Production 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, value-added food additives, from hydroxycinnamic acids. The gene encoding PAD from *Lactobacillus brevis* was cloned and expressed in *Escherichia coli*. The 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. As the PAD previously described, *L. brevis* enzyme 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 *B. subtilis* and *B. pumilus*, but different from the *L. plantarum* and *P. pentosaceus* PAD. Amino acid differences among these proteins could explain the differences observed. The substrate specificity showed by *L. brevis* PAD indicated a high potential for the synthesis of high-added food additives.

Keywords: Ferulic acid, phenolic acid decarboxylase, phenolic acids, vinyl phenol, *p*-coumaric acid

1. Introduction

Phenolic acids are abundant molecules in vegetable foods. Cinnamoyl ester hydrolases 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 (Cavin, Barthelmebs, & Divies, 1997a; Cavin, Dartoid, & Divies, 1998; Rodríguez, Landete, Curiel, de las Rivas, Mancheño, & Muñoz, 2008). These enzymes catalyze the conversion of ferulic or *p*-coumaric acids into the corresponding volatile compounds 4-vinyl guaiacol or 4-vinyl phenol (Fig. 1), considered as intermediates of vanillin (4-hydroxy-3-methoxybenzaldehyde) production (Koseki, Ito, Furuse, Ito, & Iwano, 1996). 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 vegetal substrates (Zheng, Sun, Bai, Wang, & Guo, 2007). Decarboxylation of ferulic acid to 4-vinyl guaiacol is a potentially profitable value addition to the starting material if the ferulic acid can be extracted efficiently from its bound polysaccharides in the biomass. Vinyl guaiacol is priced some 40 times more than ferulic acid, and it can be biotransformed further to acetovanillone, ethylguaiacol, and vanillin (Mathew and Abraham 2006; Rosazza, Huang, Dostal, Volm, & Rousseau, 1995). The activity of PAD enzymes on hydroxycinnamic acids, *p*-coumaric and ferulic acids, resulted in the production of 4-vinyl guaiacol or 4-vinyl phenol, both compounds are considered to be food additives and are approved as flavouring agents by regulatory agencies (JECFA 2001).

Four bacterial PAD from *Lactobacillus plantarum* (Cavin et al. 1997a; Rodriguez et al. 2008), *Pediococcus pentosaceus* (Barthelmebs, Lecomte, Divies, & Cavin, 2000),

Bacillus subtilis (Cavin et al. 1998), and *Bacillus pumilus* (Zago, Degrassi, & Bruschi, 1995) were expressed in *Escherichia coli*, and their activities on *p*-coumaric, ferulic, and caffeic acid were compared. Although these four enzymes displayed 61% amino acid sequence identity, they exhibit different activities for phenolic acid metabolism. Among lactic acid bacteria, in addition to *L. plantarum* and *P. pentosaceus*, it have been described that *Lactobacillus brevis* strains are able to decarboxylate hydroxycinnamic acids (Chatonnet, Duborurdieu, & Boidron, 1995; Cavin, Andioc, Etievant, & Divies, 1993; Van Beek and Priest 2000; Couto, Campos, Figueiredo, & How, 2006; Bloem, Bertrand, Lonvaud-Funel, & de Revel, 2007; De las Rivas, Rodríguez, Curiel, Landete, & Muñoz, 2009, Curiel, Rodríguez, Landete, de las Rivas, & Muñoz, in press). However, PAD enzymes from *L. brevis* strains have not been biochemically characterized. From the analysis of the available complete genome sequence of *L. brevis* ATCC 367 (NC_008497), a protein annotated as PAD (LVIS_0213) could be identified. As substrate specificity and catalytic activity depends on the PAD sequence, in this work we biochemically characterize LVIS_0213 protein from *L. brevis* in order to broad the range of enzymes useful for generating value-added food additives.

