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Characterization of Bacteriocins Based on Amino Acid Composition and Molecular Weight

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ABSTRACT

Background and Aims: Bacteriocins are antimicrobial peptides produced by bacteria that hold great potential as natural preservatives and bioactive compounds. This study aimed to characterize bacteriocins produced by *Bacillus paramycoides* strain MCCC 1A04098 (BSH1), *Bacillus albus* strain MCCC 1A02146 (BSH2), and *Bacillus cereus* strain IAM 12605 (BSH3), isolated from cincalok, a traditional fermented shrimp product.

Materials and Methods: High-performance liquid chromatography (HPLC) was used to analyze amino acid profiles, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was employed to determine molecular weights.

Results: Bacteriocins from BSH1, BSH2, and BSH3 had molecular weights of 55.02 kDa, 51.15 kDa, and 50.12 kDa, respectively, classifying them as class III bacteriocins (>30 kDa). Amino acid analysis revealed the dominance of L-cystine (33–45%) and L-proline (9–20%), along with notable amounts of glutamic acid and leucine. These amino acids are essential for structural stability and antimicrobial activity.

Conclusion: The findings suggest that bacteriocins derived from cincalok not only broaden the understanding of bacteriocin diversity from local fermented foods but also demonstrate strong potential as natural antimicrobial agents for applications in food preservation and healthcare industries.

Keywords: Bacteriocins, Amino Acid Composition, Molecular Weight, Antimicrobial Peptides, Characterization

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1. Introduction

Fermentation is a traditional food preservation technique that has been widely practiced across many regions of the world, including Indonesia. Beyond extending shelf life, fermentation also generates a variety of bioactive compounds that enhance the value of food products. One of the key compounds produced through microbial metabolism during fermentation is bacteriocin. Bacteriocins are ribosomally synthesized antimicrobial peptides produced by various bacteria **(1)**, particularly lactic acid bacteria and certain *Bacillus* species. These peptides inhibit the growth of pathogenic and spoilage bacteria, making them highly promising as safe, natural, and environmentally friendly preservatives for the food industry **(2)**.

Bacteriocins offer unique advantages over other antimicrobials, including high specificity toward target bacteria, relatively low toxicity to humans, and minimal impact on beneficial microbiota **(3)**. Their potential applications extend beyond food preservation to pharmaceuticals and medicine. Importantly, the molecular characteristics of bacteriocins—such as amino acid composition and molecular weight—play a crucial role in determining their stability, mechanism of action, and antimicrobial efficacy **(4)**.

Bacteriocins are generally classified into several groups based on structure and molecular weight. Class I (lantibiotics) are small bacteriocins (<5 kDa) that undergo post-translational modifications and contain rare amino acids such as lanthionine **(5)**. Class II consists of unmodified, small bacteriocins, while Class III includes large bacteriocins (>30 kDa) that are typically heat-labile **(6)**. Molecular weight can be determined using SDS-PAGE electrophoresis, whereas amino acid profiling is commonly performed using mass spectrometry or liquid chromatography **(7)**. These analyses are essential for understanding bacteriocin activity and evaluating their potential applications.

Traditional Indonesian fermented foods, such as cincalok, made from small shrimp (*Acetes* spp.), represent an underexplored source of bacteriocins. Cincalok fermentation occurs spontaneously without a starter culture, producing a complex microbial community that includes bacteria capable of generating antimicrobial compounds **(8)**. Several studies suggest that such spontaneous fermentations provide valuable microbial isolates with strong bioactive properties **(9)**.

Previous work by our research team isolated bacteriocin-producing bacteria from cincalok prepared with *Acetes* shrimp. Three species were identified: *Bacillus paramycoides* strain MCCC 1A04098 (BSH1), *Bacillus albus* strain MCCC 1A02146 (BSH2), and *Bacillus cereus* strain IAM 12605 (BSH3) **(8)**. These isolates produced bacteriocins with activity against pathogenic bacteria, including *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium* **(9)**. Stability tests showed that the bacteriocins tolerated extreme temperatures (−40 °C to 121 °C), a broad pH range (pH 2-11), and resistance to proteolytic enzymes such as proteinase K **(9)**. These characteristics indicate strong potential for development as natural preservatives that remain effective under diverse conditions. However, while their antimicrobial activity and stability have been demonstrated, detailed molecular characterization—particularly amino acid profiles and molecular weights—remains unavailable. Such information is critical for understanding their mechanism of action, potential structural interactions, and suitability for modification to enhance efficacy. It also provides the scientific basis for bacteriocin classification and for developing peptide-based products that meet industry regulatory standards **(10)**.

