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Quantitative proteomic analysis of bacterial enzymes released in cheese during ripening

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ABSTRACT

Due to increasingly available bacterial genomes in databases, proteomic tools have recently been used to screen proteins expressed by micro-organisms in food in order to better understand their metabolism in situ. While the main objective is the systematic identification of proteins, the next step will be to bridge the gap between identification and quantification of these proteins. For that purpose, a new mass spectrometry-based approach was applied, using isobaric tagging reagent for quantitative proteomic analysis (iTRAQ), which are amine specific and yield labelled peptides identical in mass. Experimental Swiss-type cheeses were manufactured from microfiltered milk using *Streptococcus thermophilus* ITG ST20 and *Lactobacillus helveticus* ITG LH1 as lactic acid starters. At three ripening times (7, 20 and 69 days), cheese aqueous phases were extracted and enriched in bacterial proteins by fractionation. Each sample, standardised in protein amount prior to proteomic analyses, was: i) analysed by 2D-electrophoresis for qualitative analysis and ii) submitted to trypsinolysis, and labelled with specific iTRAQ tag, one per ripening time. The three labelled samples were mixed together and analysed by nano-LC coupled on-line with ESI-QTOF mass spectrometer. Thirty proteins, both from bacterial or bovine origin, were identified and efficiently quantified. The free bacterial proteins detected were enzymes from the central carbon metabolism as well as stress proteins. Depending on the protein considered, the quantity of these proteins in the cheese aqueous extract increased from 2.5 to 20 fold in concentration from day 7 to day 69 of ripening.

1. Introduction

Various bacteria – lactic acid bacteria, bifidobacteria, and dairy propionibacteria – are used in dairy processing for their technological or probiotic properties. Whole genome sequencing allows the detection of genes of technological interest but cannot inform us about their real expression in situ in food. For that purpose, transcriptomic, and above all, proteomic approaches are required.

They rely respectively on the extraction from food of bacterial RNA or bacterial proteins: RNA is fragile and bacterial proteins are in general in a dynamic range largely lower than the proteins of the food itself, which make both extractions complicated. Both approaches were developed to explore bacterial metabolism in cheeses: transcriptomic whole genome expression for lactococci in UF model cheeses, metabolic activity and free proteins released through lysis by thermophilic lactic starters in Swiss type cheeses (Cretenet et al., 2011; Falentin et al., 2012; Gagnaire et al., 2004, 2009). In these Swiss cheese, like in Cheddar (Sheehan et al., 2005) a mixture of lysed bacteria, and bacteria still metabolically active was observed. Regarding proteins, the identification of the most predominant was achieved from 2D gel electrophoresis after an enrichment procedure, but until yet, without any quantification.

The last ten years have been rich in emergent quantitative technologies based either on gel electrophoresis or on chromatographic separations and mass spectrometry analysis. In order to circumvent the difficulty of quantifying proteins between gels, different mass spectrometry-based approaches have been developed using isotopic labelling for protein or peptide quantification. Based on liquid chromatographic separations, a number of advantages over 2DE can be distinguished. In contrast to proteins, peptides are more soluble, have similar size and are more easily ionised in the mass spectrometer ion source (Delahunty and Yates, 2005). Moreover, peptide separations by LC actually exhibit greater sensitivity, superior dynamic range, are more easily automated and faster than 2DE (O'Donnell et al., 2004).

