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ORIGINAL PAPER

Gene cloning, expression, and characterization of phenolic acid decarboxylase from *Lactobacillus brevis* RM84

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Abstract Phenolic acid decarboxylase (PAD) catalyzes the synthesis of vinyl phenols from hydroxycinnamic acids. The gene encoding PAD from *Lactobacillus brevis* was cloned and expressed as a fusion protein in *Escherichia coli*. The recombinant PAD enzyme is a heat-labile enzyme that functions optimally at 22°C and pH 6.0. The purified enzyme did not show thermostability at temperatures above 22°C. *L. brevis* PAD is able to decarboxylate exclusively the hydroxycinnamic acids, such as *p*-coumaric, caffeic, and ferulic acids, with K_m values of 0.98, 0.96, and 0.78 mM, respectively. The substrate specificity exhibited by *L. brevis* PAD is similar to the PAD isolated from *Bacillus subtilis* and *B. pumilus*, but different from that of *L. plantarum* and *Pediococcus pentosaceus*. As the C-terminal region may be involved in determining PAD substrate specificity and catalytic capacity, amino acid differences among these proteins could explain the differences observed. The substrate specificity shown by *L. brevis* PAD shows promise for the synthesis of high-added value products from plant wastes.

Keywords *p*-Coumaric acid · Ferulic acid · Phenolic acid decarboxylase · Phenolic acids · Vinyl phenol

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Introduction

Phenolic acids are abundant naturally occurring molecules that contribute to the rigidity of plants by linking the complex lignin polymer to the hemicelluloses and cellulose of plant cell walls. Hydroxycinnamic acids, such as ferulic, sinapic, caffeic, and *p*-coumaric acids, are found both covalently attached to the plant cell wall and as soluble forms in the cytoplasm. Esters and amides are the most frequently reported types of conjugates, whereas glycosides occur only rarely [14]. Enzymes capable of cleaving hydroxycinnamate esters, namely, the cinnamoyl ester hydrolases, have been isolated from a large number of microorganisms [29], and although they exhibit different substrate specificities, they are specific for the hydrolysis of hydroxycinnamoyl esters. These enzymes release ferulic and/or *p*-coumaric acid from plant cell walls which, in their free form, become substrates of phenolic acid decarboxylase (PAD) enzymes, which convert the former compounds into their vinyl phenol derivatives [5, 7, 24]. PAD enzymes catalyze the conversion of ferulic or *p*-coumaric acids into the corresponding volatile compounds 4-vinyl guaiacol (3-methoxy-4-hydroxystyrene) or 4-vinyl phenol (4-hydroxystyrene) (Fig. 1), which are considered to be the precursors of vanillin (4-hydroxy-3-methoxybenzaldehyde) production [19]. This biosynthetic pathway has attracted a growing interest due to the potential for application in the industrial-scale production of natural vanillin, the most commonly used flavoring in foods, beverages, perfumes, and pharmaceuticals, by biotransformation of plant waste [31]. Vinyl guaiacol has a 40-fold higher economic value than ferulic acid, and it can be biotransformed further to acetovanillone, ethylguaiacol, and vanillin [22, 25]. As a styrene-type molecule, vinyl guaiacol can be polymerized, and the resultant oligomer [poly(3-methoxy-4-hydroxystyrene)] has

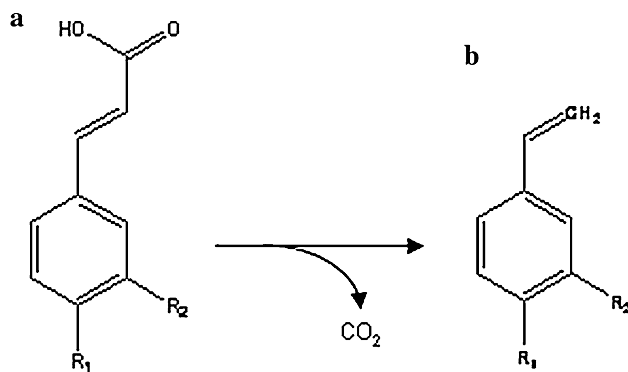

Fig. 1 Schematic representation of the reaction catalyzed by the phenolic acid decarboxylase (PAD) enzyme. **a** *p*-Coumaric acid and **b** vinyl phenol when R_1 represents (OH) and R_2 represents (H). **a** Caffeic acid and (**b**) vinyl catechol when R_1 represents (OH) and R_2 represents (OH). **a** Ferulic acid and **b** vinyl guaiacol when R_1 represents (OH) and R_2 represents (OCH_3)