Accordingly, this study focuses on the structural characterization of bacteriocins produced by *B. paramycoides* BSH1, *B. albus* BSH2, and *B. cereus* BSH3 isolated from cincalok. Two key parameters were analyzed: amino acid profile and molecular weight. The amino acid profile provides insight into chemical composition and potential structural properties, while molecular weight helps classify the bacteriocins and detect possible post-translational modifications. The objective of this study is to analyze the amino acid profiles and determine the molecular weights of bacteriocins from these isolates. The findings are expected to broaden understanding of bacteriocin diversity from local biological sources and provide a foundation for developing bacteriocins as safe, effective, and sustainable natural preservatives for the food industry.

2. Materials and Methods

2.1 Sample Collection and Isolation

Cincalok, a traditional fermented product made from fresh *Acetes* shrimp, was obtained from South Sorong, Southwest Papua, Indonesia. It is typically prepared with shrimp, white rice, and salt. In this study, three cincalok samples were prepared with different salt concentrations: BSH1 (2%), BSH2 (4%), and BSH3 (6%). Each sample contained 15% cooked white rice. From each sample, 1 g was collected and serially diluted up to 10^{-3}. Aliquots (0.1 mL) were plated onto de Man Rogosa Sharpe Agar (MRSA) using

the pour plate method and incubated at 29 °C for 24 hours. Colonies with distinct morphology were selected for further isolation and identification. The isolates were identified as *Bacillus paramycoides* strain MCCC 1A04098 (BSH1), *Bacillus albus* strain MCCC 1A02146 (BSH2), and *Bacillus cereus* strain IAM 12605 (BSH3) **(8)**.

2.2 Bacterial Culture and Characterization

2.2.1 Gram Staining

Gram staining was performed to confirm the purity of bacteriocin-producing cultures. Bacterial isolates Sukmawati et al **(8)** in 2024 were suspended on a glass slide, heat-fixed, stained with crystal violet, rinsed, treated with Lugol's iodine, and rinsed again. Decolorization was carried out with 96% ethanol, followed by counterstaining with safranin. The slides were rinsed with distilled water and examined under an immersion oil objective microscope.

2.2.2 Media Preparation and Re-culture

Isolates were re-cultured on MRS agar supplemented with 0.5% $CaCO_3$, sterilized before use. The plates were incubated at 29 °C for 24 hours.

2.2.3 Bacteriocin Production

Each isolate (*B. paramycoides* BSH1, *B. albus* BSH2, *B. cereus* BSH3) was inoculated into 1000 mL of MRS broth containing 0.5% $CaCO_3$ and incubated at 29 °C for 24 hours in a shaker incubator at 80 rpm. Cultures were centrifuged at 4500 rpm for 15 minutes to separate supernatants, which were then filtered through 0.22 µm Millipore membranes to obtain crude bacteriocin extracts **(11)**.

2.2.4 Amino Acid Composition Analysis

The composition of 18 amino acids (L-alanine, L-arginine, L-aspartic acid, glycine, L-glutamic acid, L-histidine, L-isoleucine, L-cystine, L-leucine, L-lysine, L-methionine, L-tryptophan, L-valine, L-phenylalanine, L-proline, L-serine, L-threonine, and L-tyrosine) was analyzed using high-performance liquid chromatography (HPLC) with photodiode array (PDA) detection, following method 18-5-63/MU/SMM-SIG.

Samples were hydrolyzed by adding 4.2 M NaOH, heated at 110 °C for 20 hours, neutralized with citrate buffer to pH 4.25, and diluted to a final volume of 50 mL. After centrifugation, the supernatant was filtered (0.45 µm) into vials for injection into the HPLC system.

Instrument conditions: Column: RP-18; Mobile phase: A = 0.0085 M sodium acetate, B = methanol; pump: isocratic; injection volume: 15 µL; detector: PDA at 280 nm; temperature: ambient.