The ICAT technology (Isotope Coded Affinity Tag) was the first isotopic labelling presented in the literature. It uses isotopic labelling of cysteine amino acid residues by a light or heavy tag containing biotinylated reagents differing by 8 Da (Gygi et al., 1999). However, the specificity of the ICAT labelling technique to cysteine amino acid residues is limited since about 15% of proteins do not contain any cysteine amino acid residues. Another technique available since 2004

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(Ross et al., 2004) is the iTRAQ™ (Isobaric Tagging for Relative and Absolute Quantitation) technique which is specific to primary amines found on the N-term end of the peptides and on the lysine and arginine side chains and permits relative quantitation of 2 to 8 samples simultaneously (Choe et al., 2007). In contrast to ICAT, peptides differentially labelled by iTRAQ appear as single peaks in MS thanks to the presence of a mass balancing moiety that render all labels isobaric. During MS/MS fragmentation, the isotope encoded reporter ions are released to allow relative quantification. The iTRAQ method has the double advantage of permitting the identification of the proteins and their quantification in a single LC-MS/MS run. The iTRAQ Method has been utilized in very different fields such as biomarker discovery (Fenselau, 2007), elucidation of cellular signalling pathways (Sui et al., 2007), post-translational modification analysis such as phosphorylation (Sachon et al., 2006), correlations between genomics and proteomics (Scherl et al., 2006), time course analysis (Cong et al., 2006; Jagtap et al., 2006), bacterial analysis (Chong et al., 2006; Danielsen et al., 2007), and membrane or sub-cellular analysis (Lund et al., 2007).

In the field of dairy products, few studies have yet been achieved using amine-reactive isobaric tagging reagents and mostly for studying protein expression in cows (Lippolis et al., 2006) or to quantify protein changes in milk fat globule membranes (Reinhardt and Lippolis, 2008).

In this work iTRAQ labelling was applied to proteins present in Swiss type cheese extracts at different points of the ripening, focusing in particular on bacterial proteins. Such a dynamic approach can contribute to a better insight on in situ starter metabolism.

2. Materials and methods

2.1. Chemicals

The following chemicals were used: Tris (Prolabo, WWR International, Fontenay sous Bois, France), DTT (plus one, GE Healthcare Bio-Sciences, Saclay, France), iodoacetamide (Sigma-Aldrich, Saint Quentin Fallavier, France), sequencing grade modified trypsin (Promega, Charbonnières, France), trifluoroacetic acid (TFA, Pierce, Touzart et Matignon, Vitry sur Seine, France), α-cyano-4-hydroxycinnamic acid matrix (CHCA, LaserBioLab, Sophia-Antipolis, France), acetonitrile Chromasolv® (Sigma-Aldrich), HPLC gradient grade water (Fisher scientific, Loughborough, Zeicestershire, UK), hydrochloric acid (HCl, Carlo Erba Reagenti, Chaussée du Vexin, France) formic acid and nitrilotriacetic acid (Fluka, Sigma-Aldrich, Saint Quentin Fallavier, France) and iTRAQ reagent kit for plasma applications (Applied Biosystems, Framingham, MA).

2.2. Swiss-type cheese manufacture

Mini Swiss-type cheeses were made in duplicate in a pilot plant (Pierre Guérin, Mauzé sur le Mignon, France). Fifty litres of raw milk were skimmed, then micro-filtered at 50 °C through a 1.4 μm pore size Sterilox membrane (Société des Céramiques Techniques, Bazet, France) in order to eliminate contaminant flora from the milk (Trouvé et al., 1991). Milk was standardised to 0.83 ratio of fat to total nitrogen using cream heat treated at 90 °C for 45 min. Controlled flora were inoculated with 10^5 cfu/mL at 32 °C for each strain, as described below. The *Streptococcus thermophilus* starter strain, Pal ITG ST20 (Standa, Caen, France), was first cultured at 43 °C for 5 h in 412 A Marstar commercial medium (Rhodia, Dangé Saint Romain, France) and inoculated at 10 mL per 10 kg of milk. The other starter strain *Lactobacillus helveticus* Pal ITG LH1 (Standa) was cultured at 42 °C for 5 h in PHAGEX commercial medium (Standa) and inoculated at 10 mL per 10 kg of milk. The main ripening flora represented by *Propionibacterium freudenreichii* subsp *freudenreichii* Pal ITG P23 (Standa) was directly inoculated into the microfiltered milk at 0.5 g of lyophilised strain per 10 kg of milk, to mimic the industrial process, corresponding to an average amount of 10^4 cfu/mL.

