Characterization of the first feruloyl esterase from *Lactobacillus plantarum*

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Running Title

LACTOBACILLUS PLANTARUM FERULOYL ESTERASE

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Lactobacillus plantarum is frequently found in the fermentation of plant-derived food products where hydroxycinnamoyl esters are abundant. _L. plantarum_ WCFS1 cultures were unable to hydrolyze hydroxycinnamoyl esters; however, cell-free extracts from this strain partially hydrolyze methyl ferulate and methyl _p_-coumarate. In order to find out whether the protein Lp_0796 is the enzyme responsible for this hydrolytic activity, it has been recombinantly overproduced and enzymatically characterized. Lp_0796 is an esterase, which among other substrates, is able to hydrolyze efficiently the four model substrates for feruloyl esterases (methyl ferulate, methyl caffeate, methyl _p_-coumarate and methyl sinapinate). A screening test for the detection of the gene encoding feruloyl esterase Lp_0796 revealed that it is generally present among _L. plantarum_ strains. The present study constitutes the first description of a feruloyl esterase activity in _L. plantarum_ and provides new insights into the metabolism of hydroxycinnamic compounds on this bacterial species.

INTRODUCTION

Phenolic acids are abundant, naturally occurring molecules that contribute to the rigidity of plant cell walls. Hydroxycinnamic acids, such as ferulic, sinapic, caffeic, and *p*-coumaric acids, are found both covalently attached to the 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 (1). Hydroxycinnamates are found in numerous plant foods and in significant quantities in agro-industrial derived by-products. The industrial use of hydroxycinammates has attracted growing interest since they and their conjugates were shown to be bioactive molecules, possessing potential antioxidant activities and health benefits. The removal of these phenolic compounds and the breakdown of the ester linkages between polymers allow numerous exploitations for industrial and food applications.

Feruloyl esterases, also known as ferulic acid esterases, cinnamic acid esterases, or cinnamoyl esterases, are the enzymes involved in the release of phenolic compounds such as ferulic, *p*-coumaric, caffeic, and sinapic acids from plant cell wall (2). In human and rumial digestion, feruloyl esterases are important to de-esterify dietary fibre, releasing hydroxycinnamates and derivatives which have been shown to have positive effects, such as antioxidant, anti-inflammatory, and antimicrobial activities (3). They are also involved in colonic fermentation where their activities in the microbiota improve the breakdown of ester bonds in hydroxycinnamates (3). The biological properties of hydroxycinnamates depend of their absorption and their metabolism. Although there exists evidence that food hydroxycinnamates are degraded by gut microbiota, only limited information on the microorganisms and enzymes involved in this degradation is currently available.

Feruloyl esterases able to hydrolyze hydroxycinnamates have been found in lactic acid bacteria isolated from foods and from human intestinal microbiota, such as some strains of *Lactobacillus gasseri* (4), *L. acidophilus* (5), *L. helveticus* (6), and *L. johnsonii* (7-9). This enzymatic activity may provide these *Lactobacillus* strains with an ecological advantage, as they are often associated with fermentations of plant materials. *Lactobacillus plantarum* is a lactic acid bacterial species that is most abundant in fermenting plant-derived raw materials and also might colonize the human gastrointestinal tract considerably better than other tested lactobacilli (10). Despite several esterase enzymes have been described in *L. plantarum* (11-20), cinnamoyl esterase activity has not been found on them yet.

Since feruloyl esterases constitute an interesting group of enzymes with a potentially broad range of applications in the food industry and in fact nowadays there exists a constant search for such enzymes with more desirable properties for novel food applications, the present study represents the first description of a feruloyl esterase enzyme in *L. plantarum*, which is widely spread among strains from this species.

MATERIALS AND METHODS

Strains and growth conditions. *Lactobacillus plantarum* WCFS1 was kindly provided by Dr. Kleerebezem (NIZO Food Research, The Netherlands). This strain is a single colony isolate of *L. plantarum* NCIMB 8826, which was isolated from human saliva. This strain survives the passage through the human stomach (21) and persists in the digestive tract of mice and humans (22). *L. plantarum* NC8 and *L. plantarum* 57/1 strains were kindly provided by Dr. L. Axelsson (Norwegian Institute of Food, Fisheries and Aquaculture Research, Norway) and Dr. J. L. Ruíz-Barba (Instituto de la Grasa, CSIC, Spain), respectively. Strains *L. plantarum* CECT 220 (ATCC 8014), CECT 221

92 (ATCC 14431), CEC 223, CECT 224, CECT 749 (ATCC 10241), CECT 4185, and
93 CECT 4645 were purchased from the Spanish Type Culture Collection (CECT). Strains
94 *L. plantarum* DSM 1055, DSM 2648, DSM 10492, DSM 13273, and DSM 20246 were
95 purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ).
96 Type strains of *L. plantarum* subsp. *plantarum* CECT 748^T (ATCC 14917^T) and *L.*
97 *plantarum* subsp. *argentorantesis* DSM 16365 were purchased from the CECT and
98 DSMZ, respectively. *L. plantarum* strains (*L. plantarum* RM28, RM31, RM34, RM35,
99 RM38, RM39, RM40, RM41, RM71, RM72, and RM73) were isolated from grape must
100 and wine samples (23). *Lactobacillus paraplantarum* DSM 10641 (ATCC 10776) and
101 DSM 10677 were purchased from the DSMZ and included in the study. *L. plantarum*
102 strains were routinely grown in MRS media (Pronadisa, Spain) adjusted to pH 6.5 and
103 incubated at 30 ºC. For degradation assays, *L. plantarum* WCFS1 strain was cultivated
104 in a modified basal and defined medium described previously for *L. plantarum* (24).
105 The basal medium was modified by the replacement of glucose by galactose. This
106 defined medium was used to avoid the presence of phenolic compounds included in
107 non-defined media. The sterilized modified basal medium was supplemented at 1mM
108 final concentration with hydroxycinnamoyl esters filter-sterilized. The *L. plantarum*
109 inoculated media were incubated at 30 ºC, in darkness. The phenolic products were
110 extracted from the supernatants twice with ethyl acetate (one third of the reaction
111 volume).
112 *Escherichia coli* DH10B was used for all DNA manipulations. *E. coli*
113 BL21(DE3) was used for expression in pURI3-TEV vector (25). *E. coli* strains were
114 cultured in Luria-Bertani (LB) medium at 37 ºC and 140 rpm. When required,
115 ampicillin was added to the medium at a concentration of 100 μg/ml.

