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Controlled permeabilization of *Lactococcus lactis* cells as a means to study and influence cheese ripening processes

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Abstract

Cells of *Lactococcus lactis* were permeabilized by perturbing the membrane structure through the delipidating action of *n*-butanol to an extent that allowed normally excluded peptidase substrates to enter the cells and be accessible to intracellular enzymes. The degree of permeabilization of cells depended on the concentration of the solvent used, the duration of the treatment, the density of the cell suspension, pH and temperature. This was indicated by the optimum or maximum activities of the intracellular peptidases aminopeptidase N and aminopeptidase X. The possible usefulness of permeabilized cells for cheese-ripening studies was demonstrated with cells that were treated with 5 ml L^{-1} *n*-butanol at pH 6.5 and 25 °C using a mixture of chymosin-generated primary cheese peptides as substrate. Production of amino acids could be correlated with conversion of peptides that had entered the permeabilized cells and reflected the course of amino acid production in cheese.

Keywords: *Lactococcus lactis*; Membrane permeabilization; Intracellular peptidase accessibility

1. Introduction

Cheese ripening is governed mainly by enzymes from rennet and starter organisms. The view that intracellular enzymes, which are responsible for amino acid production and the generation of cheese flavour, are ineffective in cheese until the cells die and lyse, is long-standing and seems widely accepted. The sooner these enzymes are released by cell lysis (i.e., partial or total cell wall removal and opening of the cell through membrane rupture), the sooner can they participate in processes like proteolysis, amino acid conversion and other flavour-generating reactions. There has been a recent revival of interest in the role of lysis of starter cells in ripening, and there have been various studies to analyze the process and to find ways of inducing and controlling the lytic event in cheese. Molecular genetic approaches to the construction of novel starters with suitable lytic features involve the exploitation of the autolytic phenotype, the lytic system of phages, and the possibility to control the induction of a prophage (Fox et al., 1996; Gasson, 1996; Ruyter de, Kuipers, Meyer, & de Vos, 1997; Boutrou, Sepulchre, Gripon, & Monnet, 1998).

Although lysis by itself is an effective way to create the conditions that promote the interactions between intracellular enzymes and their extracellular substrates, it may not be the most obvious approach for two reasons. First, the incidence of bursting of cells in the cheese subsequent to the action of a lytic system may be questioned. This was pointed out more than 15 years ago by Thomas and Pritchard (1987) and their arguments have not been disproved today. Our knowledge of the fate of osmotically sensitized (spheroplasted) or completely stripped (protoplasted) cells in cheese is still very poor. Both the semi-solid gel structure in which cells are embedded and the osmotic stability provided by the high solute concentrations in the moisture phase of the cheese may effectively protect sphero- or protoplasts

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against bursting (rupture) unless lysis involves the emergence of a highly unstable membrane sac that has lost its coherency. Second, if lysis of cells and subsequent release and diffusion of intracellular enzymes or enzyme systems into the cheese matrix occurs, it probably has not the same impact as that resulting from efficiently structured and optimally functioning intracellular enzyme systems because of the inevitable dislocation and dilution of the released enzyme systems.

These considerations imply that permeabilization of the cell membrane may be more effective than cell lysis in realizing the full ripening potential of the enzymes. It may allow peptides, amino acids, and other low molecular weight substrates to enter the cell freely to interact with intracellular enzymes which may still be physiologically organized with respect to location, concentrations, existence of functional complexes, availability, and regeneration (or synthesis) of cofactors, etc. Moreover, such a relatively undisturbed physiological system may remain stable for a longer period. If this hypothesis is correct, lysis of the cell would not then result in a further improvement of the contribution of intracellular enzymes to the ripening process, in fact the opposite would be the case.

When considering protection of cells in cheese against lysis, it may be assumed that in those cases of supposed increased "lysis" in cheese, as established on account of increased intracellular activities in cheese extracts, the results could be mainly attributed to induced burst and release caused by the extraction method applied. Furthermore, if significant effects on proteolysis and flavour development have been established as well, this could very well have been the result of permeabilization/perforation of the membrane rather than lysis and release.

These considerations prompted us to develop a method to permeabilize the membrane of cheese-starter bacteria by limited delipidation in a way that preserves cell-bound activities. Such permeabilized cells could be used in model systems to study cheese-ripening processes in a relatively short period of time under conditions prevailing in cheese, or in cheese to influence ripening. In the present study, the model system involves primary cheese peptides that are generated by chymosin and used by starter cells to produce amino acids.

2. Materials and methods

2.1. Bacterial strain, growth, and harvest of cells

Lactococcus lactis subsp. *lactis* MG1363 was used. The strain harbours the plasmid pNZ563, which codes for a mutant cell envelope proteinase Lactocepin (EC 3.4.21.96) (hereafter designated PrtP) in which the wild-type amino acid sequence AKT 137–139 in the substrate-binding region has been substituted by GLA. This substitution resulted in an unique specificity that modifies the breakdown of chymosin-generated primary cheese peptides under cheese conditions (Exterkate, Slangen, & Siezen, 2001). Cells were grown and harvested as previously described (Exterkate et al., 2001). They were resuspended in 50 mM imidazole/10 mM $CaCl_2$, pH 6.5, using an amount of buffer that, after 60-fold dilution, gave an optical density at 650 nm (OD_{650}) of 0.5.

2.2. Treatment of cells with n-butanol

Cells were treated with *n*-butanol (analytical grade, J.T. Baker Chemicals, Deventer, Holland) either by adding the solvent (0–100 μL) to the above cell suspension (1 mL) and thorough mixing, or by resuspending cells in *n*-butanol/buffer mixtures. After the incubation period at the indicated temperature, the cells were washed five times with (each time) 8 mL 50 mM imidazole/10 mM $CaCl_2$, pH 6.5, and finally resuspended in this buffer or in 50 mM sodium acetate/10 mM $CaCl_2$, pH 5.2 (2 mL). In the cases that diluted suspensions were used for cell treatment, the treated and washed cells were resuspended in proportional smaller volumes in order to obtain suspensions with equal final densities for enzyme activity assays.

2.3. Assay of enzyme activities

Aminopeptidase N (PepN), aminopeptidase X (PepX) and PrtP activities were measured with, respectively, lysyl–*p*-nitroanilide (Lys–*p*NA), glycyl(or alanyl)–prolyl–*p*NA [Gly (or Ala)–Pro–*p*NA] (these substrates from Sigma Chemical Co., St. Louis, MO, USA) and succinyl–alanyl–glutamyl–prolyl–phenylalanyl–*p*NA (S–Glu) (Bachem AG, Bubendorf, Switzerland) at pH 6.5 and 25 °C by following the release of *p*-nitroaniline under steady-state conditions (Exterkate & Alting, 1999). In the case of PepN and PepX, 100 μL of the treated cell suspension was diluted with 400 μL 50 mM imidazole/10 mM $CaCl_2$, pH 6.5, and the reaction was started by adding 500 μL 2 mM substrate in 50 mM imidazole, pH 6.5.

PrtP activity was measured with 1 mL 1:99-diluted cell suspension in 50 mM imidazole/10 mM $CaCl_2$, pH 6.5, containing 1 mM S–Glu.

2.4. Incubation of permeabilized cells with primary cheese peptides (secondary proteolysis)

A mixture of primary cheese peptides was obtained and incubated with untreated or *n*-butanol-treated cells as described elsewhere (Exterkate et al., 2001). Reversed-phase HPLC analysis of the conversion of these peptides and analysis of the amino acids in the