homologous decarboxylases from different bacteria (Fig. 4) showed that the main differences between these proteins were located in the N- and C-terminal regions, and several highly conserved signature sequences (e.g., NGWEYE, DYRIH and SWTEPTGTDV) were identified at the middle section. These conserved sequences may play key roles in the catalytic process of these decarboxylases.

Phylogenetic analysis

A phylogenetic tree (Fig. 5) was constructed based on the deduced peptide sequences of decarboxylases from *Enterobacter* sp. Px6-4 and other bacteria by the MEGA 4.0 program package. From this tree (Fig. 5), these decarboxylases were found to separate into two clades (clade A and clade B). Clade A consisted of two well-supported minor clades, with those from *Bacillus* spp. clustered into one minor clade and the decarboxylases from *Enterococcus faecium* (EAN10835), *Pediococcus pentosaceus* (CAC16794), *L. plantarum* (CAD65735 and AAC45282), and *Lactobacillus brevis* (ABJ63379) forming another minor clade. Similarly, clade B also consisted of two well-supported minor clades, the decarboxylases from *Saccharopolyspora erythraea* (CAM05375) and *Methylobacterium* sp. 4-46 (ACA19578) were clustered into a minor clade and those from *Enterobacter* sp. Px6-4, *Enterobacter sakazakii* (ABU78196), and *Erwinia carotovora* (CAG72990) formed another minor clade.

Expression and purification of FADase

The ORF sequence of FADase was subcloned into the expression vector pET28a and introduced into *E. coli* BL21. Under the 0.2 mM IPTG incubation overnight at 30 °C, FADase was expressed as a soluble protein in *E. coli* BL21.

The recombinant FADase was isolated and the purification process was summarized in Table 2, including

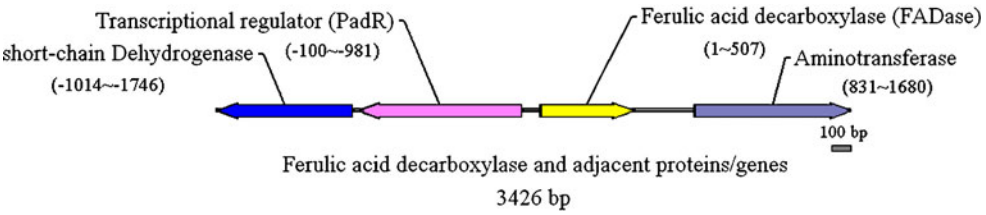

Fig. 1 Sketch of ferulic acid decarboxylase and adjacent proteins/genes. The *arrow* showed the transcription orientation and four ORFs

