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

José María Landete • Héctor Rodríguez • José Antonio Curiel • Blanca de las Rivas • José Miguel Mancheño • Rosario Muñoz

J. M. Landete • H. Rodríguez • J. A. Curiel • B. de las Rivas • R. Muñoz
Departamento de Microbiología, Instituto de Fermentaciones Industriales, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain.

J. M. Mancheño
Grupo de Cristalografía Molecular y Biología Estructural, Instituto de Química-Física "Rocasolano", CSIC, Serrano 119, 28006 Madrid, Spain

R. Muñoz (correspondence to)
e-mail: rmunoz@ifi.csic.es

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 functioned optimally at a temperature of 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 *p*–coumaric, caffeic and ferulic, with K_m values of 0.98, 0.96, and 0.78 mM, respectively. The substrate specificity exhibited by *L. brevis* PAD was similar to the PAD from *Bacillus subtilis* and *B. pumilus*, but different from the *L. plantarum* and *Pediococcus pentosaceus* PAD. As the C–terminal region might be involved in determining PAD substrate specificity and catalytic capacity, amino acid differences among these proteins could explain the differences observed. The substrate specificity showed by *L. brevis* PAD shows promise for the synthesis of high–added value products from plant wastes.

Keywords Ferulic acid • Phenolic acid decarboxylase • Phenolic acids • Vinyl phenol • *p*–Coumaric acid

Introduction

Phenolic acids are abundant molecules in nature 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 acid, 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 rarely occurs [14]. Enzymes capable of cleaving hydroxycinnamates esters, cinnamoyl ester hydrolases, have been isolated from a large number of microorganims [29]. Although these enzymes exhibit different substrate specificities they are specific for the hydrolysis of hydroxycinnamoyl esters. These enzymes released 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 these compounds into their vinyl phenol derivatives [5, 7, 24]. These 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), considered as precursors of vanillin (4–hydroxy–3–methoxybenzaldehyde) production [19]. This has led to a growing interest in the production of natural vanillin, the most commonly used flavour in foods, beverages, perfumes or pharmaceuticals by biotransformation of plant wastes [31]. Vinyl guaiacol is priced around 40 times more 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; the resultant oligomer [poly(3–methoxy–4–hydroxystyrene)] was found to be easily biodegradable [16]. In addition, the activity of PAD enzymes on hydroxycinnamic acids, *p*–coumaric and ferulic acids, resulted in the production of 4–vinyl

66 guaiacol or 4–vinyl phenol, both compounds are considered to be food additives and are
67 approved as flavouring agents by regulatory agencies [17].
68 Four bacterial PAD from *Lactobacillus plantarum* [5, 24], *Pediococcus pentosaceus*
69 [1], *Bacillus subtilis* [7], and *Bacillus pumilus* [30] were expressed in *Escherichia coli*, and
70 their activities on *p*–coumaric, ferulic, and caffeic acid were compared. Although these four
71 enzymes displayed 61% amino acid sequence identity, they exhibit different activities for
72 phenolic acid metabolism. To elucidate the domain(s) responsible for these differences,
73 chimeric PAD proteins were constructed and expressed in *E. coli* by exchanging their
74 individual carboxy–terminal portions [2]. Analysis of the chimeric enzyme activities
75 suggest that the C–terminal region may be involved in determining PAD substrate
76 specificity and catalytic capacity [2]. Among lactic acid bacteria, in addition to *L.*
77 *plantarum* and *P. pentosaceus*, *L. brevis* strains have been reported to be able to
78 decarboxylate hydroxycinnamic acids [3, 4, 8, 9, 10, 13, 28]. However, biochemical and
79 molecular properties of *L. brevis* PAD have not been characterized yet. From the analysis
80 of the available complete genome sequence of *L. brevis* ATCC 367 (NC_008497), a protein
81 annotated as PAD (LVIS_0213) could be identified.
82 The knowledge of new PAD enzymes to broad the range of enzymes useful for
83 generating value–added products from lignin degradation is an important issue for the
84 biotechnological industry, especially those showing high activity on ferulic acid. In the
85 already known PAD enzymes, it has been described that their C–terminal region is involved
86 in determining substrate specificity and catalytic capacity. As substrate specificity and
87 catalytic activity depends on the PAD sequence, in this work we decided to biochemically
88 characterize LVIS_0213 protein from *L. brevis* in order to expand the range of enzymes
89 useful for generating value–added products from 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 at the Instituto de Fermentaciones Industriales–CSIC. This strain was taxonomically identified by PCR amplification and DNA sequencing of their 16S rDNA. *Escherichia coli* DH5α and *E. coli* JM109 (DE3) were purchased from Promega (Madison, WI, USA). *E. coli* DH5α was used for all DNA manipulations. *E. coli* JM109 (DE3) was used for expression in pURI3 vector [12]. *L. brevis* strain was grown in MRS medium at 30 ºC without shaking. *E. coli* strains were cultured in Luria–Bertani (LB) medium at 37 ºC and 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, USA) (*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