Hydrolysis of hydroxycinnamoyl esters by *L. plantarum* cell-free extracts. In order to prepare cell-free extracts, *L. plantarum* WCFS1 strain was grown in 500 ml of MRS media at 30 ºC until an OD_{600nm} 0.5 was reached (10^5-10^6 cells/ml). The cultures were induced by adding 3 mM methyl ferulate and further incubated for 3 h; uninduced cultures were grown in the absence of the hydroxycinnamoyl ester. After induction, the cells were harvested by centrifugation (7500 g x 10 min, 4 ºC) and washed three times with sodium phosphate buffer (50 mM, pH 7), and subsequently resuspended in the same buffer (10 ml) for cell rupture. Bacterial cells were disintegrated twice by using the French press at 1500 psi pressure. The disintegrated cell suspension was centrifuged at 17400g for 40 min at 4 ºC in order to sediment cell debris. The supernatant containing the soluble proteins was filtered aseptically using sterile filters of 0.22 μm pore size (Sarstedt, Germany).

To determine whether uninduced or induced *L. plantarum* cells possessed enzymes able to hydrolyze hydroxycinnamoyl esters, cell-free extracts were incubated in the presence of the four model substrates for feruloyl esterase activity (methyl ferulate, methyl caffeate, methyl *p*-coumarate and methyl sinapinate) (Apin Chemicals, Oxfordshire, UK) at 1 mM final concentration. *L. plantarum* cell-free extracts (2 mg/ml of total protein) were incubated during 16 h at 30 ºC in the presence of each hydroxycinammoyl ester. The reaction products were extracted twice with ethyl acetate (Lab-Scan, Ireland) for subsequent analysis by HPLC.

Production and purification of recombinant *L. plantarum* esterase. The gene *lp_0796* from *L. plantarum* WCFS1 coding putative esterase/lipase was PCR-amplified with HS Prime Start DNA polymerase (Takara) by using the primers 703 (*GGTGAAAACCTGTATTTCCAGGGCatg*atgctgaaacaaccggaaccgt) and 704 (*ATCGATAAGCTTAGTTAGCTATTAtca*tttataaatagtttttaaatat) (the nucleotides pairing

141 the expression vector sequence are indicated in italics, and the nucleotides pairing the
142 *lp_0796* gene sequence are written in lowercase letters). The pURI3-TEV vector
143 encodes expression of a leader sequence containing a six histidine affinity tag. The
144 corresponding 831 pb purified PCR product was then inserted into the pURI3-TEV
145 vector by using a restriction enzyme- and ligation-free cloning strategy (25). *E. coli*
146 DH10B cells were transformed and the recombinant plasmids were isolated. Those
147 containing the correct insert, as identified by restriction-enzyme analysis, were further
148 verified by DNA sequencing and used for transformation of *E. coli* BL21 (DE3) cells.
149 *E. coli* cells carrying the recombinant plasmid pURI3-TEV-0796, were grown at
150 37 ºC in LB media containing ampicillin (100 µg/ml) and induced by adding 0.4 mM
151 IPTG. After induction, the cells were grown at 22 ºC during 20 h and harvested by
152 centrifugation (7500*g* for 15 min at 4 ºC). Cells were resuspended in 50 mM sodium
153 phosphate buffer, pH 7.0 containing 300 mM NaCl. Crude extracts were prepared by
154 French Press lysis of cell suspensions (three cycles at 1100 psi). The lysate was
155 centrifuged at 17400*g* for 40 min at 4 ºC.
156 The supernatant obtained was filtered through a 0.22 µm filter (Millipore) and
157 gently mixed for 20 min at room temperature with 1 ml TALON resin (Clontech). The
158 resin was washed with 50 mM sodium phosphate buffer pH 7.0 containing 300 mM
159 NaCl and 10 mM imidazole. The recombinant His6-tagged protein was eluted with 50
160 mM sodium phosphate pH 7.0 containing 300 mM NaCl and 150 mM imidazole. The
161 eluted His6-tagged Lp_0796 was dialysed overnight at 4 ºC against 50 mM sodium
162 phosphate buffer, pH 7.0 containing 300 mM NaCl. The purity of the enzyme was
163 determined by 12.5% sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-
164 PAGE) in Tris-glycine buffer.

HPLC analysis of feruloyl esterase activity. Feruloyl esterase activity was measured against four model substrates, methyl ferulate, methyl caffeate, methyl *p*-coumarate and methyl sinapinate (Apin Chemicals, Oxfordshire, UK). The assays for the methyl esters of hydroxycinnamic acids were carried out in a final volume of 1 ml at 37 ºC in 50 mM sodium phosphate buffer pH 7.0, 1 mM substrate, and 100 µg of protein. The reaction was terminated with ethyl acetate after 16 hours reaction time.

The reaction products were extracted twice with one third of the reaction volume of ethyl acetate (Lab-Scan, Ireland). The ethyl acetate was directly injected onto the column and analyzed by HPLC-DAD. A Thermo (Thermo Electron Corporation, Waltham, Massachussetts, USA) chromatograph equipped with a P4000 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} cartridge (25 cm x 4.0 mm i.d., 4.6 µm particle size) 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. Samples were injected onto the cartridge after being filtered through a 0.45 µm PVDF filter. Detection of the substrates and the degradation compounds was performed by scanning from 220 to 380 nm. 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.