Amino acid concentrations were calculated using the calibration curve equation $Y = bx + a$.

$$CTrp = \frac{(A_{spl}/b) \times FP \times V_a}{W_{(spl\ \text{or}\ V_{spl})}}$$

Where:
Aspl = peak area of sample; *a* = intercept of calibration curve; *b* = slope of calibration curve; *FP* = dilution factor; *Va* = final solution volume (mL); *Wspl* = test portion weight (g); *Vspl* = pipetted volume (mL) **(12)**.

2.2.5 Molecular Weight Determination of Bacteriocin

Bacteriocin samples were lysed in the presence of protease inhibitors and separated using 10% (v/v) SDS–polyacrylamide gel electrophoresis (SDS-PAGE). For each sample, 20 µL was mixed with 7 µL of loading buffer containing 60 mM Tris-HCl (pH 6.8), 25% (w/v) glycerol, 2% (w/v) SDS, 14 mM β-mercaptoethanol, and 0.1% (w/v) bromophenol blue. The mixtures were denatured at 100 °C for 3 minutes before loading onto the gel.

Electrophoresis was performed at 85 V and 50 mA for 180 minutes. After separation, the gel was carefully removed from the casting tray, and the migration distance of the bromophenol blue dye front was measured relative to the separating line. The gel was immersed in staining solution and gently agitated for 2 hours, then soaked in 0.1 M citrate buffer (pH 6.0) at 55 °C for 2 hours.

For zymogram visualization, the gel was immersed in 0.1% Congo red solution for 30 minutes, rinsed with NaCl solution, and soaked in 0.5% acetic acid. Protein banding patterns were then visualized to estimate the molecular weight of bacteriocins **(13)**.

3. Results

3.1 Amino Acid Profile

The amino acid composition analysis of bacteriocins produced by three bacterial isolates—*Bacillus paramycoides* strain MCCC 1A04098 (BSH1), *Bacillus albus* strain MCCC 1A02146 (BSH2), and *Bacillus cereus* strain IAM 12605 (BSH3)—revealed both shared features and distinct differences in their respective profiles (Figure 1a–c). Across all isolates, L-cystine and L-proline consistently emerged as the dominant amino acids, suggesting a conserved structural role. However, the proportions of other amino acids varied significantly, providing insight into isolate-specific adaptations.

In BSH1 (*B. paramycoides*), the profile was dominated by L-cystine (9682.02; 40%) and L-proline (2183.71; 9%), followed closely by L-aspartic acid (1984.61; 8%) and L-

glutamic acid (1836.30; 8%). Additional contributors included L-serine (6%), leucine (4%), phenylalanine (4%), and glycine (4%). Notably, both tryptophan and tyrosine were absent. This amino acid distribution indicates that the BSH1 bacteriocin is particularly rich in sulfur-containing residues and polar non-essential amino acids, a feature that may enhance its secondary structural stability and tolerance to environmental stress.

In BSH2 (*B. albus*), L-cystine was even more abundant (5730.17; 45%), accompanied by L-proline (2505.71; 19%). Together, these two amino acids comprised nearly two-thirds of the total profile. The remaining amino acids were present in modest amounts, including leucine (4%), glycine (4%), valine (3%), lysine (3%), and methionine (3%). Aspartic acid and histidine were either absent or nearly undetectable. This simplified composition suggests a structurally less diverse but highly disulfide-stabilized bacteriocin, with cystine-derived disulfide bridges likely conferring strong conformational stability.

In BSH3 (*B. cereus*), the amino acid profile was more balanced. L-cystine accounted for 4196.84 (33%), and L-proline for 2519.19 (20%), but additional residues contributed significantly, including L-glutamic acid (887.19; 7%) and leucine (639.71; 5%). Glycine (5%), alanine (5%), and aspartic acid (2%) were also detected, while histidine and tryptophan were absent. Compared with BSH1 and BSH2, this broader distribution suggests that the bacteriocin from BSH3 may possess greater conformational flexibility and potentially a wider antimicrobial spectrum.