Expression vector pURI3 was previously constructed in our laboratory [12] to avoid the enzyme restriction and ligation steps during the cloning. The pURI3 vector was created using the pT7–7 vector as template and 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 phenolic acid decarboxylase or PAD (LVIS_0213 in the *L. brevis* ATCC 367 strain) from *Lactobacillus 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 by using a restriction enzyme–and ligation–free cloning strategy described previously [12]. Expression vector pURI3 was constructed based on the commercial expression vector pT7–7 (USB) but containing 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* phenolic acid decarboxylase

Cells carrying the recombinant plasmid, pURI3–0213, were grown at 37 °C in Luria–Bertani media containing ampicillin (100 μg/ml) until an optical density at 600 nm of 0.4 was reached and then induced by adding IPTG (0.4 mM final concentration). After induction,

the cells were grown at 22 °C for 20 h and collected by centrifugation (8,000*g*, 15 min, 4 °C). Cells were resuspended in 20 mM Tris–HCl, pH 8.0, containing 0.1 M NaCl. Crude extracts were prepared by French press lysis of the cell suspension (three times at 1100 psi). The insoluble fraction of the lysate was removed by centrifugation at 47,000*g* for 30 min at 4 °C. The supernatant was filtered through a 0.45 µm filter and applied to a His–Trap–FF crude chelating affinity column (GEHealthcare, Uppsala, Sweden) equilibrated with 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 cuttoff). The purity of the enzyme was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) in 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 (München, Germany) with bovine serum albumin as standard.

Enzymatic activity determination and assay of kinetics of the *L. brevis* phenolic acid decarboxylase

PAD activity was assayed in a total volume of 1 ml of reaction solution containing substrate at 4 mM in 25 mM phosphate buffer (pH 6.5) and incubating at 30 °C for 20 min. The assay time was under the linear range of enzyme reaction. The reaction was terminated by extracting twice with ethyl acetate. The reaction products extracted with ethyl acetate were analyzed by 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 the buffer. Kinetic analysis were performed under conditions of pH 6.5 and 30 °C for 20 min in 25 mM phosphate buffer containing 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* phenolic acid decarboxylase

Activities of *L. brevis* PAD were measured at 4, 16, 22, 30, 37, 52, and 70 °C to determine the optimal 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 pH 3–5, phosphate buffer (100 mM) for pH 6–7, Tris–HCl buffer (100 mM) for pH 7–8, and 100 mM glycine–KOH buffer for pH 9 and 10. The optimal temperature was assayed by incubating the purified PDC in 25 mM