Enzyme activity assays. Esterase activity was determined by a spectrophotometric method using *p*-nitrophenyl butyrate (Sigma-Aldrich) as the

190 substrate. The rate of hydrolysis of *p*-nitrophenyl butyrate for 10 min at 37 ºC was
191 measured in 50 mM sodium phosphate buffer pH 7.0 at 348 nm in a spectrophotometer
192 (UVmini-1240 Shimadzu). The reaction was stopped by chilling on ice.
193 In order to carry out the reaction (1 ml), a stock solution of 25 mM of *p*-
194 nitrophenyl butyrate was prepared in acetonitrile/isopropanol (1:4, v/v) (26) and mixed
195 with 50 mM sodium phosphate buffer (pH 7.0) to obtain a 1 mM substrate final
196 concentration. Control reactions containing no enzyme were utilized to account for any
197 spontaneous hydrolysis of the substrates tested. Enzyme assays were performed in
198 triplicate.
199 **Substrate specificity.** To investigate the substrate specificity of Lp_0796,
200 activity was determined using different *p*-nitrophenyl esters of various chain lengths
201 (Sigma-Aldrich): *p*-nitrophenyl acetate (C2); *p*-nitrophenyl butyrate (C4); *p*-nitrophenyl
202 caprylate (C8); *p*-nitrophenyl laurate (C12); *p*-nitrophenyl myristate (C14) and *p*-
203 nitrophenyl palmitate (C16) as substrates. A stock solution of each *p*-nitrophenyl ester
204 was prepared in acetonitrile/isopropanol (1/4 v/v). Substrates were emulsified to a final
205 concentration of 0.5 mM in 50 mM sodium phosphate buffer, pH 7.0, containing 1.1
206 mg/ml Arabic gum and 4.4 mg/ml Triton X-100 (18). Reaction mix consisted of 990 µl
207 of emulsified substrate and 10 µl of enzyme solution (1 µg protein). Reactions were
208 carried out at 37 ºC in a spectrophotometer (UVmini-1240 Shimadzu) as described
209 above.
210 The enzymatic substrate profile of purified protein was determined by using an
211 ester library described previously (27). *p*-Nitrophenol was used as pH indicators to
212 monitor ester hydrolysis colorimetrically. The screening was performed in a 96-well
213 Flat Bottom plate (Sarstedt) where each well contains a different substrate (1 mM) in
214 acetonitrile (1%). A buffer/indicator solution containing 0.44 mM of *p*-nitrophenol in 1

mM sodium phosphate buffer pH 7.2 was used as pH indicator. Esterase solution 10 μg (20 μl in 1 mM sodium phosphate buffer pH 7.2) was added to each well and reactions were followed by measuring the decrease in absorbance at 410 nm for 2 h at 37 ºC in a Synergy HT BioTek microplate spectrophotometer. Blanks without enzyme were carried out for each substrate and data were collected in triplicate and the average activities were quantified. Results are shown as means ± standard deviations.

Effect of temperature, pH, and additives on esterase activity. In order to investigate temperature effect, reactions were performed in 50 mM sodium phosphate buffer (pH 7.0) at 20, 30, 37, 40, 45, 55 and 65 ºC. Effect of pH was studied by assaying esterase activity in a range of pH values from 3.0 to 9.0. Buffers (100 mM) used were acetic acid-sodium acetate buffer (pH 3-5), sodium phosphate buffer (pH 6-7), Tris-HCl buffer (pH 8) and glycine-NaOH buffer (pH 9). For temperature stability measurements, the esterase was incubated in 50 mM sodium phosphate buffer (pH 7.0) at 20, 30, 37, 45, 55 and 65 ºC for 5 min, 15 min, 30 min, and 1, 2, 3, 4, 6 and 20 h. After incubation, the residual activity was measured as described above. To test the effect of metals and ions on the activity of the esterase, the enzyme was incubated in the presence of different additives at a final concentration of 1 mM during 5 min at room temperature. Then, the substrate was added and the reaction was incubated at 37 ºC. The additives analyzed were $MgCl_2$, KCl, $MnCl_2$, $FeCl_2$, $CuCl_2$, $NiCl_2$, $CaCl_2$, $HgCl_2$, $ZnCl_2$, DEPC, Cysteine, SDS, DTT, Triton-X-100, Urea, Tween 80, Tween 20, EDTA, DMSO, pyridoxal-5-phosphate, PMSF and β-mercaptoethanol. In all cases, each analysis was performed in triplicate.

Bacterial DNA extraction and PCR detection of *lp_0796*. Bacterial chromosomal DNA was isolated from overnight cultures. Briefly, *L. plantarum* strains grown in MRS broth were pelleted by centrifugation and resuspended in TE solution

(10 mM Tris-HCl, pH 8.0; 1 mM EDTA) containing 10 mg/ml of lysozyme (Sigma, Germany). Cells were lysed by adding SDS (1%) and proteinase K (0.3 mg/ml). Crude DNA preparation was purified by performing two phenol/chloroform/isoamyl alcohol (25:24:1) extractions and one chloroform/isoamyl alcohol (24:1) extraction. Chromosomal DNA was precipitated by adding 2 volumes of cold ethanol. Finally, the DNA precipitate was resuspended in TE solution.

The *lp_0796* gene encoding esterase Lp_0796 was amplified by PCR using 10 ng of chromosomal DNA. PCR reactions were performed in 0.2 ml centrifuge tubes in a total volume of 25 µl containing 1 µl of template DNA (approximately 10 ng), 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 1 U of Ampli*Taq* Gold DNA polymerase, and 1 µM of each primer. The reactions were performed using oligonucleotides 703 and 704 to amplify the *lp_0796* gene. The reactions were performed in a Personnel Eppendorf thermocycler using the following cycling parameters: initial 10 min at 98 ºC for enzyme activation, denaturation at 94 ºC for 1 min, annealing at 50 ºC for 30 s, and extension at 72 ºC for 30 s. The expected size of the amplicon was 0.8 kb. PCR fragments were resolved on 0.7% agarose gel.