Taken together, the amino acid profiles demonstrate that while cystine and proline are universally dominant, secondary amino acids differ between isolates. These variations may influence not only the peptides' antimicrobial activity but also their stability against heat, enzymatic degradation, and their capacity to penetrate target membranes. The consistently high cystine content across all isolates highlights the probable role of disulfide bonding in stabilizing bacteriocin structure, while proline-rich segments likely contribute to β-turns and loop conformations, supporting functional activity.

3.2 Molecular Weight Analysis of Bacteriocins

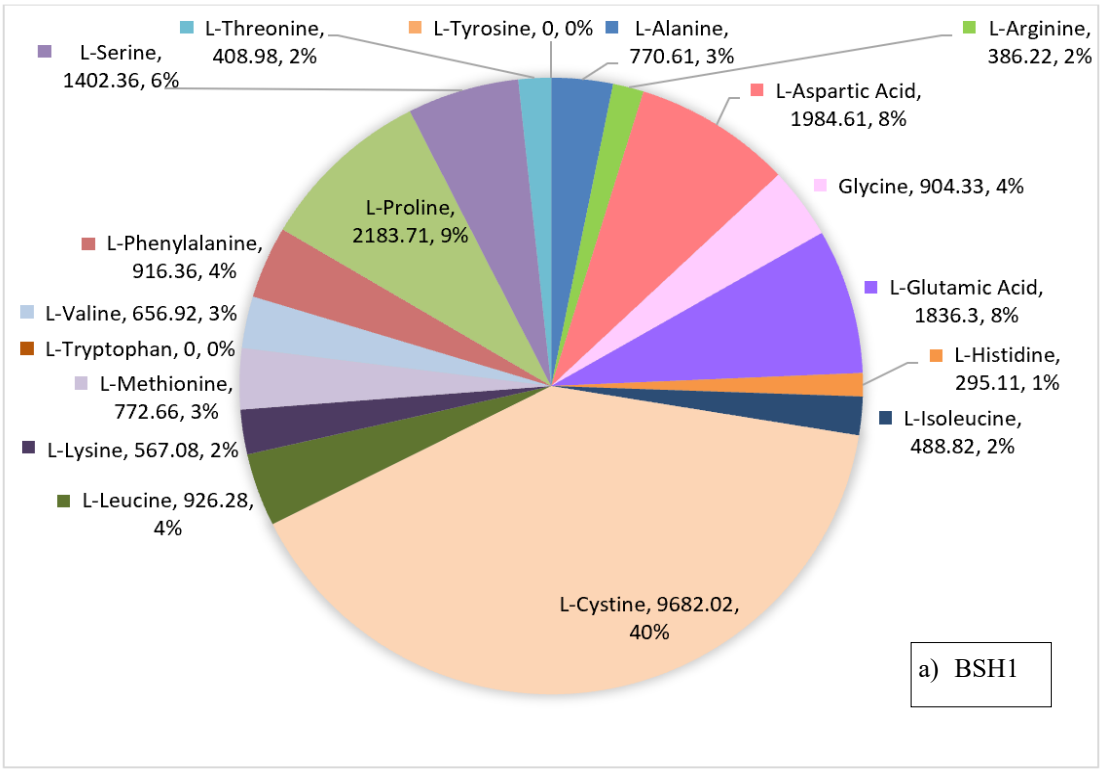
SDS-PAGE analysis confirmed the presence of bacteriocins of approximately 50 kDa in all three isolates (Figure 2). Distinct protein bands were consistently observed, with estimated molecular weights of 55.02 kDa for BSH1, 51.15 kDa for BSH2, and 50.12 kDa for BSH3.

