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Recombinant Production of Caspian Trout (*Salmo caspius*) Hepcidin in *Escherichia coli* and Evaluation of its Antibacterial Activity

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Abstract

Antimicrobial peptides are promising therapeutic agents due to their broad-spectrum activity against microbial pathogens. In this study, the recombinant antimicrobial peptide hepcidin from Caspian trout (*Salmo caspius*) was produced using an *Escherichia coli* expression system. The coding sequence of the mature CtHep peptide was codon-optimized for *E. coli*, cloned into the pET32c(+) vector, and expressed as a fusion protein. The recombinant protein was expressed as inclusion bodies, purified using a HisTALON™ affinity column, and refolded under optimized conditions. The thioredoxin-CtHep fusion protein was cleaved using TEV protease, and the recombinant CtHep peptide was isolated and analyzed. The antimicrobial activity of CtHep was evaluated against *Bacillus subtilis*, *Micrococcus luteus*, and *Escherichia coli* using the microdilution method. The results demonstrated that recombinant CtHep exhibited significant inhibitory effects on both Gram-positive and Gram-negative bacteria. These findings highlight the potential of recombinant CtHep as an antimicrobial agent for applications in aquaculture and other industries. Furthermore, the optimized conditions for *E. coli* cultivation and the methods used for the production and refolding of the recombinant CtHep peptide in this study can be applied in future research on the recombinant production of antimicrobial peptides in *E. coli*.

Introduction

Due to the overuse of antibiotics in recent decades, bacteria have developed increasing resistance to commonly used antibiotics (Ahmed et al., 2024; Solanki and Das, 2024; Dadgostar, 2019; Morar and Wright, 2010). Antibiotic resistance has become a major health concern and is considered one of the greatest threats to human health, with numerous cases of resistant bacteria causing serious infections in hospitals (Tacconelli et al., 2018; O'Neill, 2016; Marr et al., 2006; Hof et al., 2001; Davies et al., 2013). Antimicrobial peptides (AMPs) degrade more readily in the environment than traditional antibiotics, which are less biodegradable and contribute to contamination, bacterial resistance, and ecosystem damage. Additionally, AMPs disrupt conserved cell membrane components rapidly and effectively, making it difficult for bacteria to develop resistance without extended mutagenesis (Zhang et al., 2024).

Invertebrates and marine fish primarily rely on their innate immune system to defend against microbial pathogens. Their survival over millions of years in environments abundant with pathogenic organisms suggests that they have evolved various chemical defense mechanisms, including antimicrobial peptides, to combat disease. Therefore, research into antimicrobial peptides from marine organisms is highly

valuable. Marine antimicrobial peptides have been reported in sponges (Porifera), cnidarians, crustaceans, mollusks, tunicates, and teleost fishes (Zhang et al., 2024; Destoumieux-Garzón et al. 2016; Tincu and Taylor, 2004; Patrzykat and Douglas, 2003; Smith et al., 2010; Otero-González et al., 2010; Sperstad et al., 2011; Sathyan, 2015). Several antimicrobial peptides have been identified and extracted from teleost fishes, including pardaxin, misgurin, pleurocidin, epinecidin-1, cathelicidin, β-defensin, piscidins, hepcidin, and histone peptides (Debbarma et al., 2024; Wang et al., 2023; Zhang et al., 2023; Liu et al., 2017; Pant et al., 2023; Contreras et al., 2020; León et al., 2020; Sathyan, 2015). Genetic engineering offers an efficient approach to produce large quantities of highly expressed AMPs, enabling their practical use in applications such as aquaculture and medicine (Gong et al., 2022).