183 phosphate buffer (pH 6.5) at different temperatures (4–90 °C) for 20 min using *p*–coumaric
184 acid (4 mM) as substrate.
185
186 Dependence of the *L. brevis* phenolic acid decarboxylase stability on temperature
187
188 For temperature stability measurements, *L. brevis* PAD was suspended in 25 mM phosphate
189 buffer, pH 6.5, and incubated at 22, 30, and 37 °C for 1, 2, 3, 5, 12, 24, and 48 h. After
190 incubation, the residual activity was measured.
191
192 Effect of additives on activity of the *L. brevis* phenolic acid decarboxylase
193
194 To test the effect of metals and ions on the stability of *L. brevis* PAD, the enzyme
195 suspended in 25 mM phosphate buffer, pH 6.5, was incubated with 1 mM concentration of
196 one of several metals or other additives ($MgCl_2$, KCl, $CaCl_2$, $HgCl_2$, SDS, Triton–X–100,
197 Urea, EDTA, DMSO, and β–mercaptoethanol). The samples were incubated with a 1mM
198 concentration of the additive and 4 mM *p*–coumaric acid in 25 mM phosphate buffer, pH
199 6.5, at 30 °C for 20 min. The activity was calculated as relative to the sample containing no
200 additives.
201
202 HPLC analysis of the *L. brevis* phenolic acid decarboxylase activity on phenolic acids
203
204 The activity of *L. brevis* PAD on several phenolic acids was assayed by incubating the
205 enzyme during 4 h at 30 °C in presence of each phenolic acid at 1 mM final concentration.
206 As control, phosphate buffer containing the phenolic acid was incubated in the same

conditions. The reaction products were extracted twice with ethyl acetate (Lab–Scan, Sowinskiego, Poland) and analyzed hy HPLC–DAD. A Thermo (Thermo Electron Corporation, Waltham, Massachussetts, USA) chromatograph equipped with a P400 SpectraSystem pump, and 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} (25 cm x 4.0 mm i.d.) 4.6μm 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 reequilibration of the column under initial gradient conditions. Detection was performed by scanning from 220 to 380 nm. Samples were injected i**n 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/ESI–MS.

Results and discussion

Sequence analysis of *L. brevis* LVIS_0213

Lactobacillus brevis strains could be isolated from spontaneous fermentation of vegetables where phenolic compounds are abundant [18, 23, 27]. These strains are adapted to grow in phenol–containing media; therefore it could be assumed that enzymatic abilities to degrade these compounds are present. Since the *L. brevis* complete genome sequence project has

been conducted [21], a DNA fragment (LVIS_0213) was annotated as a putative phenolic acid decarboxylase (PAD). Analysis of the deduced product of *L. brevis* LVIS_0213 indicated that PAD is a protein of 178 amino acid residues, 20.7 kDa, and p*I* of 4.6. BLAST databases searches of the translated *L. brevis* PAD sequence identified high–scoring similarities with phenolic acid decarboxylase sequences that catalyze the decarboxylation of 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. The alignment of the PAD protein sequences is shown in Figure 2. The highest sequence identity was shown between *L. brevis* and PAD from lactic acid bacteria, 89% to *P. pentosaceus* PAD and 85–88% 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 Figure 2, the identity is highest in the central portion of the enzymes, which contains several highly conserved regions. It has been shown that the C–terminal region of PAD could be involved in enzyme substrate specificity [2]. As the C–terminal region from the *L. brevis* PAD showed significant sequence differences, it could exhibit different substrate specificity from the previously characterized PAD enzymes.

Enzymatic activity of *L. brevis* LVIS_0213

To confirm that 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.** Cell extracts were used to detect the presence of hyperproduced proteins by SDS–PAGE analysis. Control cells containing the pURI3 vector plasmid alone did not show expression, whereas expression of additional 20 kDa protein was apparent

with cells harbouring pURI3–0213 (Figure 3). As the poly–His tag–modified protein was cloned, *L. brevis* PAD was purified on a His–Trap–FF chelating column and eluted with a stepwise gradient of imidazole. Highly purified PAD protein was obtained from pURI3–0213 (Figure 3). The eluted protein was dialyzed to eliminate the imidazole and checked for its PAD activity on *p*–coumaric acid.

As reported, the biochemical characterization of pure *L. brevis* PAD was performed by using a standard assay with *p*–coumaric acid as substrate. Figure 4a shows that *L. brevis* PAD presented an optimal activity at 22 °C, being high active among 16 and 30 °C. At 37 °C PAD activity sharply decreases to only 12% of the maximal activity. The effect of pH is depicted in Figure 4b, which shows an optimal pH around 6.0, being also active among pH 5–5–7.0.