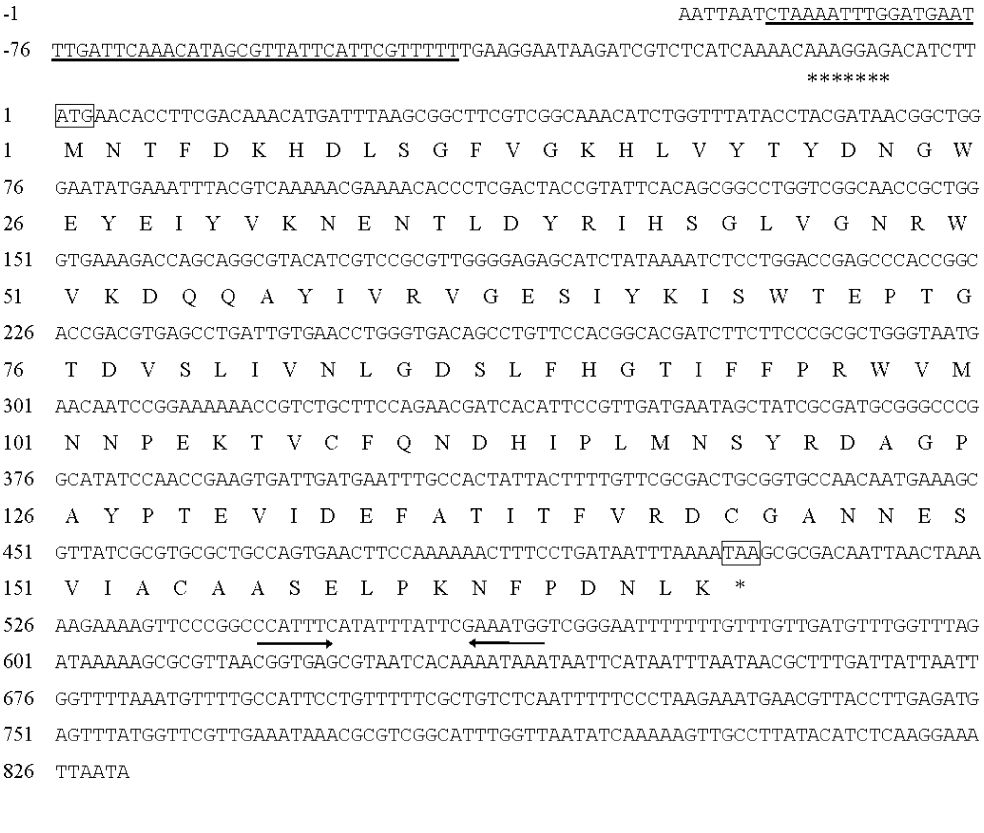

Fig. 2 Nucleotide sequence of the *fad* gene. *Numbers on the left* refer to the nucleotide positions. The number of nucleotides (*left*) starts at the predicted translational initiation ATG codon (the A in ATG being +1). The predicted amino acid sequence for the *fad* gene is given *below* the DNA sequence. A putative promoter is *underlined*, and a putative ribosome-binding site region was marked with *multiple asterisks*. The initiation and stop codons are enclosed in *boxes*. *Opposing arrows* indicate inverted repeats of a possible rho-independent terminator

