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Caseins from bovine colostrum and milk strongly bind piscidin-1, an antimicrobial peptide from fish

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ABSTRACT

A model system of bovine colostrum and piscidin, a fish-derived antimicrobial peptide, was developed to study potential interactions of antimicrobial peptides in colostrum. We did not detect any antimicrobial activity of colostrum using the radial plate diffusion assay; in fact colostrum completely abrogated activity of added piscidin. This could not be explained by degradation of piscidin by colostrum, which was less than ten percent. We found that colostrum even protected piscidin against degradation by added proteases. We further observed that colostrum and milk rapidly quenched the fluorescence of fluorescein-piscidin but not that of fluorescein. This effect was not seen with BSA and the specific quenching of fluorescein-piscidin by colostrum was saturably inhibited with unlabeled piscidin. Size exclusion chromatography indicated that fluorescein-piscidin bound to casein micelles with no apparent binding to IgG or whey proteins. Further, addition of pure caseins was able to quench fluorescence of fluorescein-piscidin and to inhibit the antimicrobial activity of piscidin. The interaction between caseins and piscidin could be dissociated by guanidine hydrochloride and recovered piscidin had antimicrobial activity against bacteria. Based on our results we propose that caseins could be carriers for antimicrobial peptides in colostrum and milk.

1. Introduction

During evolution all organisms have evolved various effective strategies to survive and combat invading pathogens, e.g. antimicrobial peptides [1,2]. Colostrum is a complex biofluid, which in addition to an ample supply of nutrients and protective factors, particularly immunoglobulins, contains antimicrobial peptides, like defensins and cathelicidins [3–7]. Recently, genome studies of indigenous Australian mammals, like wallabies [8] and echidnas [9], have revealed expression of cathelicidin genes and other antimicrobial peptides during lactation and point to strong evolutionary selection for these antimicrobial peptides as important defense mechanisms. Antimicrobial peptides display activity against a wide spectrum of pathogens, like bacteria, fungi, parasites and viruses [10].

Although organisms have evolved antimicrobial peptides as part of their innate immune system to provide protection against pathogens, bacteria have also evolved various strategies to resist antimicrobial peptides, like secretion of proteases, modification of cell membrane components or pumping out the antimicrobial peptides [11,12]. For example, in vitro tests with bacterial proteases show that several antimicrobial peptides are susceptible towards enzymatic degradation [13].

Antimicrobial peptides are produced by specialized cells in epithelia [14], such as mammary epithelium that produces colostrum and later milk. A protective transport vehicle in mammalian organisms for antimicrobial peptides could therefore be the casein micelles, which are the major proteins in colostrum and milk. This would ensure that antimicrobial peptides produced in colostrum are at least partly protected and delivered to the newborn.

Caseins are extensively phosphorylated on serine residues in the Golgi complex [15]. Kumosinski and his co-workers have shown through various molecular modeling techniques that all five negatively charged phosphoserine residues located in the N-terminal part of β-casein could potentially interact with positively charged components [16]. α_{S1}-Casein has eight and κ-casein one phosphoserine residue [17,18]. The phosphoserine residues in caseins bind calcium and calcium phosphate and form large molecular micelles in milk and colostrum to encapsulate and to deliver these important macro elements [15]. It is unclear, however, if caseins could function as vehicles for other endogenous

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components. It has been shown that caseins bind exogenous plant-derived polyphenols through hydrophilic and hydrophobic interactions [19]. Furthermore, β-casein micelles can encapsulate cancer drugs to provide stable combinations for orally consumed medicines for cancer patients [20]. Spray-dried cross-linked casein nanoparticles have been shown to trap alfuzosin hydrochloride for delayed release of the drug [21]. Caseins have also been utilized to protect against enzymatic degradation of small peptides, like insulin in vitro [22].

We have studied the binding and protection of piscidin by bovine caseins as a model to explore the potential of caseins and antimicrobial peptides as a defense mechanism. Piscidins can be obtained from fish gills, skin or mucus and could possibly be used to combat pathogens of humans and other higher vertebrates. Piscidin-1 contains 22 amino acids of which seven are positively charged at neutral pH [23]. The two main characteristics of piscidin, positively charged residues and amphipathic helical structure, cause bacterial cell disruption [24,25].

We surmised that positively charged antimicrobial peptides might bind strongly to negatively charged casein molecules in colostrum and milk and that this interaction might protect the antimicrobial peptides from enzymatic degradation. These peptides could subsequently be released, e.g. through microbial action, in the lower gastrointestinal tract.

2. Materials and methods

2.1. Materials

Reagents and chemicals were purchased from the following vendors: Sigma-Aldrich, MO., USA (Guanidine hydrochloride; Fluorescein; Bovine Serum Albumin; SIGMA*FAST*™ Protease Inhibitor Tablets); Fluka Analytical, MO., USA (LB broth Miller); Invitrogen, UK (Agarose, electrophoresis grade); Pfizer Italiana, Italy (Synulox); AppliChem GmbH, Germany (Trifluoroacetic acid, spectroscopy grade); Merck, Germany (Pronase E, a mixture of proteases from *Streptomyces griseus*); Bruker Daltonics, Germany (α-cyano-4-hydroxycinnamic acid); CASLO Laboratory ApS, Lyngby, Denmark (synthetic amidated piscidin-1 (FFHHIFRGIVHVGKTIHRLVTG-NH2) and fluorescein-piscidin-1 (N-terminally labeled). Throughout the text piscidin-1 and fluorescein-piscidin-1 are abbreviated as piscidin and fluorescein-piscidin, respectively.