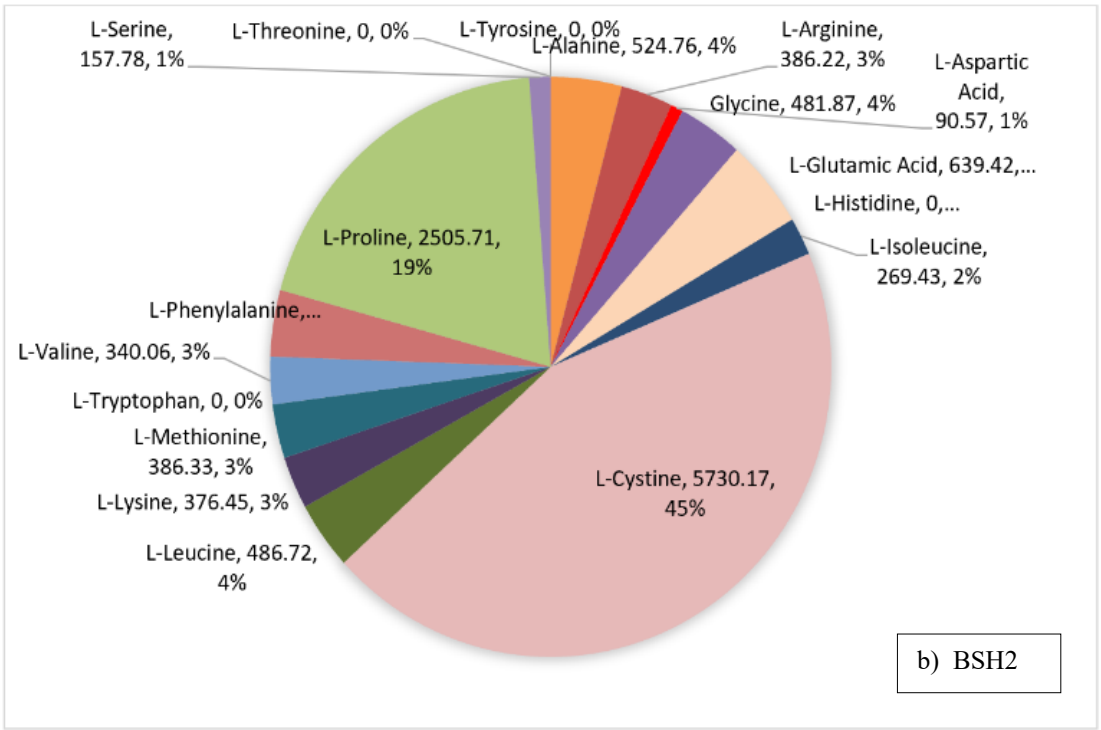
These values place the bacteriocins firmly in class III (>30 kDa). The relative uniformity of the bands suggests a conserved molecular size despite differences in amino acid composition. The consistent appearance of ~50 kDa bands across all isolates implies a shared structural framework, though their activity spectra may differ due to compositional variations.

Compared with bacteriocins typically reported from the *Bacillus* genus—ranging from very small peptides (<5 kDa, such as safencin E from *B. safensis*) to medium-sized molecules (10–30 kDa, such as subtilin from *B. subtilis*)—the bacteriocins identified in this study are unusually large. This size difference may reflect either large single-chain proteins or multimeric complexes not fully dissociated during electrophoresis.

3.3 Integrated Interpretation

The combination of amino acid and SDS-PAGE data reveals both shared and unique characteristics among the three isolates. While cystine and proline dominance suggests conserved structural requirements for stability and function, the variable presence of glutamic acid, leucine, and other residues points to isolate-specific adaptations. Moreover, the unusually high molecular weights establish these bacteriocins as rare examples of class III peptides within the *Bacillus* genus.


L-Threonine, 408.98, 2%
L-Tyrosine, 0, 0%
L-Alanine, 770.61, 3%
L-Arginine, 386.22, 2%
L-Serine, 1402.36, 6%
L-Aspartic Acid, 1984.61, 8%
Glycine, 904.33, 4%
L-Proline, 2183.71, 9%
L-Phenylalanine, 916.36, 4%
L-Glutamic Acid, 1836.3, 8%
L-Valine, 656.92, 3%
L-Histidine, 295.11, 1%
L-Tryptophan, 0, 0%
L-Methionine, 772.66, 3%
L-Isoleucine, 488.82, 2%
L-Lysine, 567.08, 2%
L-Leucine, 926.28, 4%
L-Cystine, 9682.02, 40%
a) BSH1


L-Serine, 157.78, 1%
L-Threonine, 0, 0%
L-Tyrosine, 0, 0%
L-Alanine, 524.76, 4%
L-Arginine, 386.22, 3%
L-Aspartic Acid, 90.57, 1%
Glycine, 481.87, 4%
L-Glutamic Acid, 639.42,...
L-Histidine, 0,...
L-Isoleucine, 269.43, 2%
L-Proline, 2505.71, 19%
L-Phenylalanine,...
L-Valine, 340.06, 3%
L-Tryptophan, 0, 0%
L-Methionine, 386.33, 3%
L-Lysine, 376.45, 3%
L-Leucine, 486.72, 4%
L-Cystine, 5730.17, 45%
b) BSH2