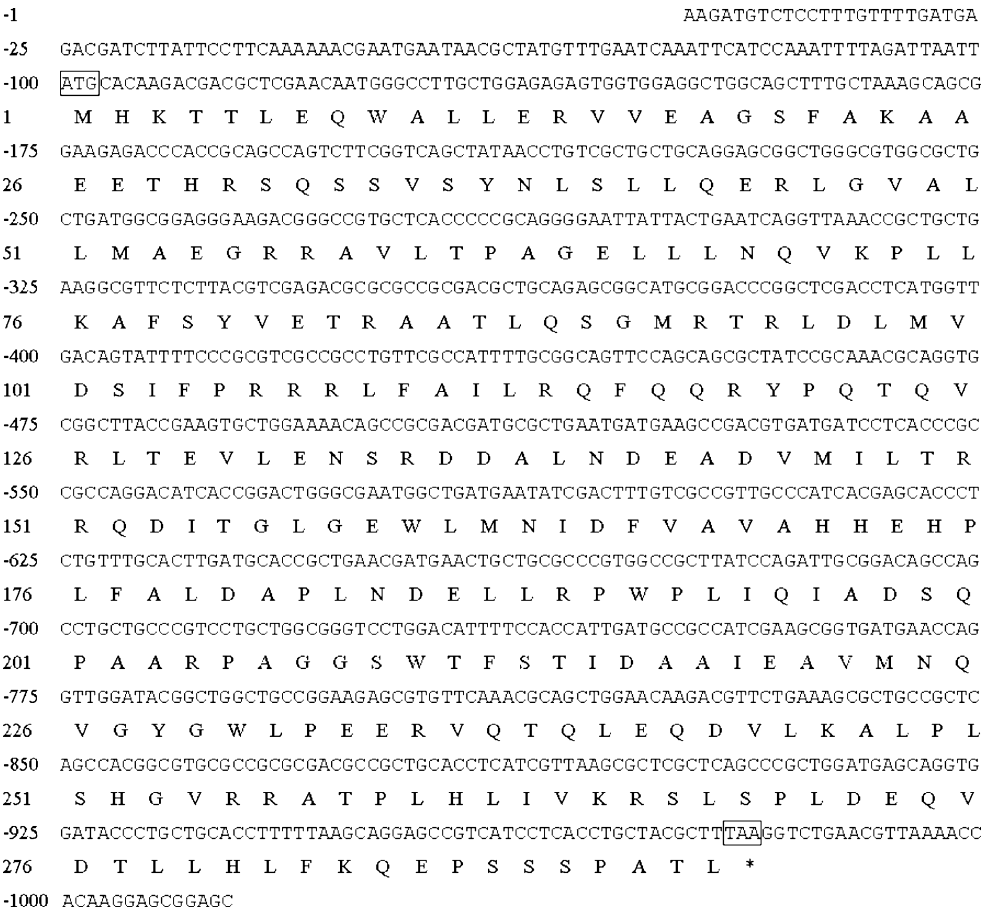

Fig. 3 Nucleotide sequence of the putative transcriptional regulator gene *padR*. *Numbers on the left* refer to the nucleotide positions (the number of nucleotides starts at the predicted translational initiation ATG codon of *fad* gene). The predicted amino acid sequence for the *padR* gene is given *below* the DNA sequence

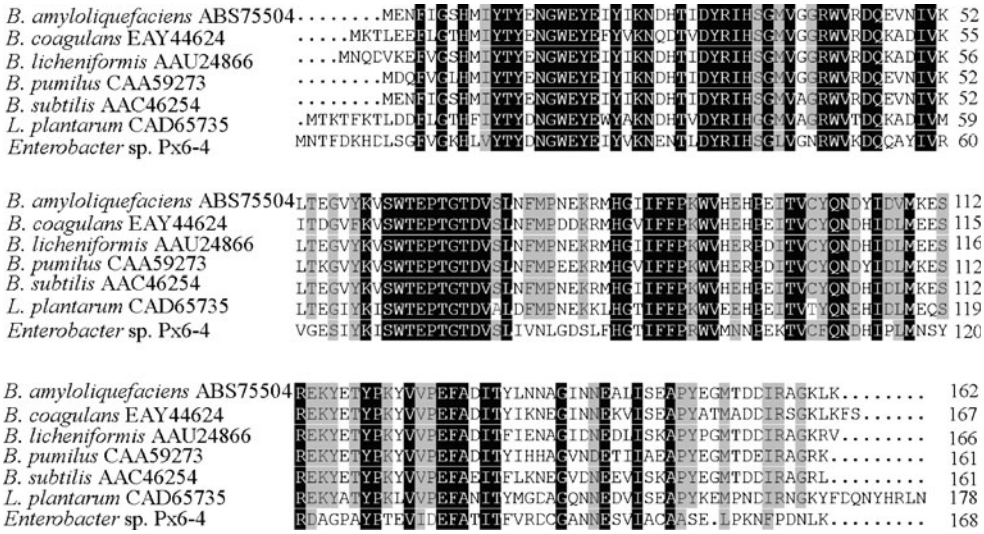

Fig. 4 Multiple sequence alignment of the deduced amino acid sequence of FADase with other phenolic/*p*-coumaric acid decarboxylases from different bacteria. The GenBank accession numbers of FADases selected were shown *after* the names of the organisms. The conserved residues are *shaded*, with the highest homology level ranging from dark black (100% identity) and gray (75% identity)

purification by chromatography using Ni-chelating column, Resource Q column, and HiLoad 16/60 Superdex column (Table 2; Fig. 6). The overall enzyme yield was 1.36%, with a 1,411.33-fold enrichment. The specific activity of the purified enzyme for the decarboxylation of ferulic acid was 410 U mg^{-1}. The molecular weight of the purified FADase was estimated to be 23 kDa by SDS-PAGE (Fig. 6).

Effect of pH, temperature, and metal ions on FADase activity

The purified FADase had activity under a broad range of temperatures from 4 to 70 °C and showed the highest activity for ferulic acid transformation at 28 °C. The enzyme also exhibited activity under a broad pH range from 4.0 to 10 with the highest activity at pH 4.0, and the

