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Enzymatic activity of lactic acid bacteria (with antimicrobial properties) isolated from a traditional Spanish cheese

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ABSTRACT

Twenty-four strains of lactic acid bacteria (LAB) isolated from a traditional Spanish cheese (Genestoso cheese) were evaluated for their enzymatic activities (acidifying and proteolytic abilities and carboxypeptidase, aminopeptidase, dipeptidase, caseinolytic and esterase activities), in order to select indigenous strains of technical interest for the manufacture of cheese. These strains were selected on the basis of their antimicrobial activity relative to five reference strains and were identified as *Lactococcus lactis* subsp. *lactis* (thirteen strains), *Leuconostoc mesenteroides* (two strains), *Leuconostoc pseudomesenteroides* (one strain), *Lactobacillus paracasei* (two strains), *Lactobacillus plantarum* (one strain) and *Enterococcus faecalis* (five strains).

Lactococcus strains were those that showed the greatest degree of acidifying and proteolytic activity. The cell-free extracts (CFE) of *L. paracasei* exhibited the highest level of aminopeptidase activity. The highest level of caseinolytic activity was shown by the CFE of one strain of *L. lactis*. High values were also obtained with the CFE of *Lactobacillus* and of several *Leuconostoc*. The highest level of dipeptidase activity was found amongst the strains of *L. lactis*. Carboxypeptidase activity was generally very low or undetectable for the majority of strains. The greatest degree of esterolytic activity was detected for *Enterococcus*.

1. Introduction

The specific characteristics of taste and aroma of craft, or traditionally made, cheese varieties may be attributed to the type of milk, its microbiological quality, the degree of acidification of the curds when moulded, the manufacturing technology or the ripening conditions. If the fact that lactic flora are crucial in liberating the specific compounds involved in the aroma and flavour of cheeses is kept in mind, there is good cause for the interest shown by cheese-makers in the type of starter cultures that must be employed. The use of indigenous lactic cultures, obtained from microorganisms isolated from craft cheeses allows the organoleptic (taste and smell) characteristics of cheese made with pasteurized milk to be brought closer to those of traditional varieties. The enzymatic potential of the strains used in the cheese-making process influences the development of the texture, aroma and flavour characteristics of cheeses. Consequently, there is an increasing interest in the design of specific indigenous starter cultures in large-scale manufacturing of traditional-type cheeses, such that they will not change the fundamental properties of the product (Pérez et al., 2003).

Proteolysis and lipolysis are biochemical events in flavour development that occur during ripening of most varieties of cheese. There is a lack of detailed information on the peptidolytic enzymes produced by non-starter lactic acid bacteria (NSLAB) (Williams et al., 1998). Moreover, only sparse information is available about the contribution of lactic acid bacteria (LAB) to lipolysis during the ripening of cheeses .

LAB strains were isolated from Genestoso cheese, a traditional cheese from the North of Spain. These strains showed antimicrobial activity in a previous study (González et al., 2007) relative to five reference strains: *Lactobacillus plantarum* CECT 748, *Listeria monocytogenes* CECT 4031, *Enterococcus faecalis* CECT 481, *Clostridium tyrobutyricum* CECT 4011 and *Staphylococcus aureus* CECT 240.

The aim of this study was to evaluate the enzymatic activities (acidifying and proteolyitic capacities and aminopeptidase, dipeptidase, carboxipeptidase, esterase and caseinolytic activities) of twenty-four strains of LAB in order to select strains of technological interest that might be used as starters in the manufacture of cheeses.

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2. Material and methods

2.1. Strains

The strains tested were selected from the LAB isolated over the course of the manufacturing and ripening processes of four batches of Genestoso cheese (Arenas et al., 2004). A total of 420 strains were isolated from different sampling points and different culture media, being identified at genus level by means of physiological and biochemical tests. Twenty-four strains were chosen on the basis of their antimicrobial activity relative to five reference strains. They were identified as *Lactococcus lactis* subsp. *lactis* (13 strains), *Leuconostoc mesenteroides* (2 strains), *Leuconostoc pseudomesenteroides* (1 strain), *Lactobacillus paracasei* (2 strains), *L. plantarum* (1 strain) and *E. faecalis* (5 strains) by API tests and genetic tests based on polymerase chain reaction amplification and DNA sequence analysis (González et al., 2007). The strains were frozen at −30 °C in MRS broth with 20% glycerol. They were cultured in the same medium at 30 °C for 16–24 h before the various assays were performed.

2.2. Enzymatic activities of whole cells

The enzymatic activities of the strains were tested using the API-ZYM test as a preliminary screening (Sacristán et al., 2006).

Extracellular quantitative proteolytic activity was determined by the o-pthaldialdehyde (OPA) method (Church et al., 1983). The results were calculated from a calibration curve obtained from the dilution of glycine in distilled water and were expressed in mM glycine L^{-1} of milk.

