Esterase activities of intracellular extracts of wild strains of lactic acid bacteria isolated from Serra da Estrela cheese

Angela C. Macedo[a,b], Tânia G. Tavares[a], F. Xavier Malcata[a,*]

[a]*Escola Superior de Biotecnologia, Rua Dr. António Bernardino de Almeida, P-4200-072 Porto, Portugal*
[b]*Instituto Superior da Maia, Avenida Carlos Oliveira Campos, P-4475-690 S. Pedro de Avioso, Castelo da Maia, Portugal*

Keywords: Dairy foods; Starter/nonstarter culture; Enzyme activity; Cheese ripening

Abstract

Lactococcus lactis subsp. *lactis* strain ESB110019 and *Lactobacillus plantarum* strain ESB5004, novel strains that were previously isolated from the wild adventitious microflora of certified Serra da Estrela cheeses, were assayed for esterase activity using, as substrates, *ortho*- and *para*-nitrophenyl derivatives of fatty acids. Both strains preferentially hydrolyzed short-chain fatty acids; *L. lactis* ESB110019 exhibited a stronger esterase activity than *Lb. plantarum* ESB5004 and cleaved the *p*-nitrophenyl adducts faster than their *o*-nitrophenyl counterparts (unlike *Lb. plantarum* ESB5004).

Introduction

Lipolysis in Serra da Estrela cheese, although not extensive, leads to a typical overall concentration of free fatty acids of ca. 8000 mg/kg in ripened cheeses. Short-chain free fatty acids tend to increase in concentration late in the ripening period, hence suggesting that they are selectively released from the cheese triglycerides by its microflora (Macedo & Malcata, 1996). Serra da Estrela cheese is manufactured from raw milk; adventitious lactic acid bacteria, which are intrinsically different from those present in commercial starters (Requena, Pelaez, & Desmazeaud, 1991), as well as psychrotrophs, micrococci and yeasts, may play a role in lipolysis (Fox, Singh, & McSweeney, 1996). This study therefore investigates (and duly compares) the esterase activities of intracellular extracts of two dominant wild strains of lactic acid bacteria, previously isolated from Serra da Estrela cheese, in attempts to assess their potential as part of a tentative starter/non starter culture, specifically designed for manufacture of this gourmet cheese.

* Corresponding author. Fax: +351-22-5090351.
E-mail address: xmalcata@esb.ucp.pt (F.X. Malcata).

Materials and methods

Preparation of biomass

The strains selected for this study, viz. *Lactobacillus plantarum* strain ESB5004 and *Lactococcus lactis* subsp. *lactis* strain ESB110019, have been isolated from Serra da Estrela cheeses, as described elsewhere (Macedo, Malcata, & Hogg, 1995), and properly identified using SDS-PAGE protein profiling analysis—ID4916 and ID5166, respectively (BCCM™/LMG, Gent, Belgium).

The harvested cells were stored at −80 °C in 30% (v/v) aqueous glycerol stock solutions. MRS and M17 broths (60 ml, from Merck, Darmstadt, Germany) were inoculated at 1%(v/v) with the stock cultures of *Lb. plantarum* ESB5004 and *L. lactis* ESB110019, respectively. Cultivation and harvesting of cells followed the procedure described by Macedo, Tavares, and Malcata (2001).

Preparation of cell extracts

Cell-free extracts were obtained as follows: after the cells were harvested, they were washed twice, then incubated with lysozyme (Sigma, St. Louis MO, USA) and mutanolysine (Sigma), and finally subjected to mechanical disruption using glass beads (150–212 μm,

from Sigma) in an ice bath, according to the method reported by Macedo et al. (2001). The nucleic acids of the intracellular extract were hydrolyzed by addition of RNAse (Sigma) and DNAse (Sigma), in the presence of $MgCl_2$ (Sigma), and then precipitated in the presence of $MnSO_4$ (Sigma) (Macedo et al., 2001). The supernatant thus obtained, termed hereafter as cell- and nucleic acid-free extract (CNFE), was kept frozen at −30 °C in several Eppendorf vials, until the enzyme assays were in order.

Determination of protein concentration

The micro protein determination kit by Sigma (Kit No. 690-A) was used to quantitate protein in the CNFE, using bovine serum albumin as standard (Ohnishi & Barr, 1978).

Determination o festerase activity

The esterase activity was measured using the following substrates, all dissolved to a final concentration of 8 mM in acetone (Merck): *o*- and *p*-nitrophenyl butyrate, *o*- and *p*-nitrophenyl caprylate, *o*- and *p*-nitrophenyl myristate, and *o*- and *p*-nitrophenyl palmitate. The reaction mixture, consisting of 1.6 ml potassium phosphate buffer (50 mM, pH 7.0), 0.2 ml substrate solution and 0.2 ml CNFE, was incubated at 30 °C for up to 6 h. Absorbance of the *o*- and *p*-nitrophenol moieties released was monitored continuously at 410 nm (UV Mini 1240, from Shimadzu, Kyoto, Japan). Enzyme and substrate blanks were monitored under the same incubation conditions so as to serve as controls. The assays were repeated for two different concentrations of CNFE. The concentrations of *o*- and *p*-nitrophenol released were estimated from calibration curves prepared in advance. The activity was calculated as the slope of the straight line fitted by linear regression to the concentration of nitrophenol-vs.-time data; the two concentrations of CNFE used gave rise to distinct datum points. The specific activity was calculated as the mole amount of substrate hydrolysed per unit time and per unit mass of protein.