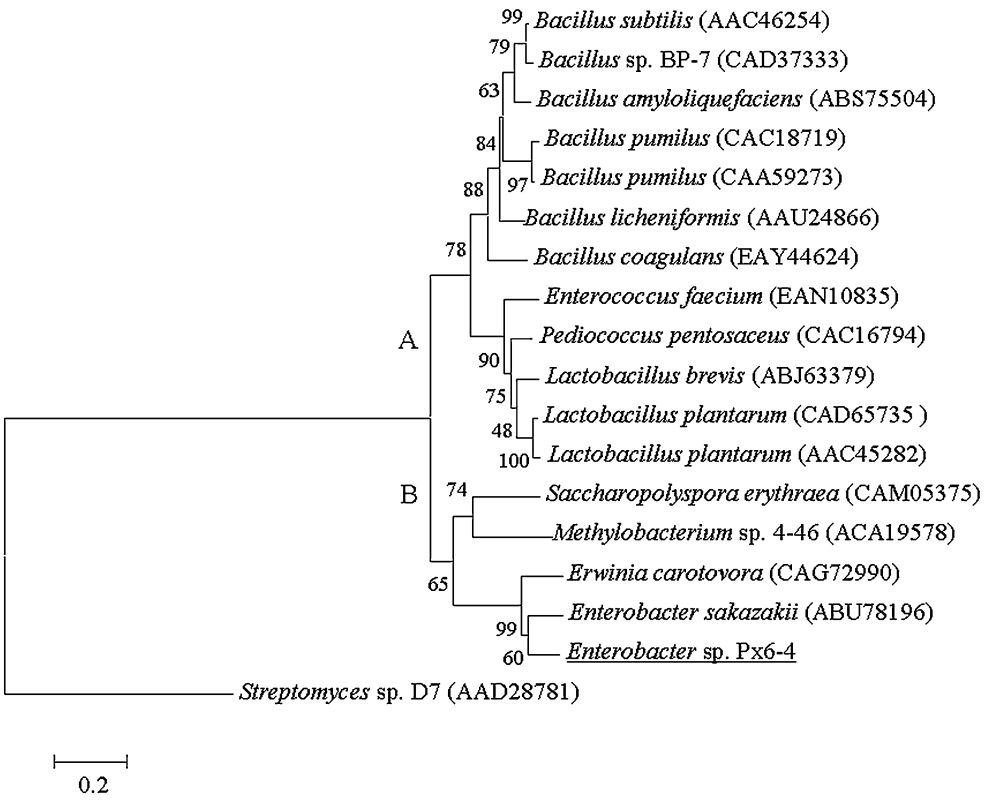

Fig. 5 Phylogenetic tree of FADases from different bacteria. The tree was constructed with MEGA version 4.0 (Tamura et al. 2007) program packages. Neighbor-joining method was used with Poisson correction for multiple-amino-acid substitution and with 1,000 random bootstrap replicates. The decarboxylase of *Streptomyces* sp. D7 was used as an outgroup in order to root the tree. The bacterium *Enterobacter* sp. Px6-4 was *underlined*

Table 2 Purification procedure of the recombinant FADase from *E. coli* BL21

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Crude extract	623.26	181.06	0.29	1	100
Ni-NTA	299.51	158.73	0.53	1.82	87.66
Resource Q	0.98	16.76	17.08	58.78	9.25
HiLoad 16/60	0.0059	2.45	410.0	1,411.33	1.36

activity was lost completely when it was pre-exposed to extreme pH environments (pH 3.0).

The effects of metal ions on the FADase activity were determined. The enzyme activity was strongly inhibited by Cu^{2+} (99%) and Hg^{2+} (99.5%). In contrast, the metal chelator (EDTA) had only a weak effect on the decarboxylase activity (5.6% inhibition, 0.5 mM). The decarboxylase activity of FADase was inhibited moderately by Fe^{2+} (17.4%) and enhanced by 0.5 mM Sr^{2+} (14.3%).

Biotransformation of ferulic acid into 4-vinyl guaiacol

The purified FADase was added to the reaction mixture consisting of 50 mM sodium phosphate buffer (pH 4.0) and 2 mM ferulic acid, the ferulic acid was converted to 4-vinyl guaiacol effectively (Fig. 7), and the transformation rate of ferulic acid reached 63.9% after a reaction for 2.5 min (the theoretical value was 77.3%).