The acidifying activity of the strains was determined in 10% reconstituted skimmed milk powder inoculated at a level of 1% (v/v) with bacterial suspensions revitalised in MRS broth at 30 °C containing 10^9 cfu mL^{-1}. After incubation at 30 °C for 6, 12 and 24 h, pH and titratable acidity were measured in accordance with the International Dairy Federation (IDF) standard 306 (IDF, 1995). Titratable acidity was expressed as g lactic acid mL^{-1} of milk.

2.3. Preparation of cell fractions

Endocellular enzymatic activities (caseinolytic, aminopeptidase, dipeptidase, carboxypeptidase and esterase activities) were tested in a cell-free extract (CFE) obtained through disrupting cells by using lysozyme.

Strains were grown in 25 mL of MRS broth (1% inoculum) at 30 °C for 16–24 h until they had reached the stationary phase. For cells grown in MRS broth up-regulation was associated with the stationary phase because aminoacids and peptide depletion would result in a removal of repression on peptidase biosynthesis leading to higher specific activities (Kenny et al., 2003).

The cultures were then centrifuged at 8160 g for 10 min at a temperature ≤ 4 °C. The sediments once dissolved in 4 mL of TRIS–HCl buffer (50 mM, pH 7.00), were incubated at 30 °C for 1 h, in order to liberate the proteinases bound to the cell wall, and afterwards centrifuged at 5,000 g for 20 min in accordance with the method proposed by Requena et al. (1993). The supernatants were frozen.

Finally the sediments were dissolved in 20 mL of TRIS–HCl buffer containing lysozyme at a concentration of 30 mg/mL and incubated for 1 h at 37 °C in order to solubilize the cells' walls. Thereafter, as recommended by Casla et al. (1996), the cells were centrifuged at 4000 g for 14 min to obtain two fractions. One of these corresponded to the supernatant or endocellular fraction, the other to the sediment made up of cellular membranes and the cell walls. The possibility of contamination between the fractions should not be excluded, so the supernatants were filtered trough 0.22 μm syringe filters (Millipore Corporation, Bedford, U.S.A.) to obtain the CFE which were stored in frozen form until their enzyme activities were determined.

The Lowry method was used to analyse the protein concentration in the CFE (Lowry et al., 1951).

2.4. Enzymatic activity of CFE

Caseinolytic activity was measured by the method of Gómez et al. (1988) using bovine casein at a concentration of 2% in phosphate buffer (50 Mm, pH 7.00) as substrate. One unit of enzymatic activity was defined as the amount of enzyme required to give an increase in absorbance of 0.01 units after a hydrolysis period of 1 h. Results were expressed as units of caseinolytic activity per mg of protein in the CFE.

Aminopeptidase activity of the CFE was determined as described by Requena et al. (1993) using p-nitroanilides (pNA) substrates such as Ala-pNA, Leu-pNA, Lys-pNA and Pro-pNA. One unit of aminopeptidase activity was defined as the amount of enzyme giving an absorbance increase of 0.001 units. Aminopeptidase activity was expressed as the number of activity units per mg of protein in the CFE per min.

Dipeptidase activity was determined by a modification of the cadmium–ninhydrin method (Doi et al., 1981), using the following dipeptides as substrates: Leu-Leu, Tyr-Leu, Ala-Ala, Leu-Gly, Ala-Phe, Lys-Leu and Phe-Ala. One unit of enzymatic activity was defined as the amount of enzyme that produced an increase in absorbance of 0.1 units at 507 nm. The results were expressed as the number of enzymatic activity units per mg of protein in the CFE per min.

Carboxypeptidase activity of CFE was measured by spectrophotometric method of El Soda and Desmazeaud, 1982 using N-carbobenzyloxy-L-Leu as substrate. One unit of enzymatic activity was defined as the amount of enzyme that produced an increase in absorbance at 570 nm of 0.01 units. The results were expressed as enzymatic units per mg of protein in the CFE per min.

The esterase activity of the cell-free extracts (CFE) was determined as described by Castillo et al. (1999) using the following β-naphthyl substrates: β-naphthyl butyrate (C4), β-naphthyl caprylate (C8), β-naphthyl myristate (C14) and β-naphthyl stearate (C18). This method is based on the spectrophotometric measurement at room temperature of hydrolysis rates of β-naphthyl derivatives. Esterase activity was expressed as μmoles of β-naphthol released per min and per mg of protein in the CFE at 560 nm.

2.5. Statistical analysis

ANOVA analysis (Statistic 5.1 computer program; Statsoft, Tulsa, OK, USA) was carried out to determine statistical differences ($p < 0.05$) between the different bacterial strains of the same genus with respect to the values of acidification and enzyme activity. The strains were tested twice for acidifying activity and three times for each enzymatic activity.

3. Results and discussion

3.1. Enzymatic activity of whole cells

3.1.1. Acidifying activity

Table 1 shows the acidifying activity of the 24 strains of LAB isolated from Genestoso cheese, selected on the basis of their antimicrobial activity. The lactococci showed a greater acidifying activity than leuconostoc and lactobacilli, with some strains reaching values around 0.7–0.8 g 100 mL^{-1} of lactic acid after 24 h