2. Materials and methods

2.1. 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 affiliated by PCR amplification and DNA sequencing of their 16S rDNA. *Escherichia coli* DH5 α and *E. coli* JM109 (DE3) were purchased from Promega. *L. brevis* strain was grown in MRS medium at 30 °C without shaking. *E. coli* strains were cultured in Luria–Bertani (LB) medium at 37 °C and 200 rpm. When required, ampicillin or chloramphenicol were added to the medium at a **concentration of 100 or 34 μ g/mL**, respectively. Chromosomal DNA, plasmid purification, and transformation of *E. coli* were carried out as described elsewhere (Sambrook, Fritsch, & Maniatis, 1988). The phenolic acids assayed were purchased from Sigma (*p*-coumaric, caffeic, ferulic, and sinapic acids), Aldrich (*m*-coumaric, cinnamic, and gentisic acids), Fluka (*o*-coumaric, syringic, and gallic acids) or Merck (benzoic and salicylic acids).

2.2. Cloning of *L. brevis* phenolic acid decarboxylase

Expression vector pURI3 was previously constructed in our laboratory (De las Rivas, Curiel, Mancheño, & Muñoz, 2007) containing 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 using the primers 369 (5'–*CATCATGGTGACGATGACGATAAGatgactaaagaattcaaaacat*) and 370 (5'–*AAGCTTAGTTAGCTATTATGCGTAttatttcgtgattcgcttgtaatta*) (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 (pURI3-0213) by using a restriction enzyme– and ligation-free cloning strategy described previously (De las Rivas et al., 2007).

2.3. Purification of the *L. brevis* phenolic acid decarboxylase

Cells carrying pURI3-0213 plasmid were grown at 37 °C in Luria-Bertani media containing ampicillin (100 µg/mL) and chloramphenicol (34 µg/mL), until they reach an optical density at 600 nm of 0.4 and induced by adding IPTG (0.4 mM final concentration). After induction, the cells were grown at 22 °C during 20 h and collected by centrifugation. 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 47,000g 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 (Amersham Biosciences) equilibrated with 20 mM Tris-HCl, pH 8.0, 100 mM NaCl containing 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 His₆-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 cutoff). The purity of the enzyme was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in Tris-glycine buffer.

2.4. SDS-PAGE and determination of protein concentration

Samples were analyzed by SDS-PAGE under reducing conditions according to Laemmli (Laemmli 1970). 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 (Germany) with bovine serum albumin as standard.

2.5. 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 μ mol 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.

2.6. Optimum temperature and optimum pH of the L. brevis phenolic acid decarboxylase

Activities of the *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 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 phosphate buffer (pH 6.5) at different temperatures (4–90 °C) for 20 min using *p*-coumaric acid (4 mM) as substrate.

2.7. Dependence of the *L. brevis* phenolic acid decarboxylase stability on temperature

For temperature stability measurements, the *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. After incubation, the remaining activity was measured.

2.8. Effect of additives on activity of the *L. brevis* phenolic acid decarboxylase

To test the effect of metals and ions on the stability of the *L. brevis* PAD, the enzyme suspended in 25 mM phosphate buffer, pH 6.5, was incubated with 1 mM concentration of one of several metals or other additives (MgCl₂, KCl, CaCl₂, HgCl₂, SDS, Triton–X–100, **Urea, EDTA, DMSO, and β**-mercaptoethanol). The samples were incubated with a 1 mM concentration of the additive 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.

2.9. HPLC analysis of the *L. brevis* phenolic acid decarboxylase activity on phenolic acids

The activity of the *L. brevis* PAD on several phenolic acids was assayed by incubating the enzyme during 4 h at 30 °C in presence of each phenolic acid at 1 mM final concentration. 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, Ireland) and analyzed by HPLC-DAD. A Thermo (Thermo Electron Corporation, Waltham, Massachusetts, 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 C₁₈ (25 cm × 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 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/ESI-MS.

