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Proteolytic activation of proteose peptone component 3 by release of a C-terminal peptide with antibacterial properties

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ABSTRACT

The milk protein proteose peptone component 3 (PP3, also known as lactophorin) is a small phosphoglycoprotein, which is exclusively expressed in the lactating mammary gland. A 23-residue synthetic peptide (lactophoricin, Lpcin S), corresponding to the C-terminal amphipathic α-helix of PP3, has previously been shown to permeabilize membranes and display antibacterial activity. Lactophorin readily undergoes proteolytic cleavage in milk and during dairy processing, and it has been suggested that PP3-derived peptides are part of milk's endogenous defense system against bacteria. Here, we report that a 26-residue C-terminal peptide (Lpcin P) can be generated by trypsin proteolysis of PP3 and that structural and functional studies of Lpcin P indicate that the peptide has antibacterial properties. The Lpcin P showed α-helical structure in both anionic and organic solvents, and the amount of α-helical structure was increased in the presence of lipid vesicles. Oriented circular dichroism showed that Lpcin P oriented parallel to the membrane surface. However, the peptide permeabilized calcein-containing vesicles efficiently. Lpcin P displayed antibacterial activity against *Streptococcus thermophilus*, but not against *Staphylococcus aureus* and *Escherichia coli*. The PP3 full-length protein did not display the same properties, which could indicate that PP3 functions as a precursor protein that upon proteolysis, releases a bioactive antibacterial peptide.

Key words: antibacterial peptide, antimicrobial activity, lactophoricin, proteose peptone component 3

INTRODUCTION

Milk is one of nature's great sources of bioactive proteins and peptides. This reflects the particular purposes to which milk is dedicated beyond nutrition in the first months of life. Milk components like lactoferrin and lactoperoxidase show a wide range of antimicrobial activities against bacteria, fungi, and viruses (Zimecki and Kruzel, 2007). Physiologically active peptides can be found encrypted in an inactive form within the sequence of the parent protein and can be released by, for example, gastrointestinal digestion and fermentation by lactic acid bacteria (Zimecki and Kruzel, 2007). Especially the whey fraction of milk represents a rich and heterogeneous source of antimicrobial peptides (AMP) and scientific and industrial interest is growing to exploit what was formerly considered a waste product (Expósito and Recio, 2006).

The whey protein proteose peptone component 3 (**PP3**, also known as lactophorin) has been isolated and characterized in the milk of several ruminants (Beg et al., 1987; Cantisani et al., 1990; Sørensen and Petersen, 1993a,b; Sørensen et al., 1997; Lister et al., 1998). Bovine PP3 is exclusively expressed in the lactating mammary gland (Groenen et al., 1995) and PP3 is not expressed in human milk or tissue (Sørensen et al., 1997; Rasmussen et al., 2002). However, a highly similar protein, glycosylation-dependent adhesion molecule 1 (GlyCAM-1), has been found in lymph nodes, the lung, cochlea, and mammary gland of mice and rats (Lasky et al., 1992; Dowbenko et al., 1993a,b; Kanoh et al., 1999) and in the ovine uterus (Spencer et al., 1999). Though the murine GLYCAM-1 sequence is 56% identical to the bovine PP3 cDNA sequence (Groenen et al., 1995; Johnsen et al., 1995), the specific expression pattern of the proteins makes it unlikely that they are functional analogs and we, therefore, refer to the protein from milk as PP3 or lactophorin.

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Proteose peptone component 3 consists of 135 AA and contains at least 2 distinct domains: a largely negatively charged N-terminal part and a C-terminal part, displaying a clear amphipathic character (Campagna et al., 1998; Bak et al., 2000). Proteose peptone component 3 is present in multimeric complexes in milk (Sørensen et al., 1997) and recently we reported that PP3 forms stable tetrameric complexes (Pedersen et al., 2012). Residues 98 to 135 have been shown to form an amphipathic membrane binding α-helix, which is oriented in plane with the membrane surface when incorporated in planar phospholipid bilayers (Bak et al., 2000). A cationic synthetic peptide modeled after residues 113 to 135 [named lactophoricin (**Lpcin**, and in this article **Lpcin S**; Figure 1B) can also form voltage-dependent channels in planar lipid bilayers (Campagna et al., 2001). It has been suggested that Lpcin S may permeabilize the membranes in a manner described as the barrel-stave mechanism (Campagna et al., 2001), in which a bundle of peptides associate to form an ion channel (Sansom, 1991). An alternative permeabilization mechanism is the toroidal model, where accumulation of parallel-oriented AMP leads to a pore with continuities between inner and outer leaflet. Thereby, the aqueous channel is lined by both AMP and lipid head groups (Matsuzaki et al., 1996). In the carpet model, parallel-oriented AMP also accumulate and destabilize the lipid bilayer via a detergent-like effect, resulting in disintegration of the membrane and subsequent liposome or cell lysis (Oren and Shai, 1998). The channel-forming activities of Lpcin S prompted further studies, which showed that the peptide limited the growth of more bacterial species in dose-response fashion and even demonstrated bactericidal activity against *Streptococcus thermophilus* (Campagna et al., 2004).