been found to be easily biodegradable [16]. In addition, the action of PAD enzymes on the hydroxycinnamic acids *p*-coumaric acid and ferulic acid results in the production of 4-vinyl guaiacol or 4-vinyl phenol, both of which are considered to be food additives and have been approved as flavoring agents by regulatory agencies [17].

In previous studies, bacterial PAD, from *Lactobacillus plantarum* [5, 24], *Pediococcus pentosaceus* [1], *Bacillus subtilis* [7], and *B. pumilus* [30], respectively, were expressed in *Escherichia coli* and their activities on *p*-coumaric acid, ferulic acid, and caffeic acid compared. Although these four enzymes were found to have 61% amino acid sequence identity, they exhibit different activities for phenolic acid metabolism. To elucidate the domain(s) responsible for these differences, chimeric PAD proteins were constructed and expressed in *E. coli* by exchanging their individual carboxy-terminal portions [2]. Analysis of the activities of these chimeric enzymes suggested that the C-terminal region may be involved in determining PAD substrate specificity and catalytic capacity [2]. In addition to *L. plantarum* and *P. pentosaceus*, other lactic acid bacteria, such as *L. brevis* strains, have also been reported to be able to decarboxylate hydroxycinnamic acids [3, 4, 8–10, 13, 28]. However, the biochemical and molecular properties of *L. brevis* PAD have not yet been characterized, although a protein annotated as PAD (LVIS_0213) has been identified from an analysis of the available complete genome sequence of *L. brevis* ATCC 367 (NC_008497).

The identification and characterization of new PAD enzymes would expand the spectrum of enzymes useful for generating value-added products from lignin degradation. As such, it is an important area of research and of particular interest to the biotechnological industry on a whole, but especially to those industries requiring enzymes highly actives on ferulic acid. The results of studies on previously identified PAD enzymes suggest that their C-terminal region is involved in determining substrate specificity and catalytic capacity. As substrate specificity and catalytic activity depend on the sequence of the specific PAD enzyme, the aim of the study reported here was to biochemically characterize LVIS_0213 protein from *L. brevis* in order to expand the range of enzymes useful for generating value-added products via lignin degradation.

Materials and methods

Bacterial strains, plasmids, enzymes, and fine chemicals

Lactobacillus brevis RM84 strain, isolated from a wine sample, was obtained from the bacterial culture collection of the Instituto de Fermentaciones Industriales–CSIC. This strain was taxonomically identified by PCR amplification and DNA sequencing of the 16S rDNA. *Escherichia coli* DH5α and *E. coli* JM109 (DE3) were purchased from Promega (Madison, WI). *E. coli* DH5α was used for all DNA manipulations, and *E. coli* JM109 (DE3) was used for expression in the pURI3 vector [12]. The *L. brevis* strain was grown in MRS medium at 30°C without shaking, and the *E. coli* strains were cultured in Luria–Bertani (LB) medium at 37°C and shaking at 200 rpm. When required, ampicillin was added to the medium at a concentration of 100 μg/ml. Chromosomal DNA, plasmid purification, and transformation of *E. coli* were carried out as described elsewhere [26]. The phenolic acids assayed were purchased from Sigma (St. Louis, MO) (*p*-coumaric, caffeic, ferulic, and sinapic acids), Aldrich (Steinheim, Germany) (*m*-coumaric, cinnamic, and gentisic acids), Fluka (Steinheim, Germany) (*o*-coumaric, syringic, and gallic acids) or Merck (Damstadt, Germany) (benzoic and salicylic acids).

