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Identification of large phosphopeptides from β-casein that characteristically accumulate during ripening of the semi-hard cheese Herrgård

Y. Ardö*, H. Lilbæk, K.R. Kristiansen, M. Zakora, J. Otte

Department of Food Science, Dairy Technology, Centre for Advanced Food Studies, The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK 1958 Frederiksberg C, Denmark

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Abstract

The peptide composition of a cheese reflects its characteristic ripening process, and in this study, large hydrophobic phosphopeptides that accumulate in semi-hard cheese, Herrgård, were identified. Anion exchange chromatography, reverse phase (RP) HPLC, liquid chromatography mass spectrometry and N-terminal amino acid sequencing were used. Milk from homozygotic cows was used to prepare β-casein A1 and A2, and peptides produced from their hydrolysis by plasmin were analysed to support identification of the peptides in cheese. Eight large phosphopeptides released by plasmin hydrolysis of β-casein were identified in the semi-hard cheese, i.e., fractions (f29–105, f29–107, f1–105, f1–107) A1 and A2. Four other peptides that accumulated in the cheese, co-eluted on RP-HPLC with the large primary plasmin-derived phosphopeptides and contributed significantly to two characteristic large peaks, i.e., one peak for each of the two genetic variants A1 and A2 of two β-casein fractions, mainly (f29–93) but also (f30–93).
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Keywords: Phosphopeptides; Semi-hard cheese; Peptide identification; LC–MS

1. Introduction

During cheese ripening casein is hydrolysed to peptides and amino acids by a broad range of enzymes. Some of the peptides accumulate because they are not easily further broken down by the enzymes present in the cheese in question. Although a number of enzyme activities are common to many cheese varieties, the peptide composition at different ages is characteristic of the cheese variety (Ardö, 2001; Ardö & Kristiansen, 2002; Coker, Crawford, Johnston, Singh, & Creamer, 2005). Reverse phase (RP) HPLC of water or pH 4.6 soluble fractions of cheese are commonly used for analysing characteristic peptide profiles. These profiles may cover over thousands of peptides derived from the four original casein molecules of different genetic and chemical variants. Some of these peptides have been identified in exploratory studies using mainly amino acid sequencing and mass spectrometry (MS) (Addeo et al., 1992, 1994; Boutrou, Mollé, & Léonil, 2001; Exterkate, Lagerwerf, Haverkamp, & van Schalkwijk, 1997; Lund & Ardö, 2004; Sousa, Ardö, & McSweeney, 2001). The peptides that typically accumulate in semi-hard cheese are produced by microbial proteolytic activity on the rennet derived peptide α_{s1}-casein (f1–23) and from β-casein (Sousa et al., 2001). A large number of small phosphopeptides derived from α_{s1}-, α_{s2}-, and β-casein have been identified in semi-hard cheese, and these were found in considerably higher amounts in semi-hard cheese as compared with an extra-hard cheese also analysed in the same investigation (Lund & Ardö, 2004). A characteristic for the semi-hard cheese Herrgård is accumulation of large hydrophobic peptides that elute in a narrow group from RP-HPLC in a similar way as the large phosphopeptides from primary plasmin activity on β-casein (Ardö & Gripon, 1995; Ardö & Kristiansen, 2002; Antonsson, Molin, & Ardö, 2003; Exterkate & Alting, 1995; Madsen & Ardö, 2001). Those large peptides are poorly separated using RP-HPLC and

*Corresponding author. Tel.: +45 3528 3193; fax: +45 3528 3190.
E-mail address: ya@kvl.dk (Y. Ardö).

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have not been properly identified in cheese extracts. By knowing the identities of the peptides that typically accumulate in a specific cheese during ripening enhance the possibilities to control the ripening process by actions taken during cheese production. This knowledge is also highly useful when assuring the authenticity of the cheese variety.

The objective of this study was to identify the hydrophobic peptides that accumulate during ripening of semi-hard cheese like Herrgård, which typically elute together in a narrow group with late retention time on RP-HPLC chromatograms. The investigations comprise identification of the large hydrophobic peptides resulting from plasmin activity on purified β-casein A1 and A2, separation of the peptides from cheese using anion exchange chromatography (AEC) and RP-HPLC, and identification of peptides using liquid chromatography–MS (LC–MS) and N-terminal amino acid sequencing.