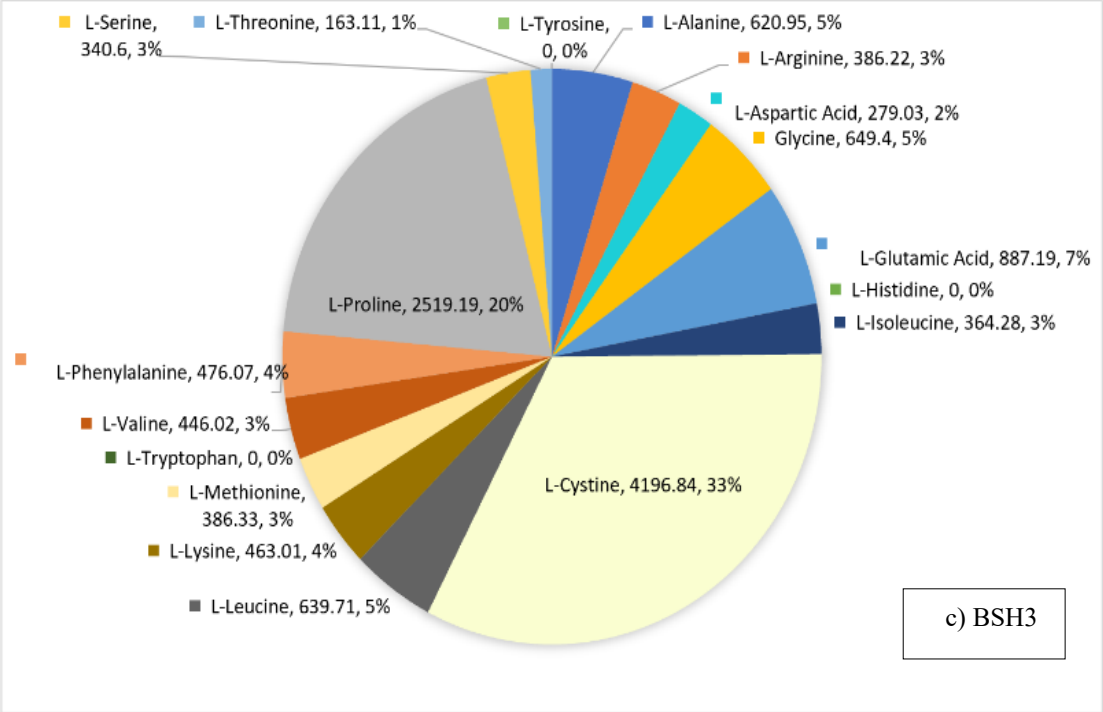


Figure 1. PRISMA flowchart demonstrating literature search and selection process Types and proportions of amino acids in bacteriocins produced by *Bacillus paramycoides* strain MCCC 1A04098 BSH1, *Bacillus albus* strain MCCC 1A02146 BSH2, and *Bacillus cereus* strain IAM 12605 BSH3 **(Prepared by Authors, 2025)**.