Construction of expression plasmid

The expression vector pURI3 had been constructed in our laboratory at an earlier date [12] to avoid the enzyme restriction and ligation steps during the cloning procedure. This vector was created using the pT7-7 vector as template, and it contains a N-terminal His-tag that allows convenient purification of the native protein directly from crude cell extracts. The gene encoding for a putative PAD (LVIS_0213 in the *L. brevis* ATCC 367 strain) from *L. brevis* RM84 was PCR-amplified by Hot-start Turbo *Pfu* DNA polymerase by using the primers 369 (5′-*CATCATGGTGACGATGACGATAAGatg*actaaagaattcaaaacat) and 370 (5′-*AAGCTTAGTTAGCTATTATGCGTA*ttatttcgtgattcgcttgtaatta) (the nucleotides pairing the expression

vector sequence are indicated in italics, and the nucleotides pairing the LVIS_0213 gene sequence are written in lowercase letters). The 0.5-kb purified PCR product was inserted into the pURI3 vector using a restriction enzyme- and ligation-free cloning strategy described previously [12]. The expression vector pURI3 was constructed based on the commercial expression vector pT7-7 (USB), but it contains a leader sequence with a six-histidine affinity tag. *E. coli* DH5α cells were transformed, recombinant plasmids were isolated, and those containing the correct insert were identified by restriction enzyme analysis, verified by DNA sequencing, and then transformed into *E. coli* JM109(DE3) cells for expression.

Purification of the His_6-tagged *L. brevis* PAD

Cells carrying the recombinant plasmid, pURI3-0213, were grown at 37°C in LB media containing ampicillin (100 μg/ml) until an optical density at 600 nm of 0.4 was reached and then induced by adding isopropyl-*β*-D-thio-galactoside (IPTG; 0.4 mM final concentration). Following induction, the cells were grown at 22°C for 20 h and collected by centrifugation (8,000 *g*, 15 min, 4°C). The cells were resuspended in 20 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl, and crude extracts were prepared by French press lysis of the cell suspension (three times at 1,100 psi). The insoluble fraction of the lysate was removed by centrifugation at 47,000 *g* for 30 min at 4°C, and the supernatant was filtered through a 0.45-μm filter and then applied to a His-Trap-FF crude chelating affinity column (GE Healthcare, Uppsala, Sweden) equilibrated with a buffer of 20 mM Tris-HCl, pH 8.0, 100 mM NaCl containing 10 mM imidazole to improve the interaction specificity in the affinity chromatography step. The bound enzyme was eluted by applying a stepwise gradient of imidazole concentration, from 20 mM Tris-HCl, pH 8.0, 100 mM NaCl containing 10 mM imidazole to the same buffer but containing 500 mM imidazole. Fractions containing the His_6-tagged protein were pooled and dialysed overnight at 4°C against 10 mM Tris-HCl, pH 8.0, containing 10 mM NaCl in a membrane (3,500 cut-off). The purity of the enzyme was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) in a Tris-glycine buffer.

SDS–PAGE and determination of protein concentration

Samples were analyzed by SDS–PAGE under reducing conditions according to Laemmli [20]. Protein bands were visualized by Coomassie blue staining. The gels were calibrated using molecular weight markers. Protein concentration was measured according to the method of Bradford using a protein assay kit purchased from Bio-Rad Laboratories (Munich, Germany) with bovine serum albumin as the standard.

Enzymatic activity determination and assay of kinetics of the *L. brevis* PAD

Phenolic acid decarboxylase activity was assayed in a 1-ml total volume of reaction solution containing substrate at 4 mM in 25 mM phosphate buffer (pH 6.5) following incubation at 30°C for 20 min. The assay time was under the linear range of the enzyme reaction. The reaction was terminated by two extractions with ethyl acetate. The reaction products extracted with ethyl acetate were analyzed by high-performance liquid chromatography (HPLC). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of 4-vinyl phenol per minute. Substrate and enzyme blanks were also prepared in which the enzyme or substrate was incubated with only the buffer. Kinetic analyses were performed under conditions of pH 6.5 and 30°C for 20 min in 25 mM phosphate buffer containing the substrate (*p*-coumaric, caffeic, or ferulic acid) at different concentrations ranging from 0.125 to 48 mM. Values of K_m were calculated by fitting the initial rates as a function of substrate concentration to the Michaelis–Menten equation.