Discussion

In our recent report, 4-vinyl guaiacol and vanillin as well as other related chemicals were identified in the culture broth of *Enterobacter* sp. Px6-4 using ferulic acid as the sole carbon source (Li et al. 2008). In this study, a gene encoding the FADase protein was isolated and expressed in *E. coli* BL21, and the biochemical properties of FADase were characterized. The *fad* gene showed approximately 50% sequence identity to other bacterial decarboxylases from *Bacillus* spp. and *L. plantarum*, suggesting that the FADase of *Enterobacter* sp. Px6-4 is a member of the bacterial phenolic acid decarboxylase (PAD) family. The calculated molecular mass of FADase was 19.2 kDa and it was expressed in *E. coli* as a protein of 23 kDa (SDS-PAGE). Moreover, no secretion signal sequence was found in the predicted amino acid sequence of FADase from *Enterobacter* sp. Px6-4, suggesting that, like other decarboxylases, the cloned enzyme is intracellular (Zago et al. 1995). FADase shared high sequence identities to decarboxylases from other bacteria with the highest amino acid sequence variability found in a region adjacent to the C-terminal portion of the proteins (Fig. 4). This region has been proposed to be involved in enzyme-substrate specificity (Barthelmebs et al. 2001).

A putative gene *padR* encoding for PadR was identified from *Enterobacter* sp. Px6-4 at the upstream of the *fad* gene (Fig. 3). The role of *padR* gene in *L. plantarum* had been investigated, the deletion of the *padR* gene leads to the constitutive over-expression of the PAD enzyme, and the specific activity of the mutant strain crude extract was about 60-fold higher than the highest specific activity previously measured in the *p*-coumaric acid-induced wild-

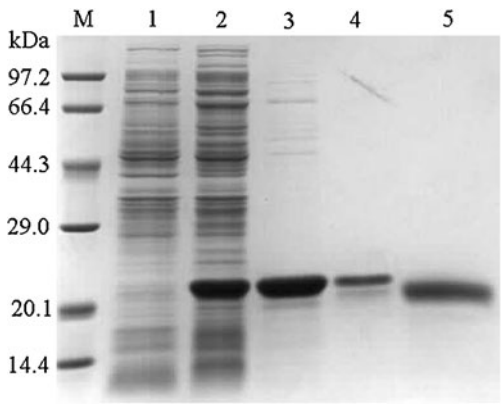

Fig. 6 SDS-PAGE analysis of the gene *fad* expressed in *E. coli* BL21. *M*, marker of proteins; *lane 1*, overall proteins of control strain without *fad* gene induced by the IPTG; *lane 2*, overall proteins induced by the IPTG; *lane 3*, proteins eluted from the Ni-chelating column; *lane 4*: proteins eluted from the Resource Q column; *lane 5*, the purified enzyme from the HiLoad 16/60 Superdex column

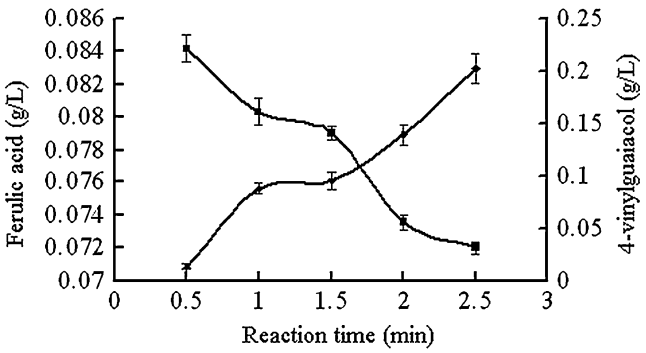

Fig. 7 Biotransformation of ferulic acid into 4-vinyl guaiacol by FADase. Changes in the levels of ferulic acid and 4-vinyl guaiacol in the reaction mixture were quantified by HPLC. *Filled square*, ferulic acid; *filled diamond*, 4-vinyl guaiacol