The obtained results indicated that, similar to *L. plantarum*, *B. pumilus*, and *Cladosporium phlei* PAD, *L. brevis* PAD is a heat–labile enzyme [11, 15, 24]. Figure 5 showed that the activity of *L. brevis* PAD was markedly decreased after incubation at a temperature of 22 °C or higher. This enzyme is even more heat–labile than the equivalent *L. plantarum* protein, as after incubation at 22 °C during 24 h *L.brevis* PAD showed only 50% of the 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 at 1 mM final concentration. Compared to the activity of the enzyme incubated in 25 mM phosphate buffer, pH 6.5, the activity of the PAD was increased by KCl and Urea (relative activity 119–123%), not significantly affected by $MgCl_2$ (relative activity104%), partially inhibited by $CaCl_2$, EDTA, and DMSO (relative activity 49–65%), and was greatly inhibited by Triton–X–100, SDS, β–mercaptoethanol, and $HgCl_2$ (relative activity 35–7%).

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280 Substrate specificity of *L. brevis* LVIS_0213

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282 Expression of the *L. plantarum*, *P. pentosaceus*, *B. subtilis*, and *B. pumilus* PAD in *E. coli*
283 reveals that *p*–coumaric acid was the main substrate for each PAD. Ferulic acid was
284 metabolized by *L. plantarum*, and *P. pentosaceus* PAD with an activity about 500–fold
285 lower than that for *p*–coumaric acid [2]. However, *B. subtilis* and *B. pumilus* PAD display
286 similar activities on either substrate. Kinetic parameters were investigated for *L. brevis*
287 PAD using *p*–coumaric, caffeic, and ferulic acids as substrates. *L brevis* PAD has K_m values
288 of 0.98, 0.96, and 0.78 mM and V_{max} values of 598, 609, and 464 µmol/h/mg for *p*–
289 coumaric, caffeic, and ferulic acids, respectively. These values indicated that kinetic
290 parameters were similar for the three hydroxycinnamic acids assayed, being this behaviour
291 similar to *B.subtilis* and *B. pumilus* PAD, but markedly different from PAD previously
292 characterized from the lactic acid bacteria *L. plantarum* and *P. pentosaceus*.
293 Chimeric enzyme construction was shown to be useful for combining properties not
294 typically found in any naturally occurring enzyme. Chimeric PAD constructed based on
295 different combinations of homologous C–terminal regions of PAD results in the formation
296 of enzymatically active chimeric species that display catalytic activities different from
297 those of the native PAD [2]. Although the chimeric PAD displayed enzymatically
298 characteristics different from those of the active enzymes, chimeric proteins from *L.*
299 *plantarum*, *L. brevis* and *B. subtilis* still displayed a greater activity on *p*–coumaric acid
300 than on ferulic and caffeic acids. However, the chimeric PAD protein constructed by the *B.*
301 *pumilus* N–terminal PAD region and *L. plantarum* C–terminal PAD region decarboxylated
302 ferulic acid with a relative activity 10–fold higher than that for *p*–coumaric acid. This

303 chimeric protein differs from the chimeric protein between *B. subtilis* and *L. plantarum* in
304 only few amino acids, being five of them conserved in the native PAD from *L. plantarum*,
305 *P. pentosaceus*, and *B. subtilis* (Arg–39, Glu–55, Asn–77, His–94, Asp–96, and His–105).
306 Most of these residues are also conserved in *L. brevis* PAD, except Glu–55 which is a
307 proline residue. The implications of this residue change in the different catalytic activity of
308 *L. brevis* PAD on ferulic acid need to be further investigated.
309 In order to know the substrate specificity of *L. brevis* PAD, seven cinnamic and five
310 benzoic acids were assayed as putative substrates for the enzyme. Among the cinnamic
311 acids assayed (*p*–coumaric, *o*–coumaric, *m*–coumaric, cinnamic, caffeic, ferulic, and sinapic
312 acid), only *p*–coumaric, caffeic, and ferulic acid were decarboxylated by *L. brevis* PAD
313 (Figure 7), similarly to the PAD enzymes previously characterized [6, 24]. The
314 decarboxylation of *p*–coumaric, caffeic, and ferulic acids originate their vinyl derivatives:
315 vinyl phenol, vinyl catechol, and vinyl guaiacol, respectively (Figure 7). These
316 decarboxylations have also been previously described from cultures of *L. brevis* strains
317 growing on the presence of these hydroxycinnamic acids [3, 4, 8, 9, 10, 12, 28]. As *L.*
318 *brevis* PAD was available, five benzoic acids (benzoic, syringic, gallic, salicylic, and
319 gentisic acids) were incubated in the presence of this enzyme. None of the benzoic acids
320 were decarboxylated by the *L. brevis* enzyme. In summary and as reported previously in *L.*
321 *plantarum* PAD, it seems that other phenolic acids without a hydroxyl group *para* to the
322 unsaturated side chain and with another substituent than –H, –OH, or $–OCH_3$ *meta* to the
323 unsaturated side chain were not metabolized [6]. The knowledge of the catalytic
324 mechanism of decarboxylation followed by PAD will open up novel biotechnological
325 possibilities for the design of novel enzymes with broadened specificities.
326