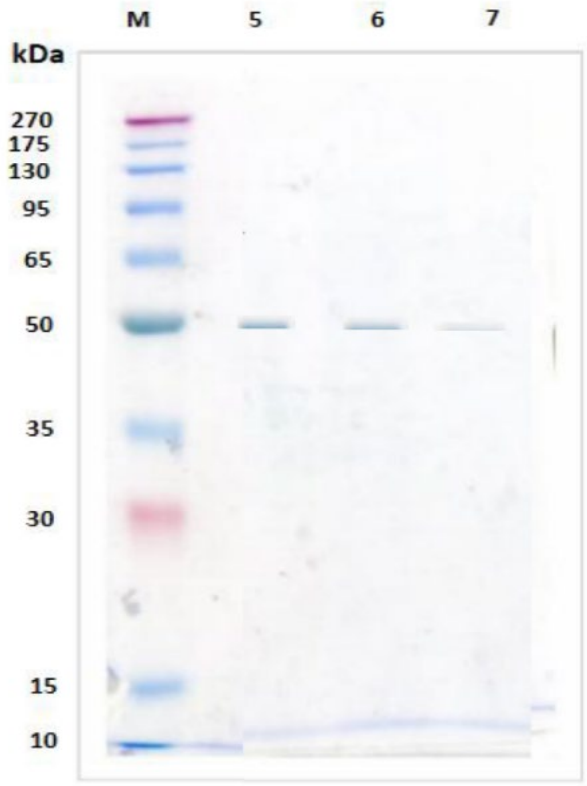


Figure 2. Molecular weight of bacteriocins produced by *Bacillus paramycoides* strain MCCC 1A04098 BSH1, *Bacillus albus* strain MCCC 1A02146 BSH2, and *Bacillus cereus* strain IAM 12605 BSH3, as determined by SDS-PAGE **(Prepared by Authors, 2025)**.

4. Discussion

This study characterized bacteriocins produced by *Bacillus paramycoides* BSH1, *Bacillus albus* BSH2, and *Bacillus cereus* BSH3, isolated from cincalok, through amino acid composition and molecular weight analysis. The results revealed that although the three isolates shared common features—particularly high levels of L-cystine and L-proline **(14)**—they also displayed distinct compositional patterns that may influence stability, antimicrobial activity, and functional range.

L-cystine was the most abundant residue, comprising 40–45% in BSH1 and BSH2, and 33% in BSH3. High cystine content is consistent with the formation of intramolecular and intermolecular disulfide bonds, which are known to confer structural rigidity and resistance to proteolytic degradation. Similar findings have been reported in Flexusin A from *Bacillus flexus*, where sulfur-rich residues contributed to remarkable stability under extreme pH and thermal conditions **(15)**. The prominence of cystine across all isolates strongly suggests that disulfide bonding is central to the structural integrity of these bacteriocins.

Proline was the second most dominant amino acid (9–20%), particularly enriched in BSH3. Proline residues are known to introduce β-turns or kinks into peptide chains, which may enhance conformational flexibility and facilitate interactions with bacterial membranes. The elevated levels of proline in BSH3, together with higher proportions of glutamic acid and leucine, may explain a potentially broader antimicrobial spectrum compared with BSH1 and BSH2. Previous studies, including those on Acidicin P from *Listeria*, have demonstrated the importance of proline and glutamic acid in enabling membrane penetration and functional versatility **(16)**.

Although arginine and lysine were present in only moderate amounts, their presence is notable. These cationic residues likely contribute to electrostatic interactions with negatively charged bacterial membranes, a mechanism commonly observed in cationic antimicrobial peptides. This feature parallels observations in Bawcin from *Bacillus wiedmannii*, where arginine and lysine were crucial for membrane disruption **(17)**. Thus, even though not abundant, their inclusion may be essential for the antimicrobial activity of these bacteriocins.

In addition, Garretto **(18)** reported that the presence of sulfur-containing amino acids such as L-Cystine plays a crucial role in the formation of disulfide bridges, which enhance the structural stability of bacteriocins under extreme environmental conditions, such as low pH and high temperatures. A study by Madhushan et al **(19)** on bacteriocins from *Bacillus cereus* also revealed a significant presence of L-Glutamic Acid and L-Aspartic Acid, supporting the findings in BSH1 and BSH3 isolates. These amino acids are known to be involved in ionic interactions with the membranes of pathogenic bacteria, which are critical to the mode of action of bacteriocins. Furthermore, Sugrue et al **(20)** emphasized that a diverse amino acid composition, such as that observed in the BSH3 isolate, contributes to a broader spectrum of antimicrobial activity, compared to bacteriocins with compositions dominated by only one or two amino acid types.

