Characterization of a cold-active and salt-tolerant esterase from *Lactobacillus plantarum* with potential application during cheese ripening

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ABSTRACT

During cheese ripening, the metabolic activity of microorganisms influences cheese flavour. *Lactobacillus plantarum* is a non-starter lactic acid bacteria which can be found during cheese ripening. An esterase-encoding gene from *L. plantarum*, *lp_3505*, was cloned and expressed in *Escherichia coli* BL21 (DE3). The biotechnologically produced Lp_3505 protein was purified as an active soluble form using His-tag affinity chromatography. The enzyme has an optimal pH and temperature of 6 and 5 ºC, respectively. The enzyme showed remarkable stability at 20 ºC, retaining more than 60% of its maximal activity after 20 h incubation at this temperature. In addition, NaCl concentrations lower than 20% increased esterase activity. The cold-activity and salt-tolerance exhibited by Lp_3505 indicated that this esterase could be an useful exogenous enzyme to be added for cheese ripening.

1. Introduction

Carboxylate esters are lipophilic molecules used as flavouring ingredients in the food industry. Ester hydrolysis and synthesis can be catalyzed by carboxyl esterases (Bornscheuer, 2002). Carboxylic ester hydrolases comprise esterases and lipases. Typically, esterases catalyze the hydrolysis of water-soluble and short- to medium-length aliphatic esters, and lipases which display high activity towards water-insoluble long-chain esters (Bornscheuer, 2002).

The flavour of mature cheese is the result of biochemical changes that occur in the curd during ripening, caused by the interaction of starter bacteria and enzymes from the milk, and coagulant, and the secondary microbiota (Collins, McSweeney, & Wilkinson, 2003). *Lactobacillus plantarum* is an important non-starter lactic acid bacteria species which can be found during cheese ripening. *L. plantarum* is a good source of esterase enzymes since lipolytic and esterase activity have been previously described in *L. plantarum* strains (Otherholm, Ordal, & Witter, 1968). Although esterases or lipases are common in *L. plantarum*, so far only few have been purified or recombinantly produced.

Although numerous genome sequences from *L. plantarum* are currently available, there is still limited information on the function of genes coding for esterases and their potential contribution to cheese aroma. In this regard, a wide study to dissect the complex array of esterase activities in *L. plantarum* cells was designed by our group. The objective of this study was to determine the functional features of the esterase Lp_3505 from *L. plantarum*, through biochemical characterization of the recombinantly produced protein, and to establish if its biochemical properties are adequate for its possible application during cheese manufacture.

2. Materials and methods

2.1. Strains, plasmids, media and materials

L. plantarum WCFS1, kindly provided by M. Kleerebezem (NIZO Food Research, The Netherlands), was grown in MRS medium (Pronadisa, Spain) adjusted to pH 6.5 and incubated at 30 ºC. *Escherichia coli* DH10B (Invitrogen, Warrington, UK) was used as host strain for all DNA manipulations. *E. coli* BL21 (DE3) was used for heterologous expression in the pURI3-TEV vector (Curiel, de las Rivas, Mancheño, & Muñoz, 2011). *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 ºC with shaking at 200 rpm. When required, ampicillin and chloramphenicol were added at a concentration of 100 or 20 μg mL^{-1}, respectively.

Plasmid DNA was extracted by a High Pure plasmid isolation kit (Roche, Manheim, Germany). PCR product was purified with a QIAquick gel extraction kit (Qiagen, Hilden, Germany). *Dpn*I and HS Prime Star DNA polymerase were obtained from TaKaRa. His-tagged protein was purified by a Talon Superflow resin (Clontech, Mountain View, Ca, USA).

2.2. Cloning of Lp_3505 esterase

Genomic DNA from *L. plantarum* WCFS1 was extracted as previously described (Vaquero, Marcobal, & Muñoz, 2004). The gene encoding a putative acetylesterase (*lp_3505*) in *L. plantarum* WCFS1 was amplified by PCR by using the primers 679 (5´-GGTGAAAACCTGTATTTCCAGGGCATGTCAATTCATAGCAATAACTTTT) and 680 (5´-

ATCGATAAGCTTAGTTAGCTATTATTAAACATTGCGCCAACCTCCCTTA). Prime Star HS DNA polymerase (TaKaRa) was used for PCR amplification. The 792-bp purified PCR product was inserted into the pURI3-TEV vector using a restriction enzyme- and ligation-free cloning strategy (Curiel et al., 2011). This vector produces recombinant proteins having a six-histidine affinity tag in their N-terminal. *E. coli* DH10B cells were transformed and, for expression, the recombinant plasmids obtained were transformed into *E. coli* BL21 (DE3).