2. Material and methods

2.1. Cheese samples

The semi-hard cheese studied, Herrgård, is a pressed, round-eyed (diameter, 5–15 mm) cheese made in low cylinders of about 12 kg. It is produced in Sweden from pasteurised cows' milk using calf rennet and a mesophilic undefined DL-starter culture including various *Lactococcus lactis* (including also citrate using strains) and *Leuconostoc* spp. (Ardö, 1993, 2004). During manufacture, the cheese grains are heated stepwise to around 40 °C and the cooking time is rather long. The cheese is brine salted to a NaCl content of 0.9–1.3%. Fat content is 28% (w/w) and moisture in non-fat substance 53–57% (w/w). The cheeses are waxed to prevent any microflora from growing on the surfaces during ripening. They are consumed from 3 months of ripening and until it is about 1 year old. The flavour is mild and aromatic with a sweet touch, and the flavour intensity increases with age.

Herrgård cheese was made at the former cheese-making pilot plant in Falkenberg, Sweden, using the conditions described above. Peptide profiles of cheeses were analysed using RP-HPLC after 24 h, 15 weeks and 21 weeks. Two 21-week old cheeses from the same vat were used for LC–MS analysis as well as fractionation by AEC. N-terminals of selected phosphopeptides, which had been partly purified using AEC and RP-HPLC, were characterised by Edman degradation and amino acid analysis.

2.2. Crude fractionation of cheese peptides

The pH 4.6 soluble fraction of all cheeses was prepared according to the method described by Ardö and Polychroniadou (1999). Frozen grated cheese samples were thawed overnight in a refrigerator and kept for 1 h at room temperature prior to weighing 10.0 g into a beaker and adding 40 mL 0.5 M tri-sodium citrate buffer (pH 8.1–8.3). The slurry was stirred at 40–45 °C for 1 h and then transferred quantitatively into a 100 or 200 mL flask that was filled with deionised water and mixed carefully. The pH was approximately 7 at this point, and 80 mL of the slurry was transferred to a 100 mL flask, to which 11.3 mL of 1 M HCl were added while shaking vigorously, and then pH was adjusted to 4.4–4.5. The volume was made up to 100 mL with deionised water and the samples were filtered through a rough ashless filter paper (Whatman 45 cat. no. 1440150, Whatman International Ltd, Maidstone, England) and subsequently a 0.45 μm filter (Millipore, Bedford, MA, USA). The filtrate of fractions diluted to 200 mL was analysed by RP-HPLC. The more concentrated fractions (diluted to 100 mL in the first step) were further fractionated by first AEC and then RP-HPLC and LC–MS. Filtered samples were kept frozen until use.

2.3. Preparation of β-casein A1 and A2 from milk of homozygotic cows

Milk from two cows that were homozygotic for β-casein A1 and A2, respectively, was collected in separate cans. Acid casein was prepared from the skimmed milk at the Swedish Agricultural University, SLU, Uppsala, Sweden, using the method of McKenzie (1970). HCl (1 M) was added to ca. 18 L milk in a stainless steel vessel with mechanical stirring at a rate to reach pH 4.6 in 30–40 min, and then stirring was continued for another 20–30 min. The acid whey was drained off the curd through a double cheesecloth, and the acid curd was washed with 5 L acid deionised water (1 mL L^{-1} 1 M HCl) at about 8 °C during stirring for 30 min. Then the cleaning water was drained off through double cheesecloth for about 1 h. The samples of acid casein were frozen and transported to the pilot plant at The Royal Veterinary and Agricultural University, KVL, Denmark, and kept there frozen at −18 °C until fractionation by AEC.

The crude wet casein of milk from each of the two cows was thawed for two days in a refrigerator and washed again with the same procedure as described above. For about 3 kg of wet casein 5 L deionised water acidified to pH 4.6 with HCl was added. The suspension was centrifuged at 1900 × g and 4 °C for 30 min. A total amount of ca. 2.4 kg wet casein (corresponding to ca. 1 kg dry casein) was dissolved in 12 L sample buffer of pH 8 under stirring for 2–3 h. The sample buffer contained 0.02 M Tris(hydroxymethyl)-aminomethane, biology grade (no. 1.4405–1), 2 mM DTT, dithiothreitol (no. 1.12013.00100) and 3.3 M urea (extra pure) (all from Merck, Darmstadt, Germany). The pH was adjusted to 6.8–7.2 with 1 M NaOH, giving a viscous solution (ca. 8% casein) that was kept at 4 °C overnight with stirring. Next morning, pH was adjusted to exactly 6.8 with 1 M NaOH, and the casein solution was filtered through cheesecloth prior to fractionation on a large size AEC column (High Load 26/10 Q-Sepharose Fast Flow column (Pharmacia, Uppsala, Sweden) during the next few days at 8–10 °C.

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