type strain of *L. plantarum* (Gury et al. 2004). Therefore, the *padR* gene likely encodes the transcriptional repressor of *padA* gene expression and the *padA* gene is under the control of a very tightly inducible promoter. Furthermore, Barthelmebs et al. (2000) also reported that *padA* gene expression in *P. pentosaceus* is regulated by bicistronic transcription of a *padR* gene located downstream of *padA*. The *padA* and *padR* genes reside in an operon in *P. pentosaceus* (Barthelmebs et al. 2000). However, the *padR* gene is divergently oriented in relation to the *padA* gene in *L. plantarum*, with only 115 bp between the two genes. Similarly, the *padR* gene is also divergently oriented in relation to the *fad* gene in *Enterobacter* sp. Px6-4, with only 99 bp between the two genes (Figs. 1 and 3). This divergent orientation was also observed for the putative *padR* gene of *B. pumilus* (Barthelmebs et al. 2000). Therefore, the putative PadR protein may also act as a transcriptional repressor of the FADase operon upstream of the *fad* gene in *Enterobacter* sp. Px6-4. However, the putative PadR protein in *Enterobacter* sp. Px6-4 showed low sequence identities to those in *L. plantarum* and *B. pumilus*: the gene *padR* in *Enterobacter* sp. Px6-4 encodes a putative polypeptide of 293 aa, but the lengths were only 185 aa and 181 aa in *L. plantarum* (Cavin et al. 1998) and *B. pumilus* (Zago et al. 1995), respectively.

Several PADs have been purified and characterized from different bacteria, such as *P. fluorescens* (Huang et al. 1994), *B. pumilus* (Degrassi et al. 1995), and *L. plantarum* (Cavin et al. 1997a, 1997b). The first PAD was purified from *P. fluorescens* UI-670, which appears to be constitutive in nature because enzyme production was not affected by the inclusion of ferulic acid in the culture medium (Huang et al. 1994). The purified enzyme displayed an optimum temperature range of 27 to 30 °C and exhibited an optimum pH of 7.3 in potassium phosphate buffer, but it had only 15% and 8% of enzyme activity at 15 and 47 °C, respectively (Huang et al. 1994). The PAD isolated from *B. pumilus* showed optimal enzyme activity at 37 °C and pH 5.5, and its enzyme activity rapidly declines at 37 °C and is lost completely at temperatures above 42 °C (Degrassi et al. 1995). Moreover, the highest activity of PAD from *Bacillus* sp. BP-7 was found at pH 5.5 and 40 °C, exhibiting 50% of the maximum activity at 24 °C. At pH 5.0 and 8.0, the cloned enzyme displayed 84% and 14% of its maximum activity, respectively, and remained stable over a pH range from 5.0 to 9.0 when incubated for 1 h at room temperature (Prim et al. 2003). In contrast to the above decarboxylases, the optimal temperature of FADase isolated here was 28 °C and there was about 80% relative activity remaining between 4 and 70 °C. The optimal pH of FADase was 4.0, and from pH 4.0 to pH 9.0 the relative activity of FADase was well above 80%. Therefore, the FADase obtained from *Enterobacter* sp. Px6-4 was more stable in a wide range of conditions, especially in high-temperature and acidic environments, which suggested that FADase might be more valuable in industrial applications.

We also determined the effects of several agents on FADase activities. The enzyme activity was strongly inhibited by Cu^{2+} and Hg^{2+}, similar to those observed for the ferulate decarboxylase from *P. fluorescens* (Huang et al. 1994). The FADase activity was enhanced by 0.5 mM Sr^{2+}, while the other ions assayed did not significantly affect the FADase activity. Moreover, the FADase activity was weakly affected by the presence of EDTA, suggesting that Ca^{2+} is not necessary for FADase activity, similar to previous reports (Degrassi et al. 1995).

FADase and other decarboxylases from different bacteria were likely evolved from a common ancestor and separated into different clades (Fig. 5). Therefore, these decarboxylases may share a common catalytic mechanism in different microorganisms. Moreover, the high degree of sequence identity (Fig. 4) also suggests that FADase may be involved in a generalized detoxifying process developed by bacterial cells to convert the toxic compounds derived from plant cell wall degradation into less toxic forms (Barthelmebs et al. 2000; Edlin et al. 1998). The proposed mechanism for the decarboxylation catalyzed by PAD involves the initial enzymatic isomerization of ferulic acid to a quinoid intermediate, which subsequently decarboxylates spontaneously (Huang et al. 1993). However, the detailed catalytic mechanism of PAD/FADase has not yet been fully elucidated. The study on the crystal structure of FADase in complex with its substrate (ferulic acid) may help clarify the catalytic mechanism of FADase.

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