Results and discussion

Lactococcus lactis ESB110019 extract exhibited the highest esterase activity on *p*-nitrophenyl butyrate (*p*NP butyrate), as concluded from inspection of Table 1. This substrate was preferentially attacked when compared with *p*NP caprylate (ca. 1.5-fold), *o*NP caprylate and butyrate (ca. 7.7-fold and 10.2-fold, respectively), and *p*NP and *o*NP myristate (ca. 233-fold and 244-fold, respectively). No esterase activity was detected on either *p*NP or *o*NP palmitate. On the other hand, *Lb. plantarum* ESB5004 extract did not exhibit esterase activity on *o*NP and *p*NP butyrate, whereas it exhibited the highest activity on *o*NP caprylate (see Table 1); this activity was higher than that on *p*NP caprylate (ca. 1.3-fold), *p*NP palmitate (ca. 7.5-fold) and *o*NP myristate (ca. 9.8-fold). No esterase activity by the strain ESB5004 was detected on *p*NP myristate or palmitate.

In addition to their active role in protein and peptide degradation during cheese ripening, it has been also shown (El Soda, Law, Tsakalidou, & Kalantzopoulos, 1995) that *Lactococcus* and *Lactobacillus* spp. play a role in fat breakdown. Both strains of lactic acid bacteria tested in this study performed in agreement with the above authors, in that they exhibited esterase activity, although at much lower levels than their peptidase counterparts (Macedo, Vieira, Poças, & Malcata, 2000). On comparing the esterase activities of those two strains, it seems, in general, that *L. lactis* ESB110019 extract is more active than *Lb. plantarum* ESB5004 extract; this observation is consistent with results reported by Brandl (1970) Jakubowska, Piatkiewicz, and Libudzisz (1978), and Jakubowska, Libudzisz, and Piatkiewicz (1980), who claimed that lactococci are significantly more esterolytic than lactobacilli.

Lactococcus lactis ESB110019 extract preferentially hydrolyzed *p*NP relative to *o*NP derivatives of fatty acids; this strain also showed preference for short- rather than long-chain fatty acid derivatives and, within the former, for *p*NP butyrate rather than caprylate. This behaviour is similar to the performance of *Lactococccus* strains reported by other authors (Chich, Marchesseau, & Gripon, 1997; Holland & Coolbear, 1996).

The substrate specificity of the esterase system of *Lb. plantarum* ESB5004 extract is, in general, similar to that exhibited by other *Lactobacillus* strains, in that caprylate derivatives are hydrolyzed at the highest rates, and

Table 1
Esterase specific activity[a] (average±standard error) of cell- and nucleic acid-free extract (CNFE) of lactic acid bacteria isolated from Serra da Estrela cheese

Substrate	Lactic acid bacteria CNFE[b]	
	Strain ESB110019	Strain ESB5004
o-Nitrophenyl butyrate	0.05±0.01	n.d.[c]
o-Nitrophenyl caprylate	0.07±0.02	0.046±0.004
o-Nitrophenyl myristate	0.002±0.001	0.005±0.002
o-Nitrophenyl palmitate	n.d.	0.006±0.004
p-Nitrophenyl butyrate	0.54±0.05	n.d.
p-Nitrophenyl caprylate	0.35±0.02	0.037±0.003
p-Nitrophenyl myristate	0.0023±0.0009	n.d.
p-Nitrophenyl palmitate	n.d.	n.d.

[a] Specific activity expressed as μmol of substrate per h and per mg of protein.
[b] ESB5004: *Lactobacillus plantarum*; ESB110019: *Lactococcus lactis* subsp. *lactis*.
[c] n.d. = Not detected.

myristate and palmitate derivatives are cleaved at negligible rates (Castillo, Requena, Fernández de Palencia, Fontecha, & Gobbetti, 1999; El Soda & Ezzat, 1993; El Soda et al., 1995; Gobbetti, Fox, & Stepaniak, 1997). However, esterase(s) from *Lb. plantarum* ESB5004 extract attack *o*NP and *p*NP butyrate at negligible rates.

Conclusions

Lactococcus lactis strain ESB110019 extract exhibits higher rates of esterolysis than *Lb. plantarum* strain ESB5004 extract. These activities are dependent on the positional isomery of the fatty acid derivative used as substrate; *p*NP derivatives are hydrolyzed faster by *L. lactis* extract, and by *Lb. plantarum* extract, at a low rate when compared with their *o*NP counterparts. Additionally, *L. lactis* extract exhibits a narrower specificity toward short-chain fatty acid derivatives, which suggests a role for this strain with regard to release of flavour compounds, especially at late stages of cheese ripening.

Acknowledgements

Thanks go to the FCT-Program PRAXIS XXI (Portugal), for the postdoctoral fellowship granted to author A. C. Macedo (ref. BPD/20158/99), and to the FCT-Program POCTI (Portugal) for funding through project MICROCHEESE (ref. POCTI/1999/BIO/36197).

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