Optimum temperature and optimum pH of the *L. brevis* PAD

Activities of *L. brevis* PAD were measured at 4, 16, 22, 30, 37, 52, and 70°C to determine the optimal reaction temperature. The optimum pH of the recombinant decarboxylase was determined by measuring activity at various pH values between 3 and 10. Citric acid–sodium citrate buffer (100 mM) was used for measurements at pH 3–5, phosphate buffer (100 mM) for those at pH 6–7, Tris-HCl buffer (100 mM) for those at pH 7–8, and 100 mM glycine-KOH buffer those at pH 9–10. The optimal temperature was assayed by incubating the purified PAD in 25 mM phosphate buffer (pH 6.5) at different temperatures (4–90°C) for 20 min using *p*-coumaric acid (4 mM) as the substrate.

Dependence of the *L. brevis* PAD stability on temperature

For the temperature stability measurements, *L. brevis* PAD was suspended in 25 mM phosphate buffer, pH 6.5, and incubated at 22, 30, and 37°C for 1, 2, 3, 5, 12, 24, and 48 h. The residual activity was measured after each incubation.

Effect of additives on activity of the *L. brevis* PAD

To test the effect of metals and ions on the stability of *L. brevis* PAD, the enzyme was suspended in 25 mM phosphate buffer, pH 6.5, then incubated with a 1 mM concentration of one of several metals or other additives [$MgCl_2$, KCl, $CaCl_2$, $HgCl_2$, SDS, Triton-X-100, urea, ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), and *β*-mercaptoethanol, respectively], and 4 mM *p*-coumaric acid in 25 mM phosphate buffer, pH 6.5, at 30°C for 20 min. The activity was calculated as relative to the sample containing no additives.

HPLC analysis of the *L. brevis* PAD activity on phenolic acids

The activity of the *L. brevis* PAD on several phenolic acids was assayed by incubating the enzyme for 4 h at 30°C in the presence of each phenolic acid at a final concentration of 1 mM. As a control, phosphate buffer containing the phenolic acid was incubated under the same conditions. The reaction products were extracted twice with ethyl acetate (Lab-Scan, Sowinskiego, Poland) and analyzed by HPLC–diode-array detection (DAD). A Thermo (Thermo Electron Corp, Waltham, MA) chromatograph equipped with a P400 SpectraSystem pump, AS3000 autosampler, and a UV6000LP photodiode array detector were used. A gradient of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 78:20:2, v/v/v) was applied to a reversed-phase Nova-pack C_{18} (internal diameter 25 cm × 4.0 mm) 4.6-μm particle size cartridge at room temperature as follows: 0–55 min, 80% B linear, 1.1 ml/min; 55–57 min, 90% B linear, 1.2 ml/min; 57–70 min, 90% B isocratic, 1.2 ml/min; 70–80 min, 95% B linear, 1.2 ml/min; 80–90 min, 100% linear, 1.2 ml/min; 100–120 min, washing 1.0 ml/min, and re-equilibration of the column under initial gradient conditions. Detection was performed by scanning from 220 to 380 nm. Samples were injected in duplicate onto the cartridge after being filtered through a 0.45-μm PVDF filter.The identification of degradation compounds was carried out by comparing the retention times and spectral data of each peak with those of standards from commercial suppliers or by LC–DAD/electrospray ionization mass spectrometry.

