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Antibacterial Activities of Peptides from the Water-Soluble Extracts of Italian Cheese Varieties

C. G. Rizzello,[1] I. Losito,[2] M. Gobbetti,[1] T. Carbonara,[2] M. D. De Bari,[2] and P. G. Zambonin[2,3]
[1]Dipartimento di Protezione delle Piante e Microbiologia Applicata,
[2]Dipartimento di Chimica, and
[3]Laboratorio di Spettrometria di Massa per la Proteomica,
Università degli Studi di Bari, 70126 Bari, Italy

ABSTRACT

Water-soluble extracts of 9 Italian cheese varieties that differed mainly for type of cheese milk, starter, technology, and time of ripening were fractionated by reversed-phase fast protein liquid chromatography, and the antimicrobial activity of each fraction was first assayed toward *Lactobacillus sakei* A15 by well-diffusion assay. Active fractions were further analyzed by HPLC coupled to electrospray ionization-ion trap mass spectrometry, and peptide sequences were identified by comparison with a proteomic database. Parmigiano Reggiano, Fossa, and Gorgonzola water-soluble extracts did not show antibacterial peptides. Fractions of Pecorino Romano, Canestrato Pugliese, Crescenza, and Caprino del Piemonte contained a mixture of peptides with a high degree of homology. Pasta filata cheeses (Caciocavallo and Mozzarella) also had antibacterial peptides. Peptides showed high levels of homology with N-terminal, C-terminal, or whole fragments of well known antimicrobial or multifunctional peptides reported in the literature: α_{S1}-casokinin (e.g., sheep α_{S1}-casein (CN) f22–30 of Pecorino Romano and cow α_{S1}-CN f24–33 of Canestrato Pugliese); isracidin (e.g., sheep α_{S1}-CN f10–21 of Pecorino Romano); kappacin and casoplatelin (e.g., cow κ-CN f106–115 of Canestrato Pugliese and Crescenza); and β-casomorphin-11 (e.g., goat β-CN f60–68 of Caprino del Piemonte). As shown by the broth microdilution technique, most of the water-soluble fractions had a large spectrum of inhibition (minimal inhibitory concentration of 20 to 200 μg/mL) toward gram-positive and gram-negative bacterial species, including potentially pathogenic bacteria of clinical interest. Cheeses manufactured from different types of cheese milk (cow, sheep, and goat) have the potential to generate similar peptides with antimicrobial activity.
(**Key words:** antibacterial peptide, Italian cheese)

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Corresponding author: M. Gobetti; e-mail: gobbetti@agr.uniba.it.

Abbreviation key: ACE = angiotensin-I converting enzyme, **ESI-IT** = electrospray ionization-ion trap, **MS** = mass spectrometry, **OPA** = o-phthaldialdehyde, **RP-FPLC** = reversed phase fast protein liquid chromatography, **TFA** = trifluoroacetic acid.

INTRODUCTION

During the last decade, fundamental studies have opened a new field of research dealing with bioactive or biogenic substances derived from foods. Numerous definitions have been given for bioactive peptides and one of the most appropriate could be the following: components (genuine or generated) of consumption-ready foods which may exert a regulatory activity in the human organism, irrespective of their nutritive functions (Meisel, 2001). Milk proteins are currently the main source of a range of biologically active peptides. Most of these bioactivities in milk are encrypted within the primary structure of milk proteins, requiring proteolysis for their release from precursors. Proteolysis may release the biogenic peptides during gastrointestinal transit or during food processing. For a comprehensive view of the physiological activities of bioactive peptides, see the reviews by Clare and Swaisgood (2000), Meisel (2001), Gobbetti et al. (2002, 2004), Korhonen and Pihlanto (2003), and Floris et al. (2003).

Compared with other bioactivities (e.g., antihypertensive peptides), only a few reports have considered the enzymatic release of antimicrobial peptides in milk and dairy products. Antimicrobial activity can be easily demonstrated by well-diffusion assay (Schillinger and Lucke, 1989), broth microdilution technique (Recio and Visser, 1999; Malkoski et al., 2001; Pellegrini et al., 2001), and during microbial growth in food matrix. This strictly correlates with their potential role in nature. After discovering lactenin in milk treated with rennet (Jones and Simms, 1930), a number of potent antimicrobial peptides have been reported in the literature, namely 1) casecidins, a group of basic, glycosylated, and high molecular mass (~5 kDa) polypeptides, released

