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 hydroxycinnamates 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 on 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. Ruiz-Barba (Instituto de la Grasa, CSIC, Spain), respectively. Strains *L. plantarum* CECT 220 (ATCC 8014), CECT 221
(ATCC 14431), CEC 223, CECT 224, CECT 749 (ATCC 10241), CECT 4185, and CECT 4645 were purchased from the Spanish Type Culture Collection (CECT). Strains L. plantarum DSM 1055, DSM 2648, DSM 10492, DSM 13273, and DSM 20246 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). Type strains of L. plantarum subsp. plantarum CECT 748\(^T\) (ATCC 14917\(^T\)) and L. plantarum subsp. argentorantensis DSM 16365 were purchased from the CECT and DSMZ, respectively. L. plantarum strains (L. plantarum RM28, RM31, RM34, RM35, RM38, RM39, RM40, RM41, RM71, RM72, and RM73) were isolated from grape must and wine samples (23). Lactobacillus paraplantarum DSM 10641 (ATCC 10776) and DSM 10677 were purchased from the DSMZ and included in the study. L. plantarum strains were routinely grown in MRS media (Pronadisa, Spain) adjusted to pH 6.5 and incubated at 30 °C. For degradation assays, L. plantarum WCFS1 strain was cultivated in a modified basal and defined medium described previously for L. plantarum (24). The basal medium was modified by the replacement of glucose by galactose. This defined medium was used to avoid the presence of phenolic compounds included in non-defined media. The sterilized modified basal medium was supplemented at 1mM final concentration with hydroxycinnamoyl esters filter-sterilized. The L. plantarum inoculated media were incubated at 30 °C, in darkness. The phenolic products were extracted from the supernatants twice with ethyl acetate (one third of the reaction volume).

Escherichia coli DH10B was used for all DNA manipulations. E. coli BL21(DE3) was used for expression in pURI3-TEV vector (25). E. coli strains were cultured in Luria-Bertani (LB) medium at 37 °C and 140 rpm. When required, 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 OD₆₀₀nm 0.5 was reached (10⁵-10⁶ 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 hydroxycinnamoyl 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 (GGTGAACCTGTATTCCAGGGCatgatgctgaaacaaccggaaccgt) and 704 (ATCGATAAGCTTAGTTAGCTATTAtcatttataatgttttaaatat) (the nucleotides pairing
the expression vector sequence are indicated in italics, and the nucleotides pairing the
lp_0796 gene sequence are written in lowercase letters). The pURI3-TEV vector
encodes expression of a leader sequence containing a six histidine affinity tag. The
corresponding 831 pb purified PCR product was then inserted into the pURI3-TEV
vector by using a restriction enzyme- and ligation-free cloning strategy (25). E. coli
DH10B cells were transformed and the recombinant plasmids were isolated. Those
containing the correct insert, as identified by restriction-enzyme analysis, were further
verified by DNA sequencing and used for transformation of E. coli BL21 (DE3) cells.
E. coli cells carrying the recombinant plasmid pURI3-TEV-0796, were grown at
37 ºC in LB media containing ampicillin (100 µg/ml) and induced by adding 0.4 mM
IPTG. After induction, the cells were grown at 22 ºC during 20 h and harvested by
centrifugation (7500g for 15 min at 4 ºC). Cells were resuspended in 50 mM sodium
phosphate buffer, pH 7.0 containing 300 mM NaCl. Crude extracts were prepared by
French Press lysis of cell suspensions (three cycles at 1100 psi). The lysate was
centrifuged at 17400g for 40 min at 4 ºC.
The supernatant obtained was filtered through a 0.22 µm filter (Millipore) and
gently mixed for 20 min at room temperature with 1 ml TALON resin (Clontech). The
resin was washed with 50 mM sodium phosphate buffer pH 7.0 containing 300 mM
NaCl and 10 mM imidazole. The recombinant His6-tagged protein was eluted with 50
mM sodium phosphate pH 7.0 containing 300 mM NaCl and 150 mM imidazole. The
eluted His6-tagged Lp_0796 was dialysed overnight at 4 ºC against 50 mM sodium
phosphate buffer, pH 7.0 containing 300 mM NaCl. The purity of the enzyme was
determined by 12.5% sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-
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 °C 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 C18 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
substrate. The rate of hydrolysis of \( p \)-nitrophenyl butyrate for 10 min at 37 °C was measured in 50 mM sodium phosphate buffer pH 7.0 at 348 nm in a spectrophotometer (UVmini-1240 Shimadzu). The reaction was stopped by chilling on ice.