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424 Figure captions

425

426 Fig. 1. Schematic representation of the reaction catalyzed by the PAD enzyme. When R_1
427 (OH) and R_2 (H), the represented compounds are *p*-coumaric acid (a) and vinyl phenol (b).
428 When R_1 (OH) and R_2 (OH), caffeic acid (a) and vinyl catechol (b). When R_1 (OH) and R_2
429 (OCH_3), the compounds are ferulic acid (a), and vinyl guaiacol (b).

430

431 Fig. 2. Comparison of PAD protein sequences from *Lactobacillus brevis* ATCC 367 (LVI)
432 (accession ABJ63379.1), *L. brevis* RM84 (RM8), *L. plantarum* LPCHL2 (LPC) (accession
433 AAC45282), *L. plantarum* WCFS1 (LP3) (accession CAD65735), *Pediococcus*
434 *pentosaceus* ATCC 25745 (PPE) (accession ABJ67585.1), *Bacillus subtilis* strain 168
435 (BSU) (accession CAB15445.1), and *Bacillus pumilus* ATCC 15884 (BPU) (accession
436 AJ278683). Asterisks represent amino acid identity, and dashes represent gaps introduced
437 to maximize similarities.

438

439 Fig. 3. SDS–PAGE analysis of the expression and purification of PAD protein from *L.*
440 *brevis* RM84 cloned into pURI3 vector. SDS–PAGE analysis of soluble cells extracts of
441 IPTG–induced cultures. *E. coli* JM109 (pURI3) (1); *E. coli* JM109 (pURI3–0213) (2);
442 fractions eluated after His–Trap^{TM}–FF crude chelating affinity column (3–7). The
443 polyacrylamide gels were stained with Coomassie blue. The positions of molecular mass
444 markers (Bio–Rad) are indicated on the left.

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 ºC. The observed maximum activity was defined as 100%.

Fig. 5. Effects of temperature on the stability of *L. brevis* PAD. Residual activities of *L. brevis* PAD after preincubation at 22 ºC (♦), 30 ºC (■), or 37 ºC (▲) in phosphate buffer pH 6.5 during 1, 2, 3, 5, 12, 24, and 48 h. The observed maximum activity was defined as 100%.

Fig. 6. Relative activity of *L. brevis* PAD when was incubated with 1 mM concentrations of the different additives (A to K) and 4 mM *p*–coumaric acid in 1 mL of 25 mM phosphate buffer, pH 6.5, at 30 ºC 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), EDTA (I), DMSO (J), and β–mercaptoethanol (K). The activity of the enzyme incubated in 25 mM phosphate buffer, pH 6.5, at 30 ºC for 20 min was defined as 100% (A).

Fig. 7. HPLC chromatograms of purified *L. brevis* PAD on hydroxycinnamic acids. PAD enzyme from *L. brevis* RM84 was incubated for 2 h in the presence of *p*–coumaric (a), caffeic (b), or ferulic acids (c). Chromatograms without protein (controls) are also showed. The chromatograms were recorded at 280 nm. *p*CA, *p*–coumaric acid; CA, caffeic acid; FA, ferulic acid; VP, vinyl phenol; VC, vinyl catechol; VG, vinyl guaiacol.