Staphylococcus aureus and *Escherichia coli* isolates from milk were obtained from Estonian University of Life Science, Tartu, Estonia. *E. coli K-12* strain was kindly provided by Ole Højberg, Department of Microbiology, AU, DK. Spray-dried colostrum powder (a mixture collected and pooled from different cows and herds) was kindly provided by Biofiber damino, Gesten, DK. Sodium caseinate was from Arla Foods, Holstebro, Denmark. Fresh colostrum and milk samples from different cows were kindly provided by Mette-Marie Løkke, Department of Food Science, AU, DK.

For practical reasons, we primarily used reconstituted colostrum prepared as a 10% (w/v) suspension of spray-dried colostrum powder in water. Some experiments required use of fresh colostrum, e.g. degradation, where the spray-drying process may have inactivated proteolytic activity. Although we have observed only minor quantitative differences in the behavior of reconstituted and fresh colostrum, we have chosen to specifically refer to our use of reconstituted colostrum as colostrum^{r} and fresh colostrum as colostrum^{f} throughout the text.

HPLC grade water (Burdick & Jackson, MI, USA) was used for peptide stock solutions. MilliQ water (Millipore Corporation, MA, USA) was used throughout the study. PBS, ACN, methanol, HCl and NaOH were of analytical grade and commercially available.

2.2. Methods

2.2.1. Bacterial cultures

Bacteria were grown overnight in LB broth at 37 °C. Next day the bacterial cultures were washed three times with PBS (pH 7.0) and diluted in LB to an OD_{600} = 0.05.

2.2.2. Radial plate diffusion assay

Agarose plates were used and growth inhibition was observed as clear zones after application and diffusion of the antimicrobial component from a small hole punched in the agarose. LB broth with 1.5% agarose was autoclaved, cooled to ∼40 °C, mixed with washed bacteria to obtain OD_{600} = 0.05 and poured onto Petri dishes. 3 mm holes were punched after solidification and filled with 10–20 μl of sample to be tested. Synulox, a mixture of amoxicillin and clavulanic acid, was used as positive control.

In experiments with guanidinium hydrochloride (GndHCl), we also added protease inhibitors (0.5 mg/ml) in the LB medium. Plates were incubated overnight at 37 °C and next day diameters of inhibition zones were measured.

2.2.3. Degradation of fluorescein-piscidin in colostrum

Colostrum^{f} samples from three different cows and fresh milk from cows in late lactation were centrifuged (∼2500*g*, 30 min, 4 °C) to remove fat. All subsequent experiments used skimmed colostrum or skimmed milk. The colostrum^{f} and colostrum^{r} or dilutions thereof were mixed with piscidin (mixture of piscidin and fluorescein-piscidin) or PBS as a control. Parallel samples were then loaded on bacterial plates for measurement of inhibition zones or incubated without bacteria overnight at 37 °C for measurement of degradation of fluorescein-piscidin by size exclusion chromatography using an ÄKTA FPLC 900 System (Amersham Biotech) equipped with column (Sephacryl S200 – 15 mm × 100 mm – internal diameter × length (Pharmacia, Sweden). Chromatographic conditions were as follows – mobile phase: PBS, pH 7.0; flow rate: 1 ml/min; UV detection at 280 nm. Fractions of 1 ml were collected and fluorescence was measured with a Synergy 2 micro plate reader (BioTek Instruments, Inc., USA) using excitation at 485 nm and emission at 528 nm. Subsequently, pronase E were added at 1 mg/ml to all fractions to release fluorescein and fluorescence was measured again after 30 min. Fluorescence was corrected for suppression by a factor of 0.7 as determined for fluorescence intensity of fluorescein before and after addition of pronase E.

2.2.4. Protection of fluorescein-piscidin by colostrum

The degradation of fluorescein-piscidin was analyzed by size exclusion chromatography. fluorescein-piscidin was incubated with colostrum^{r} or colostrum^{f} for 60 s at room temperature. Then pronase E was added and samples were incubated for an additional 60 s under the same conditions. Control samples were prepared under the same conditions, but switching the order of additions. Additional controls with BSA at the same concentration as colostrum^{f} were performed to rule out a general protective effect of protein against degradation of fluorescein-piscidin by pronase E. Samples were then immediately injected and separated by FPLC as described in Section 2.2.3.

2.2.5. Binding of fluorescein-piscidin by colostrum and milk

Binding of piscidin to colostrum or milk was studied using fluorescence quenching. The rapid binding of fluorescein-piscidin was carried out with colostrum^{r}, colostrum^{f} and milk. The signal of fluorescein-piscidin was measured in a quartz cuvette with an LS50B spectrometer (Perkin Elmer, UK) using excitation at 490 nm and emission at 520 nm. After 60 s, colostrum or milk was added and quenching of fluorescein-piscidin was followed for another 60 s.