In order to carry out the reaction (1 ml), a stock solution of 25 mM of \( p \)-nitrophenyl butyrate was prepared in acetonitrile/isopropanol (1:4, v/v) (26) and mixed with 50 mM sodium phosphate buffer (pH 7.0) to obtain a 1 mM substrate final concentration. Control reactions containing no enzyme were utilized to account for any spontaneous hydrolysis of the substrates tested. Enzyme assays were performed in triplicate.

**Substrate specificity.** To investigate the substrate specificity of Lp_0796, activity was determined using different \( p \)-nitrophenyl esters of various chain lengths (Sigma-Aldrich): \( p \)-nitrophenyl acetate (C2); \( p \)-nitrophenyl butyrate (C4); \( p \)-nitrophenyl caprylate (C8); \( p \)-nitrophenyl laurate (C12); \( p \)-nitrophenyl myristate (C14) and \( p \)-nitrophenyl palmitate (C16) as substrates. A stock solution of each \( p \)-nitrophenyl ester was prepared in acetonitrile/isopropanol (1/4 v/v). Substrates were emulsified to a final concentration of 0.5 mM in 50 mM sodium phosphate buffer, pH 7.0, containing 1.1 mg/ml Arabic gum and 4.4 mg/ml Triton X-100 (18). Reaction mix consisted of 990 μl of emulsified substrate and 10 μl of enzyme solution (1 μg protein). Reactions were carried out at 37 °C in a spectrophotometer (UVmini-1240 Shimadzu) as described above.

The enzymatic substrate profile of purified protein was determined by using an ester library described previously (27). \( p \)-Nitrophenol was used as pH indicators to monitor ester hydrolysis colorimetrically. The screening was performed in a 96-well Flat Bottom plate (Sarstedt) where each well contains a different substrate (1 mM) in 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 MgCl₂, KCl, MnCl₂, FeCl₂, CuCl₂, NiCl₂, CaCl₂, HgCl₂, ZnCl₂, 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 MgCl₂, 200 μM of each dNTP, 1 U of AmpliTaq 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 °C 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 one 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
the correct molecular mass eluted from the TALON resin by washing with a buffer containing 150 mM imidazole (Fig. 2). The eluted protein was then dialyzed against sodium phosphate buffer (50 mM, pH 7.0) to remove the imidazole, which may interfere in the feruloyl esterase enzymatic activity assays.

Feruloyl esterase activity of pure *L. plantarum* Lp_0796 was performed by using the four hydroxycinnamoyl esters (methyl ferulate, methyl caffeate, methyl *p*-coumarate, and methyl sinapinate) as substrates at 1 mM final concentration. Fig. 3 shows that the four hydroxycinnamoyl esters were fully hydrolyzed by Lp_0796 in our experimental conditions, revealing Lp_0796 as a feruloyl esterase.

**Biochemical properties of Lp_0796.** An ester library was used to test the substrate range of Lp_0796. This ester library consisted of esters, which were chosen to identify acyl chain length preferences of the esterase, and also the ability of Lp_0796 to hydrolyze hindered or charged substrates (27). In addition, the activity of Lp_0796 against *p*-nitrophenyl esters of various chain lengths, from C2 (*p*-nitrophenyl acetate) to C16 (*p*-nitrophenyl palmitate) was assayed. The highest hydrolytic activity was observed on phenyl acetate, followed by methyl phenyl acetate and ethyl and methyl bromoacetate (Fig. 4A). Moreover, the ester library confirmed that the four model substrates for feruloyl esterases were efficiently hydrolyzed by Lp_0796 (methyl caffeate, methyl *p*-coumarate, methyl ferulate, and methyl sinapinate). Other esters substrates were also hydrolyzed, although less efficiently (methyl mandelate, vinyl propanoate, vinyl acetate, vinyl benzoate, vinyl butanoate, methyl benzoate, butyl acetate, and isopropenyl acetate, among others). Regarding the *p*-nitrophenyl esters assayed, Lp_0796 showed maximum activity against the short acyl chain esters, *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate (Fig. 4B), although activity against *p*-nitrophenyl caprilate (C8) and *p*-nitrophenyl palmitate (C16) was also observed.
Therefore, according to the substrates hydrolyzed, it can be concluded that Lp_0796 is a feruloyl esterase with a relatively wide specificity spectrum, not described previously in any other esterase from lactic acid bacteria. Since feruloyl esterases are enzymes with a broad range of applications, and Lp_0796 is the first feruloyl esterase described in *L. plantarum*, its biochemical properties have been characterized. Fig. 5 shows the optimum pH, temperature and the thermal stability of Lp_0796 determined using *p*-nitrophenyl butyrate as substrate. Lp_0796 displays an optimal activity at 30-37 °C, showing marginal activity at 45 °C (14% of the maximal activity) (Fig. 5A). In fact, Lp_0796 can be classified as a heat-labile enzyme since its activity decreased drastically after incubation for a few minutes at 45 °C or after 20 hours incubation at 22 °C where the esterase showed only 60% of its maximal activity.