Results and discussion

Sequence analysis of *L. brevis* LVIS_0213

Lactobacillus brevis strains could be isolated from the spontaneous fermentation of vegetables, where phenolic compounds are abundant [18, 23, 27]. These strains are adapted to grow in phenol-containing media; therefore, we assumed that enzymatic abilities to degrade these compounds are present. Based on the results of the *L. brevis* complete genome sequence project [21], a DNA fragment (LVIS_0213) had been annotated as a putative PAD. Analysis of the deduced product of *L. brevis* LVIS_0213 indicated that the PAD is a 20.7-kDa protein of 178 amino acid residues, with a p*I* of 4.6. BLAST database searches of the translated *L. brevis* PAD sequence identified high-scoring similarities with PAD sequences that catalyze the decarboxylation of various hydroxycinnamic acids. The predicted sequence of the *L. brevis* PAD protein was aligned with PAD from lactic acid bacteria and from species of the *Bacillus* genera (Fig. 2). The highest sequence identity was found between *L. brevis* and the PAD from lactic acid bacteria, with an 89% sequence identity to *P. pentosaceus* PAD and an 85–88% sequence identity to *L. plantarum* PAD. In addition, PAD from *L. brevis* showed a 71 and 67% identity to PAD from *B. subtilis* and *B. pumilus*, respectively. As shown in Fig. 2, the identity is highest in the central portion of the enzymes, which contains several highly conserved regions. The C-terminal region of PAD may be involved in enzyme substrate specificity [2]. As the C-terminal region from the *L. brevis* PAD showed significant sequence differences, it may have a substrate specificity that is different from that of previously characterized PAD enzymes.

Enzymatic activity of *L. brevis* LVIS_0213

To confirm that the LVIS_0213 gene from *L. brevis* encodes a functional PAD, we expressed this gene from *L. brevis* RM84 in *E. coli* under the control of the T7 RNA polymerase-inducible Φ10 promoter. The cell extracts were analyzed by SDS–PAGE to detect the presence of hyperproduced proteins. Control cells containing the pURI3 vector plasmid alone did not show expression, whereas the expression of an additional 20-kDa protein was apparent in cells harboring pURI3-0213 (Fig. 3). As the poly-His tag-modified protein was cloned, *L. brevis* PAD could be purified on a His-Trap-FF chelating column and eluted with a stepwise gradient of imidazole. Highly purified PAD protein was obtained from cells transformed with pURI3-0213 (Fig. 3). The eluted protein was dialyzed to eliminate the imidazole and checked for its enzymatic activity as a PAD on *p*-coumaric acid.

The biochemical characterization of pure *L. brevis* PAD was performed using a standard assay with *p*-coumaric acid as substrate. Figure 4a shows that *L. brevis* PAD had an optimal activity at 22°C and a high activity between 16 and 30°C. At 37°C, PAD activity sharply decreased to only 12% of the maximal activity. The effect of pH is shown in Fig. 4b. The PAD enzyme had an optimal activity at a pH

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LVI   MTKEFKTLDDFLGTHFIYTYDNGWEYEWYAKNDHTVDYRIHGGMVAGRWVTDQAANIVML  60
RM8   ************************************************************  60
LPC   ***T*************************************************K*D****  60
LP3   ***T*************************************************K*D****  60
PPE   *E*T**********************************************K**E*H*A**  60
BSU   -------MEN*I*S*M****E******I*I*****I*****S********R**EV***K*  53
BPU   -------M*Q*I*L*M****E******I*I*****I*****S***G****R**EV***K*  53

LVI   VPGIYKVAWTEPTGTDVALDFVPNEKKLNGTIFFPKWVEEYPEITVTYQNEHIDLMEESR 120
RM8   ****************************************H******************* 120
LPC   TE****IS*************M******H***********H****************Q** 120
LP3   TE****IS*************M******H***********H****************Q** 120
PPE   TE**************************************H******F************ 120
BSU   TE*V***S*********S*N*M****RMH*I*******H*H*****C***D*****K*** 113
BPU   TK*V**IS*********S*N*M*E**RMH*V*******H*R*D***C***DC****K*** 113