RESULTS

Hydrolysis of hydroxycinnamoyl esters by *L. plantarum* WCFS1. In order to find out whether *L. plantarum* WCFS1 has the ability to hydrolyze hydroxycinnamoyl esters, two different experimental approaches were followed. First, *L. plantarum* cultures were grown for 7 days in the presence of the four model substrates for feruloyl esterases (methyl ferulate, methyl caffeate, methyl *p*-coumarate, and methyl sinapinate) at 1 mM final concentration. In the case *L. plantarum* cells were able to metabolize the hydroxycinnamoyl esters assayed, the end-products could be detected in the culture

media. In addition, cell-free extracts, from methyl ferulate-induced and non-induced cultures, were incubated at 30 ºC during 16 h in the presence of 1 mM of each of the four model substrates.

The results indicated that *L. plantarum* WCFS1 cell cultures were unable to hydrolyze any of the four model substrates tested (data not shown). However, interestingly, methyl ferulate and methyl *p*-coumarate were partially hydrolyzed by *L. plantarum* WCFS1 cell-free extracts (Fig. 1). No significant differences in the hydrolysis were observed among methyl ferulate-induced extracts or uninduced extracts, indicating that the enzymatic activity involved is not inducible by the presence of methyl ferulate in the culture media in our experimental conditions.

Identification of Lp_0796 as a feruloyl esterase. *L. plantarum* WCFS1 cell extracts partially hydrolyzed methyl ferulate and methyl-*p*-coumarate, therefore an enzyme possessing feruloyl esterase activity should be present. In this regard, numerous ORFs encoding putative esterases can be identified from the genomic information of *L. plantarum* WCFS1 and on of such ORF is precisely *lp_0796*. To check the working hypothesis that Lp_0796 may be a functional feruloyl esterase therefore we decided to clone the corresponding ORF.

The gene *lp_0796* from *L. plantarum* WCFS1 has been expressed in *E. coli* under the control of an inducible promoter. Cell extracts were used to detect the presence of overproduced proteins by SDS-PAGE analysis. Whereas control cells containing the pURI3-TEV vector plasmid did not show protein overexpression, an overexpressed protein with an apparent molecular mass around 28 kDa was apparent with cells harbouring pURI3-TEV-0796 (Fig. 2). Since the cloning strategy would yield a His-tagged protein variant, *L. plantarum* Lp_0796 could be purified on an immobilized metal affinity chromatography (IMAC) resin. As expected, a protein with

290 the correct molecular mass eluted from the TALON resin by washing with a buffer
291 containing 150 mM imidazole (Fig. 2). The eluted protein was then dialyzed against
292 sodium phosphate buffer (50 mM, pH 7.0) to remove the imidazole, which may
293 interfere in the feruloyl esterase enzymatic activity assays.
294 Feruloyl esterase activity of pure *L. plantarum* Lp_0796 was performed by using
295 the four hydroxycinnamoyl esters (methyl ferulate, methyl caffeate, methyl *p*-
296 coumarate, and methyl sinapinate) as substrates at 1 mM final concentration. Fig. 3
297 shows that the four hydroxycinnamoyl esters were fully hydrolyzed by Lp_0796 in our
298 experimental conditions, revealing Lp_0796 as a feruloyl esterase.
299 **Biochemical properties of Lp_0796.** An ester library was used to test the
300 substrate range of Lp_0796. This ester library consisted of esters, which were chosen to
301 identify acyl chain length preferences of the esterase, and also the ability of Lp_0796 to
302 hydrolyze hindered or charged substrates (27). In addition, the activity of Lp_0796
303 against *p*-nitrophenyl esters of various chain lengths, from C2 (*p*-nitrophenyl acetate) to
304 C16 (*p*-nitrophenyl palmitate) was assayed. The highest hydrolytic activity was
305 observed on phenyl acetate, followed by methyl phenyl acetate and ethyl and methyl
306 bromoacetate (Fig. 4A). Moreover, the ester library confirmed that the four model
307 substrates for feruloyl esterases were efficiently hydrolyzed by Lp_0796 (methyl
308 caffeate, methyl *p*-coumarate, methyl ferulate, and methyl sinapinate). Other esters
309 substrates were also hydrolyzed, although less efficiently (methyl mandelate, vinyl
310 propanoate, vinyl acetate, vinyl benzoate, vinyl butanoate, metyl benzoate, butyl
311 acetate, and isopropenyl acetate, among others). Regarding the *p*-nitrophenyl esters
312 assayed, Lp_0796 showed maximum activity against the short acyl chain esters, *p*-
313 nitrophenyl acetate and *p*-nitrophenyl butyrate (Fig. 4B), although activity against *p*-
314 nitrophenyl caprilate (C8) and *p*-nitrophenyl palmitate (C16) was also observed.

315 Therefore, according to the substrates hydrolyzed, it can be concluded that Lp_0796 is a
316 feruloyl esterase with a relatively wide specificity spectrum, not described previously in
317 any other esterase from lactic acid bacteria.

318 Since feruloyl esterases are enzymes with a broad range of applications, and
319 Lp_0796 is the first feruloyl esterase described in *L. plantarum*, its biochemical
320 properties have been characterized. Fig. 5 shows the optimum pH, temperature and the
321 thermal stability of Lp_0796 determined using *p*-nitrophenyl butyrate as substrate.
322 Lp_0796 displays an optimal activity at 30-37 ºC, showing marginal activity at 45 ºC
323 (14% of the maximal activity) (Fig. 5A). In fact, Lp_0796 can be classified as a heat-
324 labile enzyme since its activity decreased drastically after incubation for a few minutes
325 at 45 ºC or after 20 hours incubation at 22 ºC where the esterase showed only 60% of its
326 maximal activity.

327 Fig. 5D shows the effect of various additives (1 mM final concentration) on the
328 enzymatic activity of Lp_0796. It can be observed that the activity of Lp_0796 is
329 greatly increased by the addition of Tween 20 and Tween 80 (250%) and $ZnCl_2$ (155%),
330 being not significantly affected by $FeCl_2$, $NiCl_2$, $MnCl_2$, cysteine, $CaCl_2$, $MgCl_2$,
331 DMSO, urea, and Triton-X-100 (relative activity 85 to 105%), only partially inhibited
332 by $CuCl_2$, DEPC, KCl, EDTA, β-mercaptoethanol, pyridoxal-5-phosphate (relative
333 activity 55-73%), and greatly inhibited by SDS, $HgCl_2$, DTT, and PMSF (relative
334 activity 12-35%). The effect of Tween 20 on Lp_0796 seems to be concentration-
335 dependent, since at concentration of 1 mM is an activating additive (250%) whereas at
336 5-10% concentration the esterase was inactivated to a significant extend.