The recombinant expression of AMPs allows for the production of sufficient amounts for a range of applications, including laboratory studies and the control of diseases in humans and animals (Qiao et al., 2023; Gong et al., 2022; Chen et al., 2020; Wei et al., 2018; Ingham and Moore, 2007). Bacteria and yeast are the most commonly used expression hosts for the recombinant production of antimicrobial peptides (Roca-Pinilla et al., 2022; Li and Chen, 2008). Among these, *Escherichia coli* is the most widely used bacterial expression system for antimicrobial peptide production (Ingham and Moore, 2007; Parachin et al., 2012). Recombinant protein production in *E. coli* is a more economical process than other expression systems for a number of reasons (Wang et al., 2023; Sorensen and Mortensen, 2005). These include its rapid growth, the availability of a wide range of commercial expression vectors, efficient protocols for *E. coli* manipulation, and the extensive knowledge base on *E. coli* genetics, biochemistry, and physiology (Roca-Pinilla et al., 2022). The pET vector is the most frequently used expression vector for the recombinant production of antimicrobial peptides in bacterial expression systems, and commonly used *E. coli* strains include BL21 (DE3), Origami, pLysS, and Rosetta (Parachin et al., 2012). In recombinant production, the antimicrobial peptide is typically attached to a carrier protein, also referred to as a fusion protein. The fusion protein has an anionic property that prevents the antimicrobial peptide from acting on the host and protects it from degradation by proteases (Parachin et al., 2012). Additionally, these proteins facilitate the production of antimicrobial peptides in a soluble form (Esposito and Chatterjee, 2006; Chatterjee and Esposito, 2006). Studies on recombinant production of antimicrobial peptides strongly support bacteria as a suitable host. Cathelicidins, defensins, and crostins are among the main classes of marine antimicrobial peptides successfully produced in the *E. coli* expression system (Sathyan, 2015).

The isolation and preparation of antimicrobial peptides from their natural sources in large quantities are complicated, time-consuming, and practically unfeasible. Additionally, extracting these peptides from organisms in nature, especially in large quantities, is not cost-effective and raises ethical concerns (Metlitskaia et al., 2004). Chemical synthesis of antimicrobial peptides is expensive and not economically viable for applications in aquaculture and other industries. The production of recombinant antimicrobial peptides in bioreactor organisms, such as bacteria and yeast, offers a practical solution to this problem. Among these, recombinant production in bacterial hosts represents the most cost-effective strategy for delivering antimicrobial peptides. To date, numerous antimicrobial peptides from various plant and animal species have been produced using recombinant methods. Examples include the production of pleurocidin in *E. coli* (Bryksa et al., 2006) and in a fish cell line (Brocal et al., 2006), oyster defensin in *E. coli* (Gueguen et al., 2006), Hepc2 from Japanese seabass in *Pichia pastoris* (Gong et al., 2022), grass carp hepcidin in *E. coli* BL21 (Wei et al., 2018), and various penaeidins in yeast (Destoumieux et al., 1999; Li et al., 2005).

The purpose of the present study is to produce the recombinant antimicrobial peptide hepcidin from Caspian trout in *E. coli* and to investigate the biological activity of the produced peptide. The hepcidin-producing gene of Caspian trout was identified and reported in our previous study (Shirdel et al., 2019). This gene has been recorded in the NCBI GenBank with the accession number MK089523.

Materials and Methods

Gene Construct and Transformation

The coding sequence of the mature part of CtHep was codon-optimized according to *E. coli* codon preferences (Figure 1). The codon-optimized sequence was then synthesized in the pET32c(+) plasmid by Eurofins Genomics Company (Ebersberg, Germany). A TEV protease cleavage site (ENLYFQG) was inserted between the thioredoxin and CtHep sequences (Figure 2) (Nallamsetty et al., 2004). The pET32c-CtHep plasmid expresses a fusion protein composed of Trx (109 amino acids), His6 (6 amino acids), the TEV recognition site (6 amino acids), and CtHep (25 amino acids) (Contreras et al., 2018). *E. coli* BL21 was transformed with the pET32c-CtHep plasmid using electroporation (voltage: 2.5 kV, resistance: 200 ohm, capacitance: 25 μF) (Choi et al., 2006).

Optimization of Culture Conditions

The transformed bacteria were cultured in Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 1% sodium chloride) under varying temperatures, durations, and IPTG concentrations. The optimal conditions for producing the highest yield of recombinant protein were determined as follows: OD_{600}: 0.6–0.8, temperature: 28°C, IPTG concentration: 1 mM, and incubation time: 6 hours after IPTG

a

CAG AGC CAC CTC TCC CTG TGC CGT TGG TGC TGC AAC TGC
TGT CAC AAC AAG GGC TGT GGC TTC TGC TGC AAA TTC TGA

b

CAG AGC CAT CTG TCG TTA TGT CGC TGG TGT TGC AAT TGC
TGT CAC AAC AAA GGT TGT GGC TTT TGC TGC AAA TTC TAA

c

QSHLSLCRWCCNCCHNKGCGFCCKF

Figure 1. (a) Original nucleotide sequence of CtHep with the stop codon underlined. (b) Codon-optimized nucleotide sequence of CtHep for *E. coli* expression; optimized codons are highlighted in green, and the stop codon is underlined. (c) Amino acid sequence of mature CtHep.

