Antibacterial function and mechanism of peptide $\beta$-casein 65 action in breast milk

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Abstract

Breast milk has significant benefits to preterm infants, including reduced risk of infection and inflammatory disorders. Many biomolecules, such as peptides, that exhibit immunomodulation function or antimicrobial activity and protect against infection have been identified in human milk. $\beta$-Casein 65, an endogenous peptide cleaved amino acids 204-225 of $\beta$-casein, is expressed at high levels in preterm breast milk. Our study demonstrates the antibacterial activity of $\beta$-casein 65 against Staphylococcus aureus ($S.\ aureus$) and Escherichia coli ($E.\ coli$) through use of an agarose filter paper diffusion and microdilution method. The LIVE/DEAD BacLight bacterial viability assay results also confirmed the antibacterial effects of $\beta$-casein 65. Destroyed membrane structures in $S.\ aureus$ and $E.\ coli$ after treatment with $\beta$-casein 65 were observed by scanning electron microscopy and transmission electron microscopy. A DNA-binding assay showed that $\beta$-casein 65 exerts antimicrobial effects independent of DNA-binding activity. Our results revealed the antibacterial activity and mechanism of peptide $\beta$-casein 65 sourced from human milk. Further studies should be carried out to determine ways to prevent and treat pathogens in animals and human neonates.

Αντιβαςτεριαλ φυνςτιον ανδ μεςηανισμ οφ πεπτιδε β-ςασειν 65 αςτιον ιν βρεαστ μιλκ

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SIGNIFICANCE PARAGRAPH

$\beta$-Casein 65 is expressed at high levels in preterm breast milk. The peptide showed antimicrobial activity against important bacteria. The peptide destroyed membrane structures in $S.\ aureus$ and $E.\ coli$. $\beta$-casein 65 exerts antimicrobial effects independent of DNA-binding activity.

Abstract

Breast milk has significant benefits to preterm infants, including reduced risk of infection and inflammatory disorders. Many biomolecules, such as peptides, that exhibit immunomodulation function or antimicrobial activity and protect against infection have been identified in human milk. $\beta$-Casein 65, an endogenous
peptide cleaved amino acids 204-225 of β-casein, is expressed at high levels in preterm breast milk. Our study demonstrates the antibacterial activity of β-casein 65 against *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) through use of an agarose filter paper diffusion and microdilution method. The LIVE/DEAD BacLight bacterial viability assay results also confirmed the antibacterial effects of β-casein 65. Destroyed membrane structures in *S. aureus* and *E. coli* after treatment with β-casein 65 were observed by scanning electron microscopy and transmission electron microscopy. A DNA-binding assay showed that β-casein 65 exerts antimicrobial effects independent of DNA-binding activity. Our results revealed the antibacterial activity and mechanism of peptide β-casein 65 sourced from human milk. Further studies should be carried out to determine ways to prevent and treat pathogens in animals and human neonates.

**Key words:** antibacterial; β-casein 65; peptide; breast milk

**Introduction**

Preterm birth is a significant global issue, affecting approximately one in ten births worldwide (Simoncic, Deguen, Enaux, Vandentorren, & Kihal-Talantikite, 2022). Infections are a major cause of morbidity and mortality in preterm infants (Brown, Meader, Wright, Cleminson, & McGuire, 2020; Wu, Fan, Gao, Wang, & Zheng, 2022). Breast milk has significant benefits for preterm infants, including reduced risk of infection and inflammatory disorders (Newton et al., 2022). Milk produced by mothers who deliver prematurely is well known to be higher in protein and immune proteins than term breast milk (Chong et al., 2022). Human milk contains a large number of antimicrobial proteins and peptides (AMPs), which provide passive immunity to the GI tract in premature infants (Newton et al., 2022; Shende & Khanolkar, 2021). AMPs are commonly composed of between 12 and 50 amino acids and show cationic, acid-stable, amphiphilic features. They exhibit broad-spectrum antimicrobial activity against various pathogens, including bacteria, viruses, fungi, and parasites (Zhang & Gallo, 2016). In addition, different physical and chemical structures of these peptides suggest different antimicrobial activity (Zharkova et al., 2023). Defensins and cathelicidins are two AMP families (Pachón-Ibáñez, Smani, Pachón, & Sánchez-Céspedes, 2017; Rončević, Puizina, & Tossi, 2019) that have been extensively studied in human breast milk. Defensin antimicrobial activity is realized through the disruption of the structure of bacterial membranes (Xu & Lu, 2020). In contrast to defensins, cathelicidins not only trigger specific defines responses in the host but also exert direct antimicrobial effects (Yang, Chertov, & Oppenheim, 2001; Zanetti, 2004); therefore, these two AMPs exert antimicrobial activity through two completely different mechanisms. There are hundreds of AMPs in human breast milk. Although some of the AMPs, and associated mechanisms, in human milk that confer protection against infection have been well studied, the complete picture and complex biology of AMP antimicrobial protection should be fully investigated.