Figure 1

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Figure 1

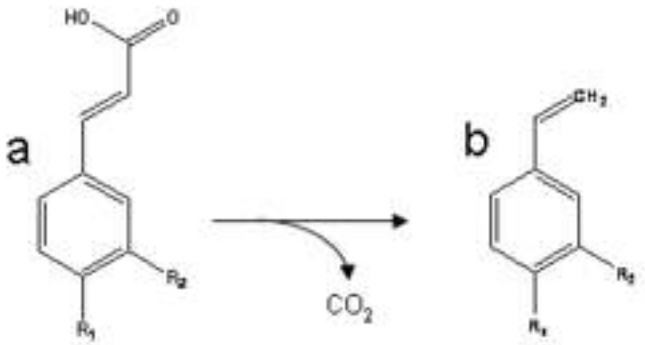

Figure 2

Figure 2

Figure 3

Figure 3

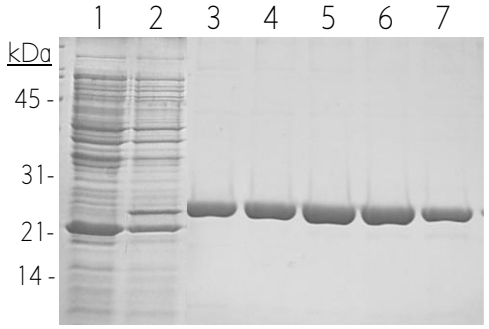

Figure 4

Figure 4

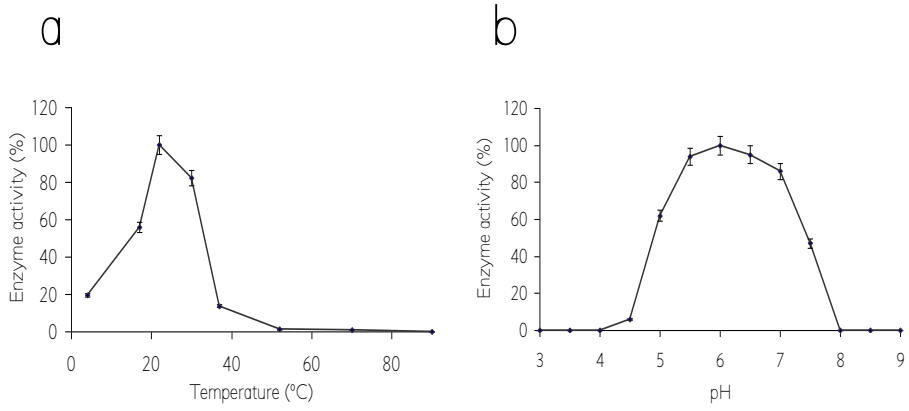

Figure 5

Figure 5

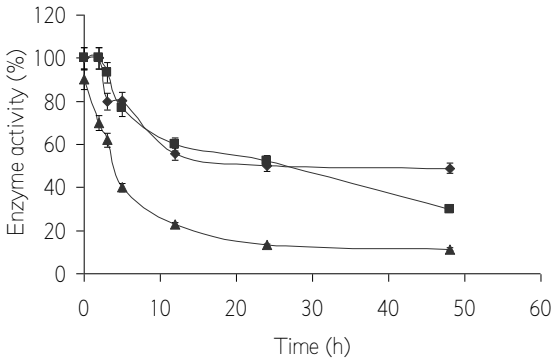

Figure 6

Figure 6

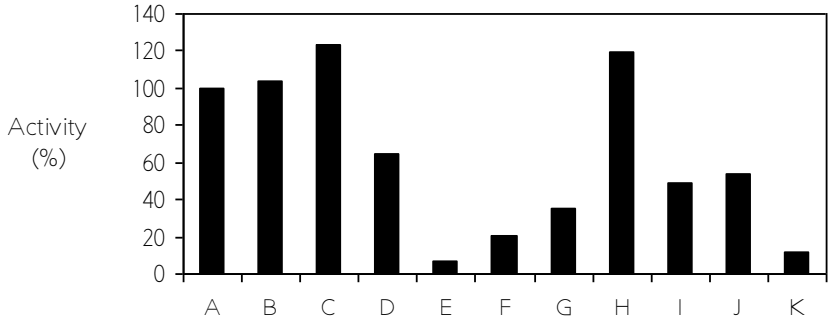

Figure 7

Figure 7