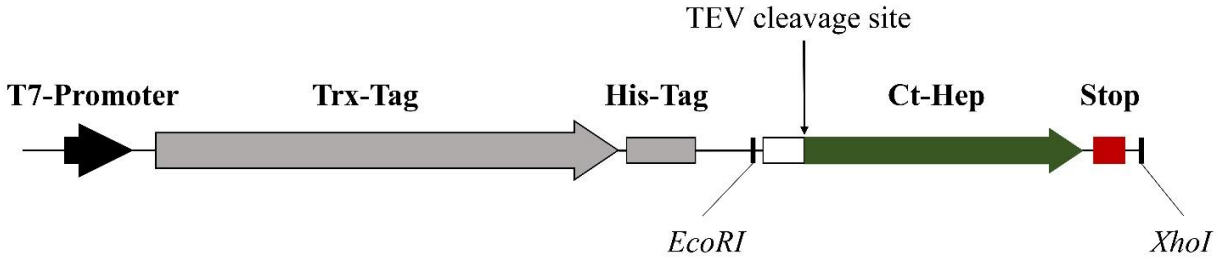

Figure 2. Schematic representation of the CtHep gene cloned into the pET32c(+) vector.

induction. The bacterial cell suspension was transferred into 50 mL Falcon tubes and centrifuged at 4°C and 5000 rpm for 15 minutes. The supernatant was discarded, and the cell pellet was stored at -20°C until protein extraction.

Extraction and Purification of Recombinant Fusion Protein

The cell pellet obtained from a 50 mL culture was resuspended in 10 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) (Aleinein et al., 2013). Lysozyme and PMSF were added to the cell suspension at final concentrations of 1 mg/mL and 1 mM, respectively, and the mixture was stirred on ice for 30 minutes. The cell suspension was then sonicated on ice (power: 20, pulser: 50%, duration: 5 minutes). The resulting lysate was centrifuged at 4°C for 30 minutes at 13,000 rpm (Contreras et al., 2018). Since the recombinant protein was produced as inclusion bodies, the supernatant (soluble fraction) was discarded, and the pellet (inclusion bodies) was retained. The inclusion bodies were solubilized in solubilization buffer containing 8 M urea (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, pH 8.0) (Contreras et al., 2018). The mixture was shaken for 10 minutes at 50°C and then for 60 minutes at room temperature (1,000 rpm). The solubilized inclusion bodies were centrifuged at 25°C for 30 minutes at 13,000 rpm, and the supernatant was filtered using a sterile 0.45 µm syringe filter. The recombinant fusion protein was purified using a HisTALON™ (Clontech) gravity column. The method provided by the manufacturer of the column was followed for the purification of the recombinant fusion protein. Briefly, 5 mL of equilibration buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, pH 8.0) was loaded into the column. Next, 10 mL of solubilized inclusion bodies was loaded onto the column. To remove unwanted proteins, the column was washed with 8 mL of equilibration buffer. The recombinant fusion protein was then eluted using an elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, 150 mM imidazole, pH 8.0), and 500 µL fractions were collected. Protein concentration was measured using the Bradford assay, with BSA as the standard (Bradford, 1976).

Refolding of the Purified Fusion Protein

To 900 µL of purified fusion protein (concentration: 1 mg/mL), DTT was added at a final concentration of 5 mM and gently stirred overnight at 4°C. The reduced sample was then divided into 300 µL aliquots, which were separately placed into 3 kDa cut-off dialysis tubes. Various methods and chemicals were tested for protein refolding, and the following procedure was found to be the most effective. The dialysis tubes were placed in a 100 mL solution containing DTT and 6 M urea (50 mM Tris, pH 8.0, 0.5 mM EDTA, 6 M urea, and 5 mM DTT) and