LVI   EKYDTYPKLVVPEFANITYMGDAGQDNEDVISEAPYAGMPDDIRAGKYFDSNYKRIKK   178
RM8   ********************************************************T*   178
LPC   ***A*****************E-**N**********KE**N***N***LIKTTIV---   174
LP3   ***A*********************N**********KE**N***N***F*Q**H*LN*   178
PPE   ***E***********T***********DE**A****E**T**********E*****N*   178
BSU   ***E****Y******E**FLKNE*V***E***K***E**T******R-----------   160
BPU   ***E****Y******D***IHH**VND*TI*A****E*LT*E****R-----------   160
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Fig. 2 Comparison of PAD protein sequences from *Lactobacillus brevis* ATCC 367 (*LVI*) (accession ABJ63379.1), *L. brevis* RM84 (*RM8*), *L. plantarum* LPCHL2 (*LPC*) (accession AAC45282), *L. plantarum* WCFS1 (*LP3*) (accession CAD65735), *Pediococcus pentosaceus* ATCC 25745 (*PPE*) (accession ABJ67585.1), *Bacillus subtilis* strain 168 (*BSU*) (accession CAB15445.1), and *B. pumilus* ATCC 15884 (*BPU*) (accession AJ278683). *Asterisks* represent amino acid identity, *dashes* indicate *gaps* introduced to maximize similarities

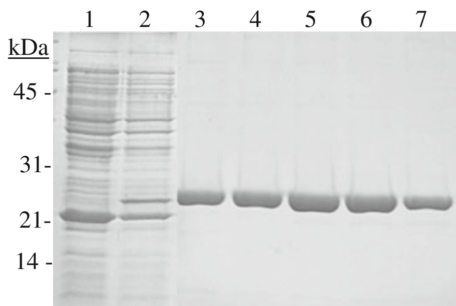

Fig. 3 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the expression and purification of PAD protein from *L. brevis* RM84 cloned into the pURI3 vector. SDS–PAGE analysis of soluble cells extracts of isopropyl-β-D-thio-galactoside (IPTG)-induced cultures. *Lanes*: *1 Escherichia coli* JM109 (pURI3), *2 E. coli* JM109 (pURI3-0213),*3–7* fractions eluted from the His-Trap-FF crude chelating affinity column. The poly-acrylamide gels were stained with Coomassie blue. The positions of molecular mass markers (Bio-Rad, Hercules, CA) are indicated on the *left*

around 6.0 and was also highly active between pH 5.5 and 7.0.

The obtained results indicated that, similar to the PAD isolated from *L. plantarum*, *B. pumilus*, and *Cladosporium phlei*, the *L. brevis* PAD is a heat-labile enzyme [11, 15, 24]. Figure 5 shows that the activity of *L. brevis* PAD decreased markedly following incubation at ≥22°C. This enzyme is even more heat-labile than the equivalent *L. plantarum* protein, as following a 24-h incubation at 22°C the *L. brevis* PAD showed only 50% activity in contrast to the 90% activity exhibited by its *L. plantarum* counterpart [24].

Figure 6 shows the results of *L. brevis* PAD activity in the presence of various additives added to the reaction mixture at a final concentration of 1 mM. Compared to the activity of the enzyme incubated in 25 mM phosphate buffer, pH 6.5, only, the activity of the PAD was increased by the addition of KCl and urea (relative activity 119–123%), not significantly affected by $MgCl_2$ (relative activity 104%), partially inhibited by $CaCl_2$, EDTA, and DMSO (relative activity 49–65%), and greatly inhibited by Triton-X-100, SDS, β-mercaptoethanol, and $HgCl_2$ (relative activity 35–7%).

Substrate specificity of *L. brevis* LVIS_0213

The expression of the *L. plantarum*, *P. pentosaceus*, *B. subtilis*, and *B. pumilus* PAD in *E. coli* reveals that *p*-coumaric acid was the main substrate for each PAD. Ferulic acid was metabolized by the *L. plantarum*, and *P. pentosaceus* PAD with an activity about 500-fold lower than that for *p*-coumaric acid [2]. However, the *B. subtilis* and *B. pumilus* PAD displayed similar activities on either substrate. The kinetic parameters of *L. brevis* PAD was investigated using *p*-coumaric, caffeic, and ferulic acids as substrates. The *L brevis* PAD was found to have K_m values of 0.98, 0.96, and 0.78 mM and V_{max} values of 598, 609, and 464 μmol/h/mg for *p*-coumaric, caffeic, and ferulic acids, respectively. These values indicate that the kinetic parameters were similar for the three hydroxycinnamic acids assayed, which is behavior similar to that of the *B. subtilis* and *B. pumilus* PAD, but markedly different from the PAD previously characterized from the lactic acid bacteria *L. plantarum* and *P. pentosaceus*.