In a previous study, we reported on 23 AMPs that were expressed more highly in preterm human breast milk than in term milk (Wan et al., 2013). Here, we focus on an AMP, named β-casein 65 (LNPTHQIYPVTQ-PLAPVHNPI5), which is found in preterm human breast milk at higher levels. We assessed β-casein 65 according to its biological characteristics, antibacterial activity and the potential mechanisms of action against common pathogens in neonatal infection.

**Methods and Materials**

2.1 Bioinformatics analysis

β-casein 65, which is expressed at higher levels in preterm human breast milk than it is in with term milk, was characterized in our previous study (Wan et al., 2013). Sequences were compared with other non-ribosomal peptide synthetase (NRPS) genes obtained from GenBank using the BLAST program. Nucleotide sequence alignment and physicochemical parameters were analysed using the ProtParam online tool (https://web.expasy.org/protparam); Helical Wheel online software (http://lbqp.unb.br/NetWheels/) and PEP-FOLD (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/).

2.2 Materials

β-casein 65 (LNPTHQIYPVTQPLAPVHNPI5) was chemically synthesized by Science Peptide Biological
Five reference bacterial strains were used in this study. *Escherichia coli* (*E. coli*, ATCC25922) and *Staphylococcus aureus* (*S. aureus*, ATCC25923) were purchased from the American Type Culture Collection. *Listeria monocytogenes* (*L. monocytogenes*), *Klebsiella pneumoniae* (*K. pneumoniae*) and *Bacillus subtilis* (*B. subtilis*) were presented by Nanjing Normal University, China. The linearized plasmid pBR322 vector from *E. coli* was obtained from New England Biolabs. The LIVE/DEAD® BacLight bacterial viability kit used for the fluorescence dye method was purchased from the Thermo Fisher company, USA. Other materials, such as cell culture reagents, were all purchased from Sigma (St. Louis, MO, USA).

2.3 Disk diffusion tests

Direct disk diffusion tests were used to evaluate the antibiotic function of β-casein 65. Log-phase bacteria were centrifuged, washed and suspended in 10 mM sodium phosphate buffer for inoculation. Yeast nitrogen base broth agar plates were each smeared with bacteria. The agar plate surface was covered with 20 μL β-casein 65 (25 μg/mL) or 20 μL ddH2O soaked paper. The inhibition zones were measured after 24 h of incubation at 37 °C.

2.4 Turbidity broth assay

The antibacterial sensitivity of β-casein 65 was assessed by the turbidity broth test. *E. coli* and *S. aureus* (1 × 10^5 CFUs per well) were each inoculated to germ-free 96-well plates. The prepared serial twofold dilutions of β-casein 65 were added to wells at final concentrations of: 0, 0.78, 1.56, 3.13, 6.25, 12.5, 25 and 50 μg/mL. The plates were incubated at 37 for 24 h, and the turbidity was measured at 600 nm by a Synergy HT multi-detection microplate reader (Synergy HT, Bio-Tek Instruments, Vermont, USA).

2.5 Bacterial viability assay

Bacterial viability assays, namely, LIVE/DEAD BacLight bacterial viability kit assays, were also used to investigate the antimicrobial activity of β-casein 65. The green fluorescent nucleic acid stain (SYTO 9) labels bacteria with intact membranes, and the red fluorescent nucleic acid stain (propidium iodide, PI) penetrates damaged membranes of bacteria. *E. coli* and *S. aureus* were grown to log-phase, and then β-casein 65 or ddH2O (the control) was added and incubated at room temperature for 1 h with mixing every 15 min. Then, the bacterial solutions were centrifuged, and the bacteria were washed, resuspended and stained following the BacLight assay kit instructions. After staining, the bacteria were viewed by a fluorescence microscope set (Zeiss, 152 Imager.A2, Germany) with excitation/emission maxima at 480/500 nm for the SYTO 9 stain and 490/635 nm for the propidium iodide (PI). All experiments were repeated three times independently, and at least three different fields were observed for each culture.