337 **Presence of Lp_0796 among *L. plantarum* strains.** In order to know the extent
338 of the presence of feruloyl esterase Lp_0796 among *L. plantarum* strains, the presence
339 of the gene *lp_0796* was studied in *L. plantarum* strains isolated from different origins.

To determine the presence of the *lp_0796* gene, chromosomal DNA was extracted and PCR amplified. A 0.8 kb gene fragment was PCR amplified using a pair of oligonuclotides designed on the basis of the *L. plantarum* WCFS1 *lp_0796* gene sequence. All the *L. plantarum* strains analyzed gave the corresponding amplicon, what indicates that Lp_0796 is generally present among *L. plantarum* strains.

DISCUSSION

Hydroxycinnamates, such as caffeic, ferulic and *p*-coumaric acids, are commonly found as esters conjugates in dietary plants. Ferulic and *p*-coumaric acids occur, ester-linked, to pectin side-chains in spinach (28), and to the arabinoxylan of cereal brans (29). *L. plantarum* is the lactic acid bacterial species most frequently found in the fermentation of plant material where hydroxycinnamoyl esters are abundant. However, nowadays, most of the *L. plantarum* metabolism on phenolic compounds remains largely unknown. From the *L. plantarum* esterases already described, only the gallic/protocatechuic acid esterase (also known as tannase) hydrolyzed phenolic compounds (18). This esterase hydrolyzes ester bonds from two hydroxybenzoic acids, gallic and protocatechuic acids. So far, no esterase acting on hydroxycinnamoyl esters from ferulic, caffeic, coumaric, or sinapic acids, has been described in *L. plantarum* strains.

L. plantarum WCFS1 cultures were unable to hydrolyze any of the four model ester substrates for feruloyl esterases, possibly due to a lack of an efficient transport system to the cell since cell-free extracts from this strain partially hydrolyze methyl ferulate and methyl *p*-coumarate. However it cannot be excluded that other natural or synthetic substrates could pass into the cell and be hydrolyzed by *L. plantarum* cells. In order to find the esterase involved in the hydrolytic activity observed by cell extracts, the published sequence of *L. plantarum* WCFS1 was analyzed and numerous ORFs

365 encoding putative esterases were found. As a considerable degree of structural diversity
366 have been described between feruloyl esterases (30), it is not possible to predict the
367 biochemical function of the esterases encoded by these *L. plantarum* ORFs. The first *L.*
368 *plantaru*m WCFS1 ORF annotated as putative esterase (carboxylesterase) is *lp_0796*.
369 While this work was in progress, Lp_0796 (Est0796) has been described (20). It was
370 demonstrated that Lp_0796 is an esterase, which showed maximum activity towards
371 short acyl chain lengths (C2-C4). However, the activity of Lp_0796 against
372 hydroxycinnamoyl esters was not analyzed. In the present study, the activity of
373 Lp_0796 against the four model hydroxycinnamoyl esters for feruloyl esterases has
374 been assayed. We demonstrate that these compounds were fully hydrolyzed by Lp_0796
375 in our experimental conditions, revealing that Lp_0796 shows feruloyl esterase activity.
376 Feruloyl esterases exhibit distinct specificity spectra concerning the release of cinnamic
377 acids, and they are in fact organized into functional classes, which take into account
378 substrate specificity against synthetic methyl esters of hydroxycinnamic acids.
379 According to the present results, Lp_0796 can be considered a type C feruloyl esterase
380 since it hydrolyses the four methyl esters of hydroxycinnamic acids generally used as
381 model substrates (31).

382 It is interesting to note that the hydrolytic activity observed in *L. plantarum* cell-
383 extracts does not perfectly correlate with the activity observed with the pure Lp_0796
384 protein, since methyl caffeate and methyl sinapinate were not hydrolyzed by the cell-
385 free extracts. As only a minor hydrolysis of methyl ferulate and methyl *p*-coumarate
386 was observed in the cell extracts, it is possible that Lp_0796 could have higher activity
387 on these substrates, and therefore, the activity on methyl caffeate and methyl sinapinate
388 was not detected. However, it is obvious that the presence in *L. plantarum* WCFS1 of

389 enzymes possessing feruloyl/*p*-coumaroyl esterase activity other than Lp_0796 cannot
390 be discarded.

391 The present study constitutes the first description of an enzyme possessing
392 hydroxycinnamoyl esterase activity from *L. plantarum*. However, among lactic acid
393 bacteria, activity against feruloylated esters have been previously described in *L.*
394 *helveticus* and *L. acidophilus* cultures (6), and in purified proteins from *L. acidophilus*
395 (5) and *L. johnsonii* (7). Activity against caffeoyl, *p*-coumaroyl, and sinapyl esters was
396 not tested on these bacteria and proteins.

397 In addition to the four model substrates for feruloyl esterase, an ester library (27)
398 was used to analyse the substrate range of Lp_0796. Based on the activity profile
399 observed, it can be concluded that Lp_0796 shows a wide substrate range that has not
400 been described in any other esterase from lactic acid bacteria. Despite chlorogenic acid,
401 a caffeoyl conjugate widely distributed in fruits and vegetables, was not hydrolysed by
402 Lp_0796, hydrolysis of chlorogenic acid was observed in *L. helveticus* (6), *L.*
403 *acidophilus* (6), and *L. gasseri* (4); moreover, feruloyl esterases from *L. johnsonii* also
404 exhibited activity against chlorogenic and rosmarinic acids (7).