from chymosin-treated CN (Lahov et al., 1971); 2) isracidin, which corresponds to the N-terminal fragment of α_{s1}-CN (Hill et al., 1974); 3) casocidin-I, isolated from acidified milk and corresponding to the fragment f150–188 of α_{s2}-CN (Zucht et al., 1995); 4) kappacin, corresponding to nonglycosylated, phosphorylated bovine caseinomacropeptide (κ-CN f106–169) (Malkoski et al., 2001); and 5) lactoferricin, isolated from a peptic hydrolysate of bovine and human lactoferrins (f17–41 and f1–47, respectively) (Bellamy et al., 1992; Liepke et al., 2001). More recently, an antimicrobial peptide, corresponding to β-CN f184–210, was synthesized by hydrolysis of human sodium caseinate with a partially purified proteinase of *Lactobacillus helveticus* (Minervini et al., 2003). Overall, the activity of antibacterial peptides is defined as a membrane-lytic activity, where they tend to assemble to form channels, with specificity for prokaryotic cell membranes (Floris et al., 2003). Many peptides have α-helical structures, are cationic, and amphipathic, but there are also hydrophobic α-helical peptides that possess antimicrobial activity (Epand and Vogel, 1999). Compared with antibiotics, antimicrobial peptides have the advantages of being able to kill target cells rapidly and having a broad spectrum of activity, including activity toward some of the most important antibiotic-resistant pathogens in clinics. Because the rate of killing is higher than the rate of bacterial multiplication, the potential to overcome drug resistance is enhanced (Bechinger, 1997).

Overall, the majority of bioactive peptides, including antimicrobial, have been identified in milk, milk hydrolysates, and fermented milks (Meisel, 2001; Gobbetti et al., 2004). Just a few reports have considered the potential of cheeses in containing biogenic substances. Meisel et al. (1997) described the presence of low-molecular-mass angiotensin-I converting enzyme- (**ACE**) inhibitory peptides in several ripened cheeses. Angiotensin-I converting enzyme inhibitory peptides were also identified in Italian cheeses characterized by short and medium ripening periods (Addeo et al., 1992; Smacchi and Gobbetti, 2000), Manchego Spanish cheese (Gomez-Ruiz et al., 2002), enzyme-modified (Haileselassie et al., 1999), and low-fat (Ryhänen et al., 2001) cheeses. Caseinophosphopeptides were isolated during ripening of cooked curd cheeses such as Comtè (Roudot-Algaron et al., 1994) or Grana Padano (Pellegrino et al., 1997). It was shown that once generated, bioactive peptides might have selective inhibitory activity toward bacterial enzymes responsible for cheese ripening (Gobbetti et al., 2002). Overall, it was demonstrated that biotechnology, type of starters and coagulants, and ripening conditions in cheese making might affect the synthesis of bioactive peptides. To our knowledge, no studies have considered cheeses as a potential source of antimicrobial peptides (Smacchi and Gobbetti, 2000).

This paper describes the antibacterial activities of the water-soluble extracts of 9 Italian cheese varieties that differ for several biotechnological traits. Peptides with high levels of homology with N-terminal, C-terminal, or whole fragments of well known antimicrobial and multifunctional peptides reported in the literature were isolated and identified from water-soluble extracts.

MATERIALS AND METHODS

Cheeses

Nine Italian cheese varieties were considered in this study: Parmigiano Reggiano, Pecorino Romano, Fossa, Canestrato Pugliese, Caciocavallo, Gorgonzola, Crescenza, Mozzarella, and Caprino del Piemonte. As shown in Table 1, these varieties differ mainly for type of cheese milk, starter, technology, and time of ripening. Based on the moisture content, cheeses are usually classified as extrahard (Parmigiano Reggiano, Pecorino Romano, Fossa, and Canestrato Pugliese), hard (Caciocavallo), or fresh (Gorgonzola, Crescenza, Mozzarella, and Caprino del Piemonte) varieties. Caciocavallo and Mozzarella are also included in the "pasta filata" cheeses, and Gorgonzola belongs to the "blue cheese" variety. Cheeses were supplied in triplicate by official cheese makers and were stored at 4°C for a few hours before preparing the water-soluble extracts.

The pH of cheeses was determined as described by the International Dairy Federation (1989). Total nitrogen (N) and pH 4.6-soluble N were determined by the microKjeldahl method.

Water-Soluble Extracts

Water-soluble extracts of cheeses were prepared according to the method of Kuchroo and Fox (1982) with some modifications. Thirty grams of cheese was suspended in 90 mL of 50 m*M* phosphate buffer pH 7.0 and treated for 10 min with a Stomacher (PBI International, Milano, Italy). The suspension was kept at 40°C for 1 h under gentle stirring (150 rpm) and centrifuged at $3000 \times g$ for 30 min at 4°C. The supernatant was filtered through Whatman no. 2 paper, and the pH of the extract was adjusted to 4.6 using 1 *N* HCl. The precipitated casein was recovered by centrifugation at $10{,}000 \times g$ for 10 min. Finally, the supernatant was filtered through a Millex-HA 0.22-μm pore size filter (Millipore Co., Bedford, MA).