Chimeric enzyme construction has been shown to be useful for combining properties not typically found in any naturally occurring enzyme. The construction of chimeric

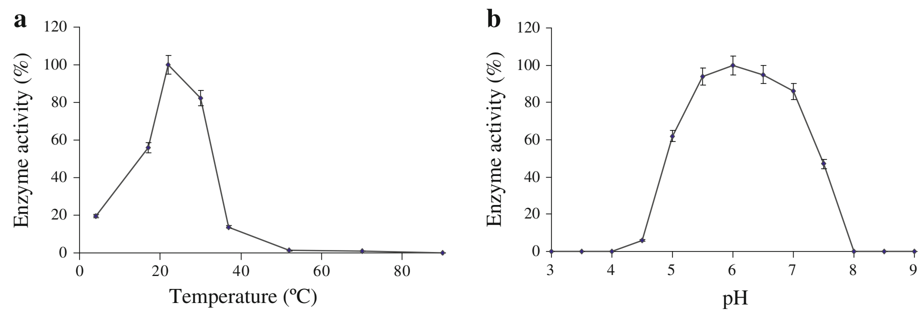

Fig. 4 Effects of temperature and pH on the activity of the *L. brevis* PAD. **a** Relative activity of *L. brevis* PAD versus temperature. Enzyme activity was assayed at pH 6.5. **b** Relative activity of *L. brevis* PAD versus pH. Enzyme activity was assayed at 30°C. The observed maximum activity was defined as 100%

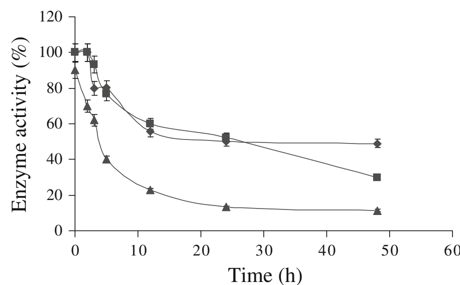

Fig. 5 Effects of temperature on the stability of the *L. brevis* PAD. Residual activities of *L. brevis* PAD after preincubation at 22°C (*filled diamond*), 30°C (*filled square*), or 37°C (*filled triangle*) in phosphate buffer, pH 6.5, for 1, 2, 3, 5, 12, 24, and 48 h. The observed maximum activity was defined as 100%

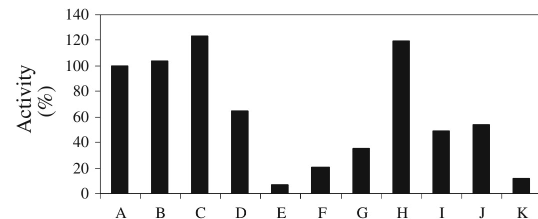

Fig. 6 Relative activity of the *L. brevis* PAD following incubation with 1 mM concentrations of different additives (*A*–*K*) and 4 mM *p*-coumaric acid in 1 ml of 25 mM phosphate buffer, pH 6.5, at 30°C for 20 min. The additives assayed were: $MgCl_2$ (*B*), KCl (*C*), $CaCl_2$ (*D*), $HgCl_2$ (*E*), SDS (*F*), Triton-X-100 (*G*), urea (*H*), ethylenediaminetetraacetic acid (*I*), dimethyl sulfoxide (*J*), and *β*-mercaptoethanol (*K*). The activity of the enzyme incubated in 25 mM phosphate buffer, pH 6.5, only at 30°C for 20 min was defined as 100% (*A*)