2.3. Enzyme production and purification

E. coli BL21(DE3) harbouring pGro7 (TaKaRa), a vector overexpressing GroES/GroEL chaperones, was transformed with the recombinant plasmid pURI3-TEV-3505. The enzyme was produced as previously described for esterase Lp_2631 (Esteban-Torres, Mancheño, de las Rivas, & Muñoz, 2014), and purified by IMAC using a Talon Superflow resin (Esteban-Torres et al., 2014).

2.4. Enzyme activity assay

Esterase activity was determined by a spectrophotometric method previously described using *p*-nitrophenyl acetate (Sigma-Aldrich, Steinheim, Germany) as the substrate (Esteban-Torres, Reverón, Mancheño, de las Rivas, & Muñoz, 2013).

The substrate specificity of Lp_3505 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 as described previously

(Brod, Vernal, Bertoldo, Terenzi, & Maissonnave Arisi, 2010; Esteban-Torres et al., 2013).

2.5. Biochemical characterization

Esterase activity was assayed in the pH range from 3.0 to 9.0, and at temperatures of 5, 20, 30, 37, 40, 45, 55, and 65 ºC as described previously (Esteban-Torres et al., 2013). Enzyme thermostability was measured by incubation of the enzyme 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, 4, 6, and 20 h. After incubation, the residual activity was measured as described above.

The effect of NaCl on Lp_3505 activity was determined by adding NaCl at concentrations ranging from 0 to 25% (w/v). For the reaction mixtures, different volumes of 25% NaCl solution were added, and the volume of the buffer was adjusted accordingly to maintain the final reaction volume. Reactions mixtures were pre-incubated for 5 min at room temperature before the substrate was added. After the reaction, the absorbance was measured.

3. Results and discussion

Numerous ORFs encoding putative esterases/lipases have been found in the genome of *L. pantarum* WCFS1. Among these, Lp_3505 esterase was recombinantly produced and characterized. The *lp_3505* gene was cloned into the pURI3-TEV expression vector (Curiel et al. 2011) and transformed into *E. coli* BL21 (DE3). SDS-PAGE analysis of cell extracts showed that there was one major band of protein, of

approximately 30 kDa, present as inclusion bodies in the insoluble fraction (data not shown). In order to obtain Lp_3505 in a soluble form, plasmid pGro7, producing GroEs/GroEL chaperones, was used. When pURI3-TEV-3505 and pGro7 plasmids were used simultaneously, Lp_3505 appeared in the intracellular soluble fraction of the cells (Fig. 1). Lp_3505 was purified by immobilized metal affinity chromatography. Routinely, about 15 mg of purified protein was obtained from 1L culture.

Purified Lp_3505 protein was biochemically characterized. Esterase activity was determined using *p*-nitrophenyl esters possessing different acyl chain lengths from C2 to C16. Except for *p*-nitrophenyl laurate (C12), Lp_3505 was active on all the substrates assayed, exhibiting a preference for *p*-nitrophenyl acetate. Recently a cold-active esterase from *L. plantarum*, Lp_2631, was characterized. Although Lp_2631 also showed preference for *p*-nitrophenyl acetate, it was unable to degrade esters possessing a chain length higher than C4 (Esteban-Torres et al., 2014). However, except for *p*-nitrophenyl laurate (C12), Lp_3505 exhibited approximately 25-30% of its maximal activity on C4 to C16 esters (data not shown). This result indicates that Lp_3505 is mainly an esterase but, in contrast to Lp_2631, also exhibits putative lipase activity.