405 It has been described that *L. johnsonii* NCC 533 cells hydrolyzed rosmarinic
406 acid, while no cinnamoyl esterase-like activity was observed in both culture and
407 reaction media. Moreover, cell-free extracts from *L. johnsonii* showed a strong increase
408 of the reaction rate as compared to nonlysed cells, suggesting that the enzyme involved
409 in the hydrolysis is presumably intracellular (8). Taking into account that the deduced
410 amino acid sequence of Lp_0796 lacked an N-terminal secretion signal sequence,
411 possibly it is also located intracellularly. Several esterases and lipases from *L.*
412 *plantarum* (14, 15) and other lactic acid bacteria such as *L. casei* (32) and *S.*
413 *thermophilus* (33) were also reported to be located intracellularly. These observations

414 suggest that cell lysis may be important for the release of these enzymes during
415 fermentation or during the gastrointestinal tract passage.

416 Most of the metabolism of phenolic compounds in lactic acid bacteria remains
417 unknown, however, the description of new enzymatic activities help to uncover it. In
418 relation to hydroxycinnamic compounds, a decarboxylase of hydroxycinnamates has
419 been previously described in *L. plantarum* (34). The subsequent actions of the feruloyl
420 esterase described in the present study (Lp_0796), and that of a vinyl reductase, which
421 remains unkown, could allow *L. plantarum* to metabolize compounds abundant in
422 fermented plant-derived food products (hydroxycinnamoyl esters). However, since the
423 components of plant cells are constituted by complex carbohydrates, the ability of
424 Lp_0796 to degrade this biological material needs further investigation.

425

426

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434

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Legends to Figures

FIG 1. HPLC analysis of the degradation of hydroxycinnamoyl esters by *L. plantarum* WCFS1 cell-free extracts. Extracts from cultures non-induced (1) or induced by 3 mM methyl ferulate (2) were incubated in the presence of 1 mM methyl ferulate (A), methyl caffeate (B), methyl *p*-coumarate (C), and methyl sinapinate (D) during 16 h. The methyl ferulate (MF), methyl caffeate (MC), methyl *p*-coumarate (M*p*C), methyl sinapinate (MS), ferulic acid (FA) and *p*-coumaric acid (*p*CA) detected are indicated. The chromatograms were recorded at 280 nm.

FIG 2. Purification of *L. plantarum* Lp_0796 protein. SDS-PAGE analysis of the expression and purification of the His_6-Lp_0796. Analysis of soluble cell extracts of IPTG-induced *E. coli* BL21(DE3) (pURI3-TEV) (1) or *E. coli* BL21(DE3) (pURI3-TEV-0796) (2), flowthrough from the affinity resin (3), or fractions eluted after His affinity resin (4-8). The arrow indicated the overproduced and purified protein. The 12.5% gel was stained with Coomassie blue. Molecular mass markers are located at the left (SDS-PAGE Standards, Bio-Rad).

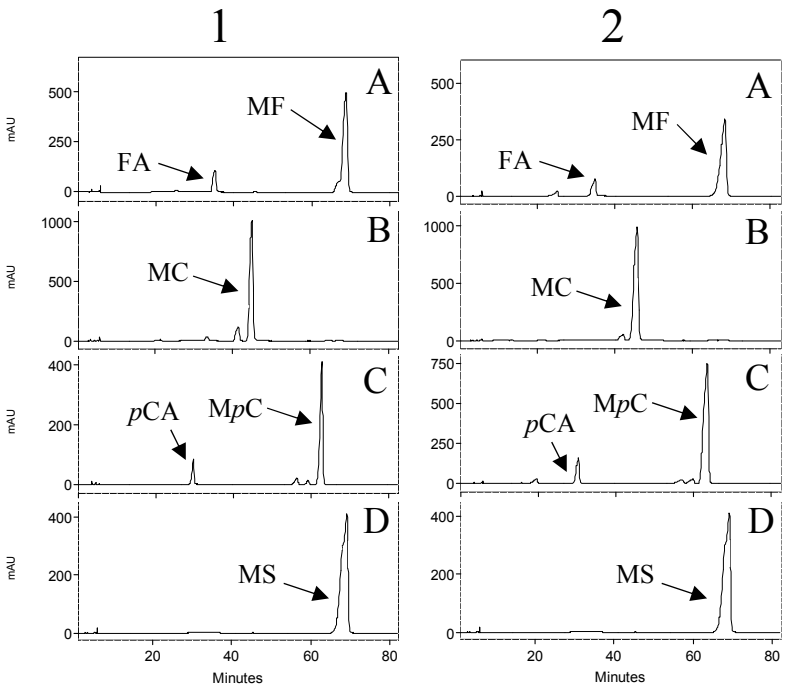
FIG 3. Enzymatic activity of *L. plantarum* Lp_0796 protein. Hydroxycinnamoyl esterase activity of purified Lp_0796 protein (2) compared with control reactions on which the enzyme was omitted (1). HPLC chromatograms of Lp_0796 (100 µg) incubated in 1 mM methyl ferulate (A), methyl caffeate (B), methyl *p*-coumarate (C), and methyl sinapinate (MS) during 10 h at 30 ºC. The methyl ferulate (MF), methyl

caffeate (MC), methyl *p*-coumarate (M*p*C), methyl sinapinate (MS), ferulic acid (FA), caffeic acid (CA), *p*-coumaric acid (*p*CA), and sinapic acid (SA) detected are indicated. The chromatograms were recorded at 280 nm.

FIG 4. Substrate profile of Lp_0796 toward (A) a general ester library or (B) against chromogenic substrates (*p*-nitrophenyl esters) with different acyl chain lengths (C2, acetate; C4, butyrate; C8, caprylate; C12, laurate; C14, myristate; C16, palmitate). The figure displays the relative specificities obtained toward different substrates, and lines on top of each bar represent the standard deviations estimated from three independent assays. The observed maximum activity was defined as 100%.