2.6 DNA-binding assay

Gel electrophoresis experiments were performed to evaluate the DNA-binding capabilities of the β-casein 65 expressed in plasmid DNA. Plasmid pBR322 vector from *E. coli* was incubated with different concentrations of β-casein 65 (from 0 to 500 μg/mL) at 37 °C for 1 h. After adding the loading solution (glycerol, Tris HCl, 1 EDTA, KCl, and BSA), the samples were placed into 1.0% agarose gel and subjected to electrophoresis at 120 V for 90 min in TBE (Tris-borate-EDTA) buffer (Huo et al., 2011).

2.7 Scanning electron microscopy of the bacteria

Morphological changes of the bacteria after treatment with β-casein 65 were observed by scanning electron microscopy (SEM). Briefly, *E. coli* and *S. aureus* were grown to the exponential growth phase, centrifuged, washed and re-suspended in PBS. *E. coli*, *S. aureus* and β-casein 65(100 μg/mL) were incubated at 37 °C for 1 h with shaking every 15 min. An equal volume of PBS was used as a control. Then, the bacteria were centrifuged, washed and fixed in 2.5% glutaraldehyde overnight. The samples were dehydrated in ethanol, dried under CO2 and stored in cool conditions. Finally, the samples were scanned by SEM (Tokyo, Japan) according to the method previously published by Taute et al (Ehmann et al., 2019; Mbuayama, Taute, Strömstedt, Bester, & Gaspar, 2022).
2.8 Transmission electron microscopy of the bacteria

Bacterial suspensions (E. coli and S. aureus) were treated with β-casein 65 for 1 h, as described above. The control was prepared from the same bacterial suspensions before β-casein 65 treatment. Then, the samples were prepared for transmission electron microscopy scanning by the method described by BO Schroeder et al. (Ehmann et al., 2019). Briefly, after centrifuging, the bacteria were coagulated in 3.5% agarose, and cut into small blocks. Then, the small blocks were fixed again in Karnovsky’s solution, cut into ultra-thin sections by an Ultracut microtome (Reichert, Wien, Austria), and analysed with a Tecnai G2 Spirit Bio TWIN transmission electron microscope (FEI, America) operating at 120 kV.

2.9 Statistical analysis

The data obtained through this study are presented as the means ± standard deviation (SD) as analysed by IBM SPSS 20. Statistical differences were defined as P < 0.05. All biological experiments were repeated three times.

Results

3.1 Characteristics of β-casein 65

The LC-MS/MS results indicated an enriched level of β-casein 65 in preterm human breast milk compared with the level in term milk (Supplementary Materials Figure S1). β-Casein 65 is composed of 22 amino acids (LNPTHQIYPVTQPLAPVHNPIS) with a molecular weight of 2436.80 Da. The ProtParam tool provided the following additional physicochemical parameters: The theoretical pI was 6.92; instability index was 51.03; aliphatic index was 101.82; grand average of hydropathicity was -0.232 and the estimated half-life was 5.5 h (in mammalian reticulocytes, in vitro) (Figure 1A). The results from the Helical Wheel analysis indicated the amphiphilic properties of β-casein 65, which suggested the capacity of β-casein 65 to interact with the bacterial membrane (Figure 1B). The structure of β-casein 65 was predicted using the PEP-FOLD platform: an online resource for peptide structure prediction (Figure 1C).

3.2 Antimicrobial activity of β-casein 65 against bacteria

Five bacterial species, including two gram-negative bacteria (Escherichia coli (E. coli) and Klebsiella pneumoniae (K. pneumoniae)) and three gram-positive bacteria (Staphylococcus aureus (S. aureus), Listeria monocytogenes (L. monocytogenes) and Bacillus subtilis (B. subtilis)), were applied for the disk diffusion assay used to evaluate the antimicrobial activity of β-casein 65 (Figure 2A). It was shown that E. coli and S. aureus were highly sensitive to the treatment of β-casein 65 (Figure 2A and 2B). Application of β-casein 65 decreased the growth of E. coli and S. aureus. The minimum inhibitory concentrations (MIC) of β-casein 65 against E. coli and S. aureus was 12.5 μg/mL (Figure 2B). These results revealed the powerful antimicrobial nature of β-casein 65 against E. coli and S. aureus.