Apart from Lp_2631, which exhibited maximal activity at 20 ºC (Esteban-Torres et al., 2014), the *L. plantarum* esterases previously described showed higher optimal temperature for enzyme activity. Lp_3505 exhibited maximum activity at 5 ºC (Fig. 2A). The enzyme is active only at low temperatures; at temperatures higher than 35 ºC, it showed only residual activity (e. g., 17% of the maximal activity at 37 ºC) (Fig. 2A). Thus, Lp_3505 is an esterase which shows maximal activity at refrigeration temperatures. This is an important property in cheese ripening, during which temperatures can be lower than 15 ºC (6-10 ºC), and esterase activity could be important even at storage temperatures (Yilmaz, Ayar, & Akin, 2005)

In order to know the thermostability of Lp_3505 enzyme, it was preincubated at different temperatures for different time intervals, before assaying the residual activity. The enzyme may be easily inactivated during milk pasteurization. However, Lp_3505 retained 80% of its maximal activity at 20 ºC after prolonged incubated time (Fig. 2B). This data confirmed the potential usefulness of Lp_3505 as an exogenous enzyme for raw milk cheeses or to be added after milk pasteurization for cheese ripening.

Low pH may reduce, or completely inactivate, the activity of enzymes that could generate either flavour components or flavor precursor compounds. Regarding the influence of pH on activity, Lp_3505 showed maximal activity at pH 6 (Fig. 2C). A similar optimal pH has been found in the cold-active Lp_2631 esterase (Esteban-Torres et al., 2014); however, in contrast to Lp_2631, which exhibited very low activity at pH 5.0, Lp_3505 showed 35% of its maximal activity at this pH, which is typical during cheese making or ripening.

In order to understand the potential role of Lp_3505 esterase activity during cheese manufacture, it is also important to study the influence of NaCl on esterase activity. *L plantarum* could be found in cheese in which a high salt concentration is present. Since *L. plantarum* is a non-halophilic bacterium, which would often encounter NaCl concentrations up to 20% in brines, it seems likely that specific proteins produced by this bacteria could be adapted to remain active under high salt concentrations. The effect on NaCl on Lp_3505 activity was assayed. It seems that concentrations commonly found in cheeses, lower than 20%, significantly increased Lp_3505 activity (Fig. 2D). The behaviour showed by Lp_3505 in relation to salt is uncommon, as only a few halophilic esterases have been discovered and characterized so far (Schreck & Grunden, 2014).

As compared to other *L. plantarum* esterases described previously, esterase Lp_3505 described in this study (i) exhibited a broad activity range, as it hydrolyzed esters

possessing a long acyl chain, (ii) showed higher enzymatic activity at pH 5, (iii) is a cold-active enzyme possessing the highest activity at refrigeration temperatures, and (iii) finally, exhibits high tolerance to salt concentrations as high as 20%.

4. Conclusion

The biotechnologically produced esterase Lp_3505 from *L. plantarum* is a cold-active esterase that exhibited maximum activity at 5 ºC. In addition, esterase Lp_3505 not only showed a good activity at low temperatures and acidic pH, but also remained active at high concentration of salt. Based on the findings reported in this study, it appears that Lp_3505 is an enzyme potentially used as an exogenous enzyme during the elaboration of raw milk cheeses or potentially added after milk pasteurization for cheese ripening.

Acknowledgements

This work was financially supported by grants AGL2011-22745 and BFU2010-17929 (MINECO), S2009/AGR-1469 (ALIBIRD) (Comunidad de Madrid), and RM2012-00004 (INIA). We are grateful to J.M. Barcenilla and M. V. Santamaría for their assistance. M. Esteban-Torres and L. Santamaría are recipient of predoctoral fellowships from the JAE-CSIC or FPI-MINECO, respectively.

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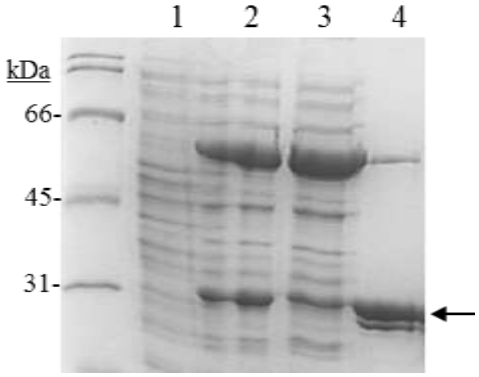
Figure captions

Fig. 1. Purification of Lp_3505 esterase from *L. plantarum* WCFS1. Analysis of soluble cell extracts of IPTG-induced *E. coli* BL21(DE3) (pGro7)(pURI3-TEV) (1) or *E. coli* BL21(DE3) (pGro7) (pURI3-TEV-3505) (2), flowthrough (3), or Lp_3505 protein eluted after His affinity resin (4). The arrow indicated the overproduced and purified protein. Molecular mass markers are located at the left (SDS-PAGE Standards, Bio-Rad).