The AA sequence of PP3 contains 4 cleavage sites for plasmin (Figure 1B) as well as several cleavage sites for trypsin and other proteases from the digestive system. In bovine milk, PP3 is known to be cleaved by plasmin, leaving a significant part of the PP3 content in milk as proteolytic peptides (Sørensen and Petersen, 1993a). The formation of these peptides in milk could potentially be a part of the endogenous defense system of milk against bacteria. If it is possible to generate peptides in a way resembling the physiological generation of AMP, the dairy industry may potentially be able to use PP3-derived peptides in regulating fermentation processes involving bacteria that are influenced by the presence such peptides, especially *Strep. thermophilus*.

Previous conformational and antibacterial studies of the synthetic Lpcin S encouraged us to investigate the peptides generated from native PP3 and examine whether they possess antibacterial effects. In vitro trypsin proteolysis of native PP3 generated a peptide covering the C-terminal 26 AA (residues 110–135, in this article called **Lpcin P**). This gave us the opportunity to investigate the structural and functional features of a biologically generated peptide analogous to the previously studied synthetic peptide Lpcin S (Campagna et al., 1998; Campagna et al., 1999; Campagna et al., 2001; Campagna et al., 2004; Barzyk et al., 2009).

In the present study, we have analyzed the secondary structure of Lpcin P by circular dichroism (**CD**), followed by oriented CD (**OCD**), to determine how the peptide orients in lipid bilayers of either 100% electrically neutral lipids or a mixture of electrically neutral and negatively charged lipids. These model membranes mimic the membranes of animals and bacteria, respectively, as the outer leaflet (surface) of bacterial membranes is more negatively charged than the outer leaflet of animal membranes (Graham and Higgins, 1997). We found that Lpcin P showed a high degree of α-helical structure in both buffered aqueous and membrane-like environments. The peptide remained on the surface of

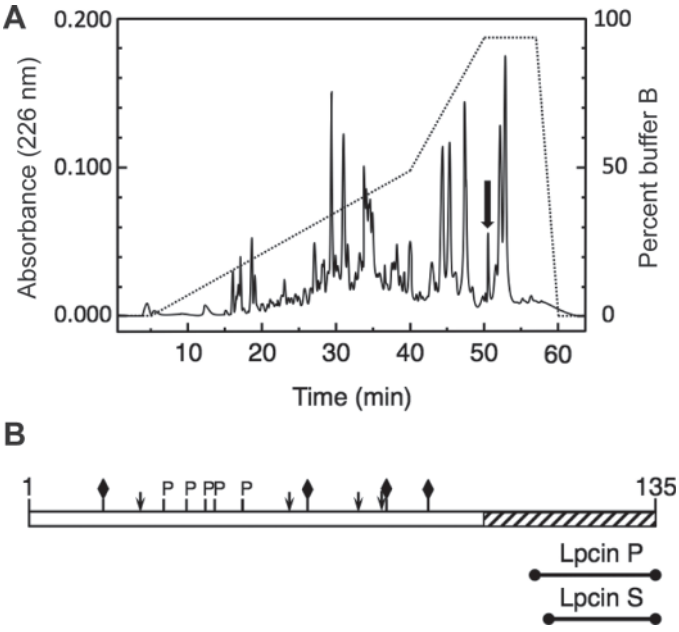

Figure 1. Purification of the peptide (Lpcin P) covering the C-terminal 26 AA (residues 110 to 135) generated by in vitro trypsin proteolysis of native proteose peptone component 3 (PP3). A) Reverse-phase HPLC elution profile of a trypsin digest of PP3. The proteolytic digest was separated on a Vydac C18 column with a gradient of 80% acetonitrile (buffer B) in 0.1% trifluoroacetic acid (buffer A) at 40°C. Detection was at 226 nm. The dashed line indicates the gradient and the peak containing Lpcin P is marked by the arrow. B) Schematic presentation of PP3, showing phosphorylation sites (P), glycosylation sites (♦), and observed plasmin cleavage sites (↓; Sørensen and Petersen, 1993a). The hatched area indicates the part constituting the potential amphipathic helix (Bak et al., 2000). The part of PP3 corresponding to the sequences of Lpcin P (26 residues) and lactophoricin [Lpcin S; 23 residues (residues 113–135)] is indicated.