3. Results and discussion

3.1. Sequence analysis of *L. brevis* LVIS_0213

Lactobacillus brevis strains could be isolated from spontaneous fermentation of vegetables where phenolic compounds are abundant (Sánchez, Palop, & Ballesteros, 2000; Karovicova and Kohajdova, 2003; Rodas, Ferrer, & Pardo, 2005). These strains are adapted to grow in a media where phenolic compounds are present; 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 (Makarova et al. 2006), 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 pI 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 showed 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 (Barthelmebs, Divies, & Cavin, 2001). As the C-terminal region from the *L.*

brevis PAD showed significant sequence differences, it could exhibit different substrate specificity from the previous characterized PAD enzymes.

3.2. Enzymatic activity of *L. brevis* LVIS_0213

To confirm that LVIS_0213 gene from *L. brevis* encodes a functional PAD, we cloned this gene from *L. brevis* RM84 in *E. coli*. 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 protein was cloned containing a purification poly-His tag, *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 (Rodriguez et al., 2008; Degrassi, Polverino, de Lureto, & Bruschi, 1995; Harada and Mino, 1976). 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 (Rodriguez et al., 2008).

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 MgCl₂ (relative activity 104%), partially inhibited by CaCl₂, EDTA, and DMSO (relative activity 49–65%), and was greatly inhibited by Triton-X-100, SDS, β -mercaptoethanol, and HgCl₂ (relative activity 35–7%).

3.3. Substrate specificity of *L. brevis* LVIS_0213

It has been shown that 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 *L. plantarum*, and *P. pentosaceus* PAD with an activity about 500-fold lower than that for *p*-coumaric acid (Barthelmebs et al., 2001). However, *B. subtilis* and *B. pumilus* PAD display similar activities on either substrate. Kinetic parameters were investigated for *L. brevis* PAD using *p*-coumaric, caffeic, and ferulic acids as substrates. *L. brevis* PAD has K_m values of 0.98, 0.96, and 0.78 mM and V_{max} values of 598, 609, and 464 μ mol/h/mg for *p*-coumaric, caffeic, and ferulic acids, respectively. These values indicated that kinetic parameters were similar for the three hydroxycinnamic acids assayed, being this behaviour similar to *B. subtilis* and *B. pumilus*

PAD, but markedly different from PAD previously characterized from the lactic acid bacteria *L. plantarum* and *P. pentosaceus*.

Chimeric enzyme construction was shown to be useful for combining properties not typically found in any naturally occurring enzyme. Chimeric PAD constructed 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 (Barthelmebs et al., 2001). Although the chimeric PAD displayed enzymatically 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 by the *B. pumilus* N-terminal PAD region and *L. plantarum* C-terminal PAD region decarboxylated ferulic acid with a relative activity 10-fold 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, being five of them 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 *L. brevis* PAD, except Glu-55 which is a proline residue. The implications of this residue change in the different catalytic activity of *L. brevis* PAD on ferulic acid need to be further investigated.

In order to know the substrate specificity of *L. brevis* PAD, seven cinnamic and five benzoic acids were assayed as putative substrates for the enzyme. Among the cinnamic acids assayed (*p*-coumaric, *o*-coumaric, *m*-coumaric, cinnamic, caffeic, ferulic, and sinapic acid), only *p*-coumaric, caffeic, and ferulic acid were decarboxylated by *L. brevis* PAD (Figure 7), similarly to the PAD enzymes previously characterized (Cavin et al., 1997b; Rodríguez et al, 2008). The decarboxylation of *p*-coumaric, caffeic, and ferulic acids