FIG 5. Some biochemical properties of Lp_0796 protein. (A) Relative activity of Lp_0796 versus temperature. (B) Relative activity versus pH. (C) Thermal stability of Lp_0796 after preincubation at 22ºC (filled diamond), 30 ºC (filled square), 37 ºC (filled triangle), and 45 ºC (filled circle) in phosphate buffer (50 mM, pH 6.5); at indicated times, aliquots were withdrawn, and analyzed as described in the Materials and methods section. The experiments were done in triplicate. The mean value and the standard error are showed. The observed maximum activity was defined as 100%. (D) Relative activity of Lp_0796 after incubation with 1 mM concentrations of different additives. The activity of the enzyme incubated in the absence of additives was defined at 100%.

Figure 1


1
2
A
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MF
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Figure 2

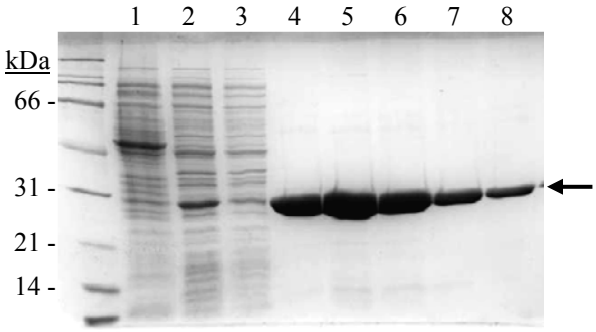


Figure 3

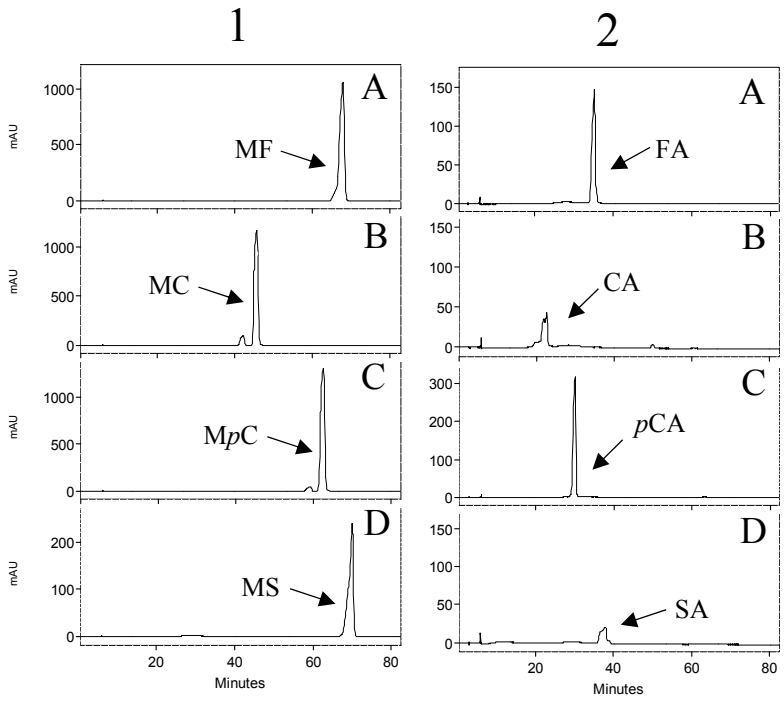
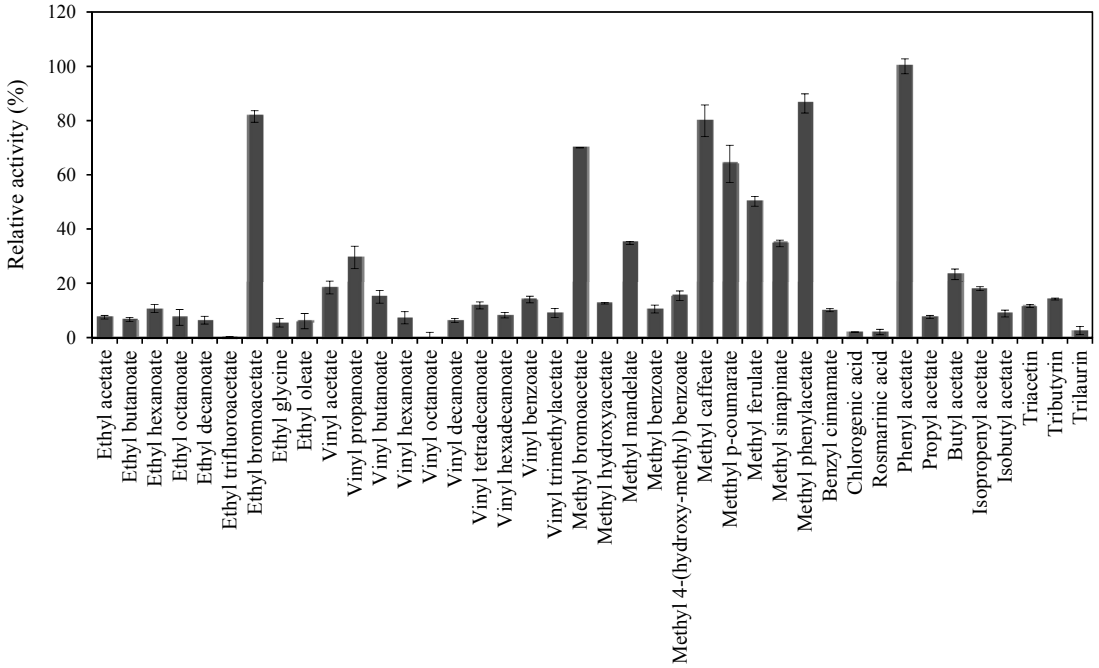


Figure 4

A


Relative activity (%)
120
100
80
60
40
20
0
Ethyl acetate
Ethyl butanoate
Ethyl hexanoate
Ethyl octanoate
Ethyl decanoate
Ethyl trifluoroacetate
Ethyl bromoacetate
Ethyl glycine
Ethyl oleate
Vinyl acetate
Vinyl propanoate
Vinyl butanoate
Vinyl hexanoate
Vinyl octanoate
Vinyl decanoate
Vinyl tetradecanoate
Vinyl hexadecanoate
Vinyl benzoate
Vinyl trimethylacetate
Methyl bromoacetate
Methyl hydroxyacetate
Methyl mandelate
Methyl benzoate
Methyl 4-(hydroxy-methyl) benzoate
Methyl caffeate
Metthyl p-coumarate
Methyl ferulate
Methyl sinapinate
Methyl phenylacetate
Benzyl cinnamate
Chlorogenic acid
Rosmarinic acid
Phenyl acetate
Propyl acetate
Butyl acetate
Isopropenyl acetate
Isobutyl acetate
Triacetin
Tributyrin
Trilaurin