Fig. 2. Biochemical properties of Lp_3505 esterase. (A) Temperature-activity profile of Lp_3505. (B) Thermal stability profile for Lp_3505 after preincubation at 22 ºC (filled diamond), 30 ºC (filled square), 37 ºC (filled triangle), 45 ºC (cross), 55 ºC (star), and 65 ºC (filled circle) in phosphate buffer (50 mM, pH 7), at indicated times, aliquots were withdrawn, and analyzed as described in the Materials and Methods section. (C) pH-activity profile of Lp_3505. (D) Activity of Lp_3505 in the presence of NaCl. The activity of the enzyme in the absence of NaCl was defined as 100%. The experiments were done in triplicate. The mean value and the standard error are showed. The percentage of residual activity was calculated by comparing with unincubated enzyme.

Figure 1



267 Figure 2

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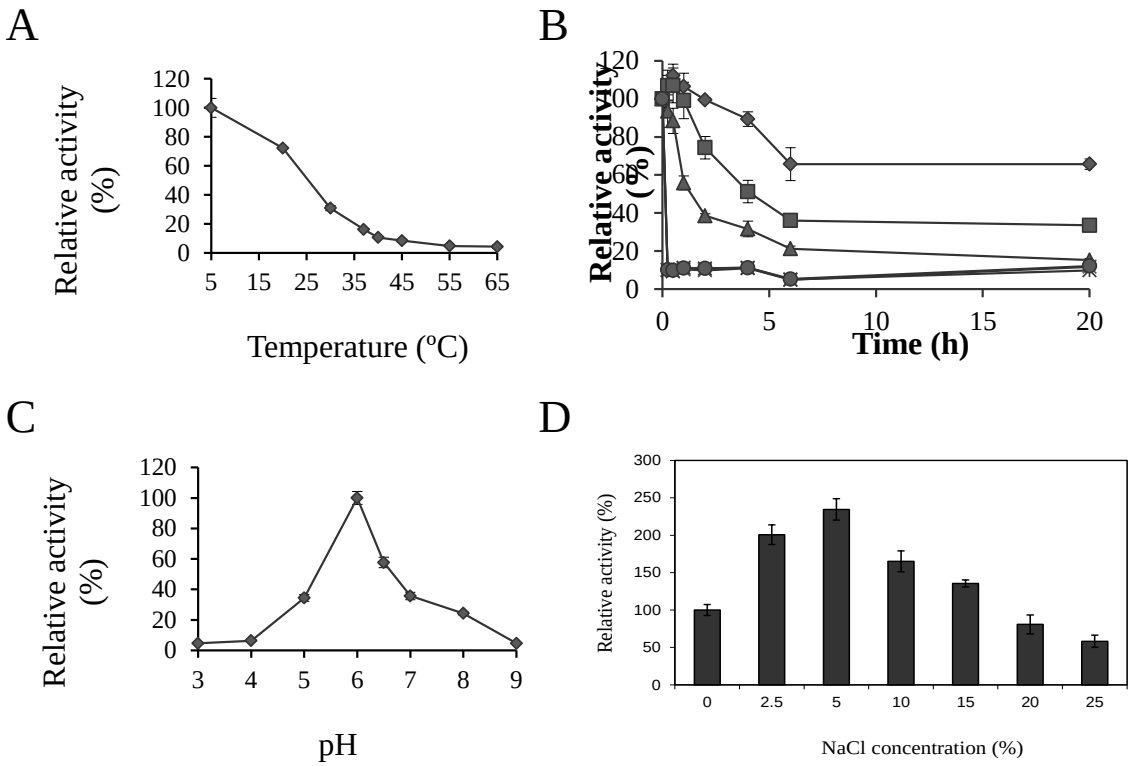

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