originate their vinyl derivatives: vinyl phenol, vinyl catechol, and vinyl guaiacol, respectively (Figure 7). These decarboxylations have also been previously described from cultures of *L. brevis* strains growing on the presence of these hydroxycinnamic acids Chatonnet et al., 1995; Cavin et al. 1993; Van Beek and Priest, 2000; Couto et al., 2006; Bloem et al. 2007; De las Rivas et al. 2009). 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 the benzoic acids were decarboxylated by the *L. brevis* enzyme. In summary and as reported previously in *L. plantarum* PAD, it seems that other phenolic acids without a hydroxyl group *para* to the unsaturated side chain and with another substituent than -H, -OH, or -OCH₃ *meta* to the unsaturated side chain were not metabolized (Cavin et al., 1997b). The knowledge of the catalytic mechanism of decarboxylation followed by PAD will open up novel biotechnological possibilities for the design of novel enzymes with broaden specificities.

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Figure captions

Fig. 1. Schematic representation of the reaction catalyzed by the PAD enzyme. When R₁ (OH) and R₂ (H), the represented compounds are *p*-coumaric acid (A) and vinyl phenol (B). When R₁ (OH) and R₂ (OH), caffeic acid (A) and vinyl catechol (B). When R₁ (OH) and R₂ (OCH₃), the compounds are ferulic acid (A), and vinyl guaiacol (B).

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 *Bacillus pumilus* ATCC 15884 (BPU) (accession AJ278683). Asterisks represent amino acid identity, and dashes represent gaps introduced to maximize similarities.

Fig. 3. SDS-PAGE analysis of the expression and purification of PAD protein from *L. brevis* RM84 cloned into pURI3 vector. SDS-PAGE analysis of soluble cells extracts of IPTG-induced cultures. *E. coli* JM109 (pURI3) (1); *E. coli* JM109 (pURI3-0213) (2); fractions eluted after His-Trap^{TM}-FF crude chelating affinity column (3-7). The polyacrylamide gels were stained with Coomassie blue. The positions of molecular mass 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)

1 Relative activity of *L. brevis* PAD versus pH. Enzyme activity was assayed at 30 °C. The
2 observed maximum activity was defined as 100%.

3
4 Fig. 5. Effects of temperature on the stability of *L. brevis* PAD. Residual activities of *L.*
5 *brevis* PAD after **preincubation at 22 °C (◆), 30 °C (■), or 37 °C (▲) in phosphate buffer**
6 pH 6.5 during 1, 2, 3, 5, 12, 24, and 48 h. The observed maximum activity was defined as
7 100%.

8
9 Fig. 6. Relative activity of *L. brevis* PAD when was incubated with 1 mM concentrations
10 of the different additives (A to K) and 4 mM *p*-coumaric acid in 1 mL of 25 mM phosphate
11 buffer, pH 6.5, at 30 °C for 20 min. The additives assayed were MgCl₂ (B), KCl (C), CaCl₂
12 (D), HgCl₂ (E), SDS (F), Triton-X-100 (G), Urea (H), EDTA (I), DMSO (J), and β-
13 mercaptoethanol (K). The activity of the enzyme incubated in 25 mM phosphate buffer, pH
14 6.5, at 30 °C for 20 min was defined as 100% (A).

15
16 Fig. 7. HPLC chromatograms of purified *L. brevis* PAD on hydroxycinnamic acids. PAD
17 enzyme from *L. brevis* RM84 was incubated for 2 h in the presence of *p*-coumaric (A),
18 caffeic (B), or ferulic acids (C). Chromatograms without protein (controls) are also showed.
19 The chromatograms were recorded at 280 nm. *p*CA, *p*-coumaric acid; CA, caffeic acid; FA,
20 ferulic acid; VP, vinyl phenol; VC, vinyl catechol; VG, vinyl guaiacol.

Figure 1

Figure 1

Figure 2

Figure 2

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	VPGIYKVAWTEPTGTDVALDFVPNEKKLNGTIFFPKWVEEYPEITVITYQNEHIDLMEEESR	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

Figure 3

Figure 3

Figure 4

Figure 4

Figure 5

Figure 5

Figure 6

Figure 6

Figure 7

Figure 7