B

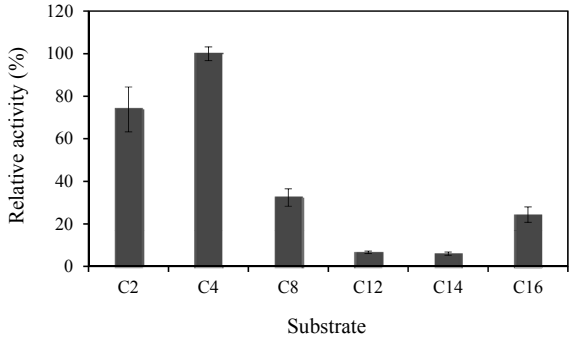
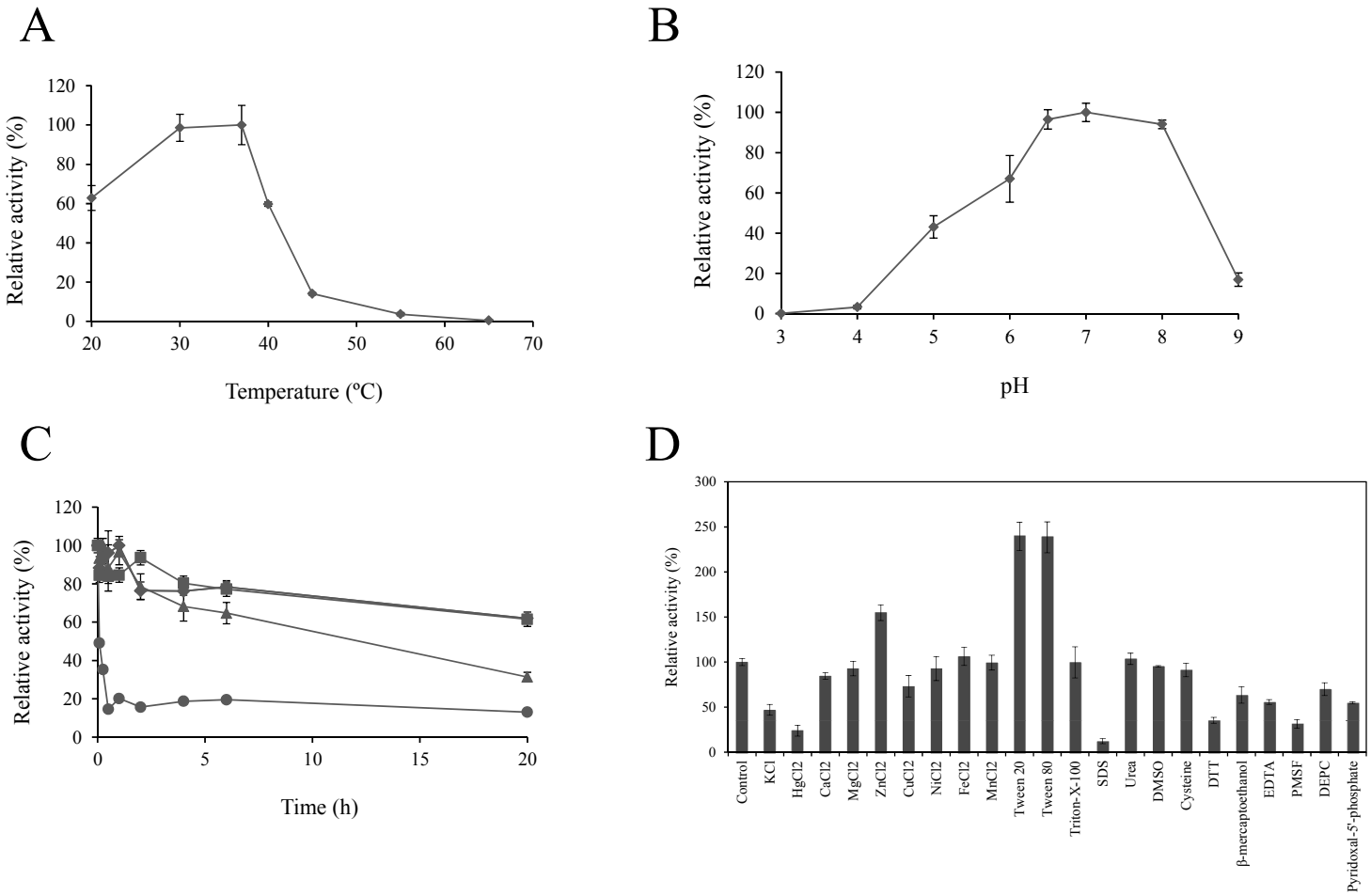

Relative activity (%)
120
100
80
60
40
20
0
C2
C4
C8
C12
C14
C16
Substrate

Figure 5


A
Relative activity (%)
120
100
80
60
40
20
0
20
30
40
50
60
70
Temperature (ºC)
B
Relative activity (%)
120
100
80
60
40
20
0
3
4
5
6
7
8
9
pH
C
Relative activity (%)
120
100
80
60
40
20
0
0
5
10
15
20
Time (h)
D
Relative activity (%)
300
250
200
150
100
50
0
Control
KCl
HgCl2
CaCl2
MgCl2
ZnCl2
CuCl2
NiCl2
FeCl2
MnCl2
Tween 20
Tween 80
Triton-X-100
SDS
Urea
DMSO
Cysteine
DTT
β-mercaptoethanol
EDTA
PMSF
DEPC
Pyridoxal-5'-phosphate