The inhibitory effect of β-casein 65 on bacterial growth may be caused by the elimination or disruption of cell division. AMPs generally affect bacterial survival to acting as antimicrobial peptides. To study the mechanism by which β-casein 65 inhibits bacterial growth, bacterial viability was evaluated. Propidium iodide (PI) is incorporated by bacteria that have lost membrane integrity, labelling them as dead. In addition, SYTO 9 accumulates only in living bacteria. Immunofluorescence was performed with these two dyes to distinguish the LIVE/DEAD bacteria in the viability assay. Fluorescence imaging showed a significantly decreased number of SYTO 9-positive E. coli and S. aureus after β-casein 65 treatment and an increased number of PI-positive bacteria (Figure 3). This result indicated that β-casein 65 acted as an antimicrobial peptide in inducing bacterial death.

3.3 DNA-binding activity

Bioinformatics predicted the DNA-binding activity of β-casein 65. ProtParam showed the potential binding sites of β-casein 65, which are designated with red plus (+) (Figure 4A). To identify the possibility that DNA binds to β-casein 65, an electrophoretic gel mobility shift assay was performed. Different concentrations of β-casein 65 (0-500 μg/mL) were mixed with the pBR322 vector DNA in 1.0% agarose and subjected to
electrophoresis. A protein-DNA interaction will result in a shift of the DNA band to a position indicating a larger size because of the increased mass of the complex. The shift assay results indicated unaltered migration of pBR322 treated with β-casein 65 (Figure 4B). These results suggest that β-casein 65 exerts antimicrobial effects independent of DNA-binding activity.

3.4 Electron microscopy experiments

Bioinformatics indicated the potential antimicrobial activity of β-casein 65 through disruptions to the integrity of the bacterial cytomembrane. To identify whether β-casein 65 suppressed the survival of E. coli and S. aureus through direct regulation, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) imaging the bacterial structure was used to observe images at subcellular resolution. The SEM results showed that β-casein 65 treatments caused significant morphological defects in both types of bacteria, including a stumpy shape of the E. coli and abnormal adhesion of S. aureus, and membrane ruffling and rupture (Figure 5A). the TEM images confirmed the effects of β-casein 65 as a disruptor to the integrity of the bacterial membrane. In addition, impaired intracellular conditions were observed in both types of bacteria (Figure 5B). These results may have been caused by membrane rupture or intracellular regulation, and therefore, it requires further study.

Collectively, these data demonstrated that β-casein 65 disrupted the integrity of the bacterial cytomembrane and may directly inhibit bacterial survival through in this effect.

Discussion

In addition to providing nutrients, breast milk confers protection to neonates against infection, such as necrotizing enterocolitis (Assad, Elliott, & Abraham, 2016; Kantorowska et al., 2016), gastrointestinal infection, and respiratory infections (Karcz, Walkowiak, Makuch, Olejnik, & Królak-Olejnik, 2019), reducing the likelihood of sepsis (Aceti et al., 2017; Adamkin, 2022). Immunocompetent potential substances in breast milk compensate for the delayed development of the immune system in infants. These substances are cleaved endogenously and produce peptides and AMPs with bioactivity that prevents bacterial survival and establishes a balanced environment composed of microbe species. The antimicrobial activities of AMPs against various viruses, fungi, bacteria, protozoa, and even transformed or cancerous cells have been thought to be conserved among species. Hundreds of AMPs have been identified in human milk (Beverly, Woonnimani, Scottoline, Lueangsakulthai, & Dallas, 2021), and extensive peptidomes of in vivo human milk revealed specific proteolysis, that indicated protective antimicrobial peptides (Auestad & Layman, 2021; Wölk, Gebauer, Hoffmann, & Milkovska-Stamenova, 2021). Interestingly, the expression profiles of breast milk from mothers with preterm infants and that of mothers with term infants differ. β-casein 65, as a novel endogenous AMP, was identified as enriched in preterm breast milk by liquid chromatography-mass spectrometry, suggesting its important role as an antimicrobial in infants. Here, we performed cellular and molecular experiments to study the antimicrobial activity and mechanism of β-casein 65.

In this study, five bacterial strains, two gram-negative (E. coli and K. pneumoniae) and three gram-positive (S. aureus, L. monocytogenes, and B. subtilis), were used. The disk diffusion assay results showed that the antimicrobial activity of β-casein 65 against E. coli and S. aureus were not dose-dependent. Even at very low concentrations of β-casein 65, E. coli and S. aureus showed significantly decreased survival. In addition, E. coli and S. aureus are both common pathogenic bacteria observed in neonatal infection. This finding indicated the beneficial potential of β-casein 65 therapeutics.