A striking finding of this study is the unusually high molecular weight of the bacteriocins. SDS-PAGE revealed consistent bands around 50 kDa for all isolates, with precise estimates of 55.02 kDa (BSH1), 51.15 kDa (BSH2), and 50.12 kDa (BSH3). These values clearly classify them as class III bacteriocins, which are relatively uncommon within the *Bacillus* genus. Most reported *Bacillus* bacteriocins fall within 2–30 kDa, such as subtilin (21 kDa), amyloliquecin (11 kDa), and safencin E (6 kDa) **(21-26)**. Even the larger *Bacillus* bacteriocins, such as ZY05-derived peptides (13–35 kDa), remain well below the ~50 kDa range observed here **(27)**. While the bacteriocin from *B. malacitensis* has a molecular weight of less than 5 kDa **(28)**. Remarkably, *Bacillus licheniformis* MKU3 produces a very small bacteriocin, approximately 1.5 kDa in size **(29)**.

Recent studies reinforce that most bacteriocins produced by various *Bacillus* species generally have low molecular weights, contrasting with your findings of bands around ±50 kDa on SDS-PAGE **(30)**. For example, *Brevibacillus laterosporus Wq-1* produces a bacteriocin with a molecular mass of approximately 12 kDa **(31)**, consistent with many leaderless bacteriocins. Similarly, Budhwan et al **(32)** reported that the bacteriocin Bawcin from *Bacillus wiedmannii* is very small, typically under 6 kDa, leaderless, and adopts a saposin-like fold.

The consistency of this molecular weight across three species suggests a conserved biosynthetic pathway, yet distinct from the well-characterized small *Bacillus* bacteriocins.

The combination of high cystine content and large molecular weight points toward a structurally stable and complex bacteriocin class. These bacteriocins may represent multimeric proteins or multidomain peptides not fully dissociated during electrophoresis. Their size and stability suggest functional diversity beyond typical pore-forming bacteriocins, possibly involving multi-step mechanisms of membrane disruption or interactions with intracellular targets. Such structural features make them particularly

interesting for further exploration as robust antimicrobial agents.

From an applied perspective, the unique molecular features of these bacteriocins enhance their potential for industrial use. The combination of stability, size, and antimicrobial activity could make them suitable for applications in food preservation, where robustness against heat and pH fluctuations is essential, and in healthcare, where resistance to enzymatic degradation is advantageous. Nevertheless, further studies are needed to fully assess their antimicrobial spectrum, mode of action, and cytotoxicity to human cells before application.

5. Conclusion

This study provides the first molecular characterization of bacteriocins produced by *B. paramycoides* BSH1, *B. albus* BSH2, and *B. cereus* BSH3 isolated from cincalok. Amino acid profiling revealed that all three bacteriocins are dominated by L-cystine and L-proline, residues that are critical for structural stability and functional activity. SDS-PAGE analysis identified consistent molecular weights of ~50 kDa, placing them in the rarely reported class III bacteriocins of the *Bacillus* genus.Compared with the smaller and more commonly described *Bacillus* bacteriocins, the unusually large size of these peptides underscores their novelty and potential importance. These findings highlight the promise of *Bacillus*-derived bacteriocins from traditional fermented foods as unique and stable antimicrobial agents. Further work should focus on sequencing, structural modeling, and antimicrobial assays to fully evaluate their potential for industrial and clinical applications.

6. Declarations

6.1 Acknowledgment

We would like to extend our gratitude for the funding support provided under the decision of the budget authority of the Directorate General of Research and Development, No. 0419/C3/DT.05.00/2025, dated May 22, 2025, regarding the acceptance of the second-phase operational assistance program for public universities in the research and community service program for the fiscal year 2025.

6.2 Ethical Considerations

All methods and experiments were approved by the Ethics Committee of Universitas Muhammadiyah Sorong, Indonesia, with registration number KEPK/UMP/107/III/2025. All participants agreed to be involved in this study.

6.3 Authors' Contributions

All methods and experiments were approved by the Ethics Committee of Universitas Muhammadiyah Sorong, Indonesia, with registration number KEPK/UMP/107/III/2025. All participants agreed to be involved in this study.

6.4 Conflict of Interests

The authors hereby declare that there are no conflicts of interest, whether financial or non-financial, that could influence the conduct of the research, data analysis, or the interpretation of the results reported in this manuscript. The entire content of this study has been prepared objectively and solely for academic and scientific purposes.

6.5 Financial Support and Sponsorship

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6.6 Using Artificial Intelligence Tools (AI Tools)

All authors declare that there is no use of AI Tools in this study, including the writing of this manuscript.

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