PAD based on different combinations of homologous C-terminal regions of PAD results in the formation of enzymatically active chimeric species that display catalytic activities different from those of the native PAD [2]. Although the chimeric PAD displayed enzymatic characteristics different from those of the active enzymes, chimeric proteins from *L. plantarum*, *L. brevis*, and *B. subtilis* still displayed a greater activity on *p*-coumaric acid than on ferulic and caffeic acids. However, the chimeric PAD protein constructed from the *B. pumilus* N-terminal PAD region and the *L. plantarum* C-terminal PAD region decarboxylated ferulic acid with a relative activity tenfold higher than that for *p*-coumaric acid. This chimeric protein differs from the chimeric protein between *B. subtilis* and *L. plantarum* in only few amino acids, with five of these conserved in the native PAD from *L. plantarum*, *P. pentosaceus*, and *B. subtilis* (Arg-39, Glu-55, Asn-77, His-94, Asp-96, and His-105). Most of these residues are also conserved in the *L. brevis* PAD, except for Glu-55, which is a proline residue. The implications of this residue change with respect to the different catalytic activity of *L. brevis* PAD on ferulic acid need to be further investigated.

In order to determine the substrate specificity of *L. brevis* PAD, we assayed seven cinnamic and five benzoic acids as putative substrates for the enzyme. Among the cinnamic acids assayed (*p*-coumaric, *o*-coumaric, *m*-coumaric, cinnamic, caffeic, ferulic, and sinapic acids), only the *p*-coumaric, caffeic, and ferulic acids were decarboxylated by *L. brevis* PAD (Fig. 7), which is similar to results obtained on previously characterized PAD enzymes [6, 24]. The decarboxylation of the *p*-coumaric, caffeic, and ferulic acids results in the production of their vinyl derivatives, namely, vinyl phenol, vinyl catechol, and vinyl guaiacol, respectively (Fig. 7). These decarboxylations have also been previously described from cultures of *L. brevis* strains growing in the presence of these hydroxycinnamic acids [3, 4, 8–10, 12, 28]. As *L. brevis* PAD was available, five benzoic acids (benzoic, syringic, gallic, salicylic, and gentisic acids) were incubated in the presence of this enzyme. None of these benzoic acids were decarboxylated by the *L. brevis* enzyme. In summary, and as reported previously for *L. plantarum* PAD, it would appear that phenolic acids without a hydroxyl group *para* to the unsaturated side chain and with a substituent other than -H, -OH, or $-OCH_3$ *meta* to the unsaturated side chain are not metabolized [6]. Knowledge of the PAD catalytic mechanism of decarboxylation will open up novel biotechnological

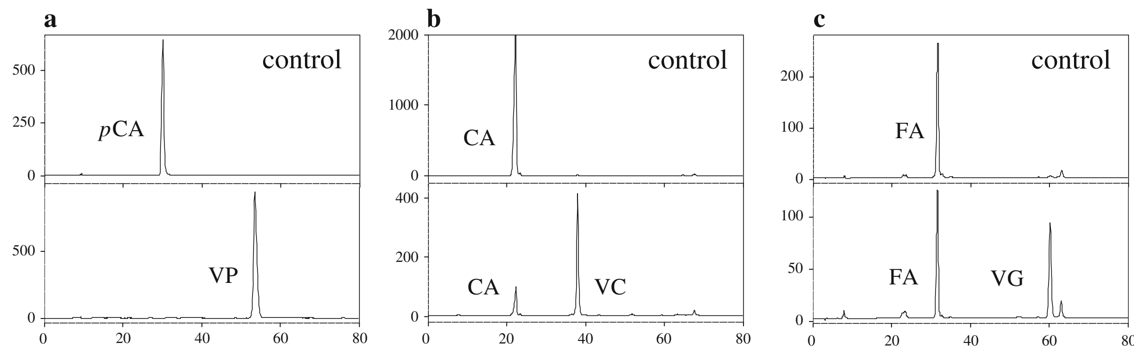

Fig. 7 High-performance liquid chromatography chromatograms of the action of purified *L. brevis* PAD on different hydroxycinnamic acids as substrate. The PAD enzyme from *L. brevis* RM84 was incubated for 2 h in the presence of *p*-coumaric acid (**a**), caffeic acid (**b**) or ferulic acid (**c**). Chromatograms without protein (*control*) are also shown. The chromatograms were recorded at 280 nm. *pCA p*-Coumaric acid, *CA* caffeic acid, *FA* ferulic acid, *VP* vinyl phenol, *VC* vinyl catechol, *VG* vinyl guaiacol

possibilities for the design of novel enzymes with broader specificities.

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