The mechanisms by which AMPs kill microorganisms have been reported: they not only alter cytoplasmic membrane (septum formation), they inhibit nucleic acid and protein synthesis (Rončević et al., 2019). Because their antimicrobial mechanisms differ from those of traditional antibiotics, AMPs have advantages against multiple antibiotic-resistant bacteria. In the present study, we revealed the mechanisms of the antimicrobial activity of β-casein 65, indicating that they likely directly disrupt the bacterial membrane. The results from a DNA shift assay suggested that β-casein 65 has low DNA-binding activity. In addition, the SEM and TEM images revealed defects in the membrane integrity of E. coli and S. aureus after treatment with β-casein 65. The bacterial cytomembrane was wrinkled and ruptured, and the cell wall was impaired. These
observations indicate that cytoplasmic membrane disruption, rather than DNA binding, may be explain the key antimicrobial activity of β-casein 65.

Conclusions
In summary, we identified β-casein 65 in preterm breast milk and found that β-casein 65 demonstrated efficient antimicrobial activity against E. coli and S. aureus by directly impairing the integrity of the bacterial membrane.

Conflicts of interest: The authors declare no conflicts of interest.

Author contributions
Xiaohui Chen and Shuping Han: conceptualization and project administration;
Jun Zhang and Min Zhang: investigation, and methodology.
Xiangyun Yan and Qinlei Yu: validation, software and visualization.
Xiaoshan Hu and Min Zhang: formal analysis and visualization.
Jun Zhang: writing – original draft, review and editing.

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References:


**Figure Legend**

**Φιγυρε 1. Χαρακτεριστικες οφ β-κασειν 65** (A) Results from the ProtParam online analysis of β-casein 65; (B) Results from the HelicalWheel software analysis of β-casein 65. Polar and basic residues are highlighted in red; polar and uncharged residues are highlighted in green; and nonpolar residues are highlighted in yellow. (C) Predicted structure of β-casein 65.

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<thead>
<tr>
<th><strong>β-casein 65</strong></th>
<th><strong>Characteristics</strong></th>
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<tr>
<td>LNPTHQIPVTQPLAPVHNPS</td>
<td><strong>Sequence</strong></td>
</tr>
<tr>
<td>22</td>
<td><strong>Number of amino acids</strong></td>
</tr>
<tr>
<td>2436.80</td>
<td><strong>Molecular weight</strong></td>
</tr>
<tr>
<td>6.92</td>
<td><strong>Theoretical pI</strong></td>
</tr>
<tr>
<td>51.03</td>
<td><strong>The instability index (II)</strong></td>
</tr>
<tr>
<td>101.82</td>
<td><strong>Aliphatic index</strong></td>
</tr>
<tr>
<td>-0.232</td>
<td><strong>Grand average of hydropathicity (GRAVY)</strong></td>
</tr>
<tr>
<td>5.5 hours (mammalian reticulocytes, in vitro)</td>
<td><strong>Estimated half-life</strong></td>
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**Φιγυρε 2. Της διαχ διφυσιον ασσαφ φορ τηε αντιμιςροβιαλ αςτιvιτψ οφ β-κασειν 65** (A) β-casein 65 (25 μg/mL) was incubated with five common bacterial species, and the diameter of the inhibition zones was measured from the centre of each culture. (B) Different concentrations of β-casein 65 were incubated with five common bacterial species, and changes in optical density (OD600) were measured after 24 h.
Figure 3. ΛΙ῞Ε/ΔΕΑΔ σελλ ασαψ ϕορ δετερμινιγ τηε αντιμιρβιαλ αςτιvιτψ οφ β-ςασειν 65 E. coli and S. aureus with intact cell membranes are stained with green fluorescence (SYTO 9), whereas bacteria with damaged membranes are stained with red fluorescence (Propidium iodide, PI).

Figure 4. ΔΝΑ-βινδινγ αςτιvιτψ οφ β-ςασειν 65 (A) ProtParam predicted binding sites designated with a red “+”. (B) Electrophoretic gel mobility of the linearized plasmid pBR322 vector.
Figure 5. SEM and TEM images of *E. coli* and *S. aureus* *E. coli* and *S. aureus* were treated with 10 mM sodium phosphate buffer (control) or 100 μg/mL β-casein 65 and investigated by scanning electron microscopy (A) and transmission electron microscopy (B).