Milk was matured for 30 min at 32 °C and renneting was carried out at the same temperature with Maxiren (DSM, Lille, France) filtered through 0.22 μm. Clotting occurred 20 to 25 min after renneting. The cutting time was determined as 1/5 of the clotting time. After cutting, the curd was gently stirred and heated from 32 to 45 °C at a rate of 0.5 °C/min and from 45 to 53 °C at a rate of 1 °C/min. Stirring was maintained for 15 min after heating. The curd grains were then drained off into the moulds and pressed at 12 kg/cheese for 4 h at 45 °C. The mould was turned first at the end of pressing, and a second time 15 h after. It was then cooled to 12 °C. The Swiss-type wheels were demoulded and brined in a saturated NaCl solution (pH 5.2) at 12 °C for 1 h. The wheels were then ripened first in a temperate room, (12 °C, with relative humidity of 85% for 13 days), then in a warm room at 22 °C with relative humidity of 75% until day 49 and finally in a cold room at 4 °C until the end of ripening at day 69. Enumeration of the different bacteria in the cheeses and the physico-chemical analyses were performed according to the methods described by Thierry et al. (1998). Two independent cheeses were manufactured.

2.3. Aqueous phase extraction

Aqueous phases were extracted from cheese aliquots after 7, 20 and 69 days of ripening, according to the method of Kuchroo and Fox (1982): 10 g grated Swiss-type cheese were dispersed in 40 g of MilliQ water (Millipore, St Quentin en Yvelines, France) and blended with a high shear batch dispenser, ultra turrax (Janke and Henkel, Bioblock, Illkirch, France) for 3 min at 20,500 rpm and further stirred for 30 min at 40 °C. Cheese samples were centrifuged at 10,000 × *g* for 10 min at 20 °C on a Beckman J2-HS centrifuge equipped with a JA-20 rotor, to separate the aqueous phase from the pelleted caseins. The aqueous phases were successively filtered through Whatman paper 541 (Prolabo, Bruchet Dano, Rennes, France) then through 1.2 and 0.45 μm pore size cellulose acetate membrane filters (Sartorius, Palaiseau, France). The cheese aqueous phases were concentrated about 10 times on Centriprep YM-10, 10 kDa MW cut off (Millipore) and stored at −20 °C until use.

2.4. Pre-fractionations steps

In order to increase the concentration of bacterial proteins in the cheese aqueous phases prior to iTRAQ quantification, two prefractionation steps were used: affinity chromatography to selectively remove most of the lactoferrin, a major bovine protein present into the cheese aqueous extracts, and size exclusion chromatography to separate bacterial proteins from residual caseins and peptides derived from casein hydrolysis throughout ripening and from whey proteins. The affinity chromatography was performed on Hi Trap Blue 1 ml (GE Healthcare) with the following buffers filtered on 0.45 μm: A: 0.05 M Tris–HCl, pH 8; B: 0.1 M NaOH and C: 2 M KBr and the following operating conditions: flow 1 ml/min and absorbance measured at 280 nm on an Äkta chain (GE Healthcare). The cheese aqueous phases were diluted to 3:4 cheese aqueous phase concentrate with 0.2 M Tris–HCl pH 8 (i.e. 0.05 M final). Two millilitres of the diluted sample were injected and the unloaded sample corresponding to aqueous phase depleted in lactoferrin was collected from 1 to 6.5 min of run with 100% A buffer. Proteins retained on Hi trap other than lactoferrin were also collected from the column with 100% B buffer for 3 min. The fractions 1 and 2 were subsequently neutralised to pH 7 with 1 M HCl and lyophilised on a Serail RP2V (SGD, Argenteuil, France). Lactoferrin was finally removed from the column by pulses of B and C buffers for 1 min each to rinse the column and remove all the lactoferrin (repeat 6 pulses). Then the column was re-equilibrated