Fig. 5D shows the effect of various additives (1 mM final concentration) on the enzymatic activity of Lp_0796. It can be observed that the activity of Lp_0796 is greatly increased by the addition of Tween 20 and Tween 80 (250%) and ZnCl₂ (155%), being not significantly affected by FeCl₂, NiCl₂, MnCl₂, cysteine, CaCl₂, MgCl₂, DMSO, urea, and Triton-X-100 (relative activity 85 to 105%), only partially inhibited by CuCl₂, DEPC, KCl, EDTA, β-mercaptoethanol, pyridoxal-5-phosphate (relative activity 55-73%), and greatly inhibited by SDS, HgCl₂, DTT, and PMSF (relative activity 12-35%). The effect of Tween 20 on Lp_0796 seems to be concentration-dependent, since at concentration of 1 mM is an activating additive (250%) whereas at 5-10% concentration the esterase was inactivated to a significant extend.

**Presence of Lp_0796 among *L. plantarum* strains.** In order to know the extent of the presence of feruloyl esterase Lp_0796 among *L. plantarum* strains, the presence 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 oligonucleotides 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
encoding putative esterases were found. As a considerable degree of structural diversity have been described between feruloyl esterases (30), it is not possible to predict the biochemical function of the esterases encoded by these *L. plantarum* ORFs. The first *L. plantarum* WCFS1 ORF annotated as putative esterase (carboxylesterase) is *lp_0796*. While this work was in progress, *Lp* _0796* (Est0796) has been described (20). It was demonstrated that *Lp* _0796* is an esterase, which showed maximum activity towards short acyl chain lengths (C2-C4). However, the activity of *Lp* _0796* against hydroxycinnamoyl esters was not analyzed. In the present study, the activity of *Lp* _0796* against the four model hydroxycinnamoyl esters for feruloyl esterases has been assayed. We demonstrate that these compounds were fully hydrolyzed by *Lp* _0796* in our experimental conditions, revealing that *Lp* _0796* shows feruloyl esterase activity. Feruloyl esterases exhibit distinct specificity spectra concerning the release of cinnamic acids, and they are in fact organized into functional classes, which take into account substrate specificity against synthetic methyl esters of hydroxycinnamic acids. According to the present results, *Lp* _0796* can be considered a type C feruloyl esterase since it hydrolyses the four methyl esters of hydroxycinnamic acids generally used as model substrates (31).

It is interesting to note that the hydrolytic activity observed in *L. plantarum* cell-extracts does not perfectly correlate with the activity observed with the pure *Lp* _0796* protein, since methyl caffeate and methyl sinapinate were not hydrolyzed by the cell-free extracts. As only a minor hydrolysis of methyl ferulate and methyl *p*-coumarate was observed in the cell extracts, it is possible that *Lp* _0796* could have higher activity on these substrates, and therefore, the activity on methyl caffeate and methyl sinapinate was not detected. However, it is obvious that the presence in *L. plantarum* WCFS1 of
enzymes possessing feruloyl/p-coumaroyl esterase activity other than Lp_0796 cannot be discarded.

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

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

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

Most of the metabolism of phenolic compounds in lactic acid bacteria remains unknown, however, the description of new enzymatic activities help to uncover it. In relation to hydroxycinnamic compounds, a decarboxylase of hydroxycinnamates has been previously described in \textit{L. plantarum} (34). The subsequent actions of the feruloyl esterase described in the present study (Lp\_0796), and that of a vinyl reductase, which remains unknown, could allow \textit{L. plantarum} to metabolize compounds abundant in fermented plant-derived food products (hydroxycinnamoyl esters). However, since the components of plant cells are constituted by complex carbohydrates, the ability of Lp\_0796 to degrade this biological material needs further investigation.

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\textbf{REFERENCES}


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_pC), methyl sinapinate (MS), ferulic acid (FA) and *p*-coumaric acid (pCA) 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 His6-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 °C. The methyl ferulate (MF), methyl
caffeate (MC), methyl p-coumarate (MPC), methyl sinapinate (MS), ferulic acid (FA),
caffeic acid (CA), p-coumaric acid (pCA), 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 22°C (filled diamond), 30°C (filled square), 37°C
(filled triangle), and 45°C (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

B

C

D

A

B

C

D

FA

MF

MC

pCA

MpC

MS

MF

MC

pCA

MpC

MS

Minutes

Minutes
Figure 2
Figure 3

1

A

MF

B

MC

C

MpC

D

MS

Minutes

2

A

FA

B

CA

C

pCA

D

SA

Minutes