gently stirred at 4°C for 12 hours. In the next step, the dialysis solution was replaced with a solution containing GSSG/GSH and 4 M urea (50 mM Tris, pH 8.0, 0.5 mM EDTA, 4 M urea, 3 mM GSH, and 0.3 mM GSSG), and the tubes were gently stirred at 4°C for 12 hours. The dialysis tubes were then transferred to a solution containing GSSG/GSH and 2 M urea (50 mM Tris, pH 8.0, 0.5 mM EDTA, 2 M urea, 3 mM GSH, and 0.3 mM GSSG) for an additional 12 hours at 4°C. In the final step, the dialysis tubes were placed in a solution without urea, which also served as the buffer required for TEV protease activity (50 mM Tris, pH 8.0, 0.5 mM EDTA), and gently stirred overnight at 4°C. After dialysis, the solution inside the dialysis tubes was removed and centrifuged at 4°C and 13,000 rpm for 30 minutes to discard unfolded and insoluble proteins. The supernatant (refolded proteins) was stored at -20°C for further experiments.

Cleaving Trx-CtHep Fusion Protein

The Trx-CtHep fusion protein was cleaved using TEV protease (Protean, Czech Republic) in TEV cleavage buffer (50 mM Tris, pH 8.0, 0.5 mM EDTA) at a TEV-to-protein ratio of 1:100 (w/w) (Contreras et al., 2018). The reaction mixture was incubated overnight at 4°C. The cleaved samples were subsequently loaded onto a 16% Tricine-SDS-PAGE gel and stained using the Blue-Silver staining method to evaluate cleavage efficiency (Candiano et al., 2004; Schägger, 2006).

Evaluation of the Antimicrobial Activity of Recombinant CtHep

The antimicrobial activity of the recombinant CtHep peptide was evaluated using the microdilution method, based on the protocol described by Wiegand et al. (2008). Since our attempts to purify CtHep from thioredoxin were not successful due to challenges in separation and the lack of equipment such as size exclusion chromatography, we used a mixture of CtHep and thioredoxin to evaluate the antimicrobial potential of recombinant CtHep. Thioredoxin was previously utilized as a negative control in the antibacterial experiment (MIC assay), and demonstrated no antimicrobial activity (Herbel et al., 2015). In the present study, bacterial cultures were adjusted to a concentration of 1×10^8 CFU/mL using physiological saline solution after overnight growth in BHI medium. The mixture of hepcidin and thioredoxin was diluted to the desired concentrations using BHI culture medium. Then, 50 µL of the bacterial suspension was added to the wells of a 96-well microplate, which was incubated at 37°C for 20 hours. Sterility and growth control groups were also included in the experiment. The minimum inhibitory concentration (MIC) of the hepcidin-thioredoxin mixture was defined as the lowest concentration at which bacterial growth was inhibited.

Results

Trx-CtHep Fusion Protein Expression

E. coli BL21 cells carrying the pET32c-CtHep plasmid successfully produced the Trx-CtHep fusion protein following induction with 1 mM IPTG. While efforts were made to optimize conditions for soluble expression, the majority of the protein was consistently expressed as inclusion bodies, with only a small fraction present in the soluble form. As achieving a higher protein yield was critical, the focus shifted to optimizing culture conditions to maximize overall protein concentration, even in the form of inclusion bodies. As shown in Figure 3, the recombinant protein was present in both the insoluble and soluble fractions, but the concentration in the soluble fraction was significantly lower compared to the insoluble fraction. The results of the SDS-PAGE analysis demonstrated the presence of a sharp band, corresponding to the Trx-CtHep fusion protein with an estimated molecular weight of 23 kDa, within the insoluble fraction of lysed IPTG-induced cells (Figure 3). The fusion protein was successfully purified using a HisTALON™ affinity chromatography column (Figure 4), yielding a protein concentration of approximately 1 mg/mL.

Enzymatic Cleavage of Trx-CtHep Fusion Protein

The sequence ENLYFQG, which serves as the recognition site for TEV (Tobacco Etch Virus) protease, is located between Trx and CtHep. Using TEV protease, the fusion protein was successfully cleaved into two segments: CtHep (2.8 kDa) and 6His-Trx (20 kDa) (Figure 5). The cleavage reaction was not complete, as indicated by the presence of the uncleaved Trx-CtHep fusion protein band on the SDS-PAGE gel. Based on the intensity of the bands observed, it can be estimated that more than 50% of the fusion protein was cleaved under the described conditions. This indicates efficient but incomplete cleavage, which could be improved in future studies by further optimizing reaction conditions.

Antimicrobial Activity of Recombinant CtHep

The antimicrobial activity of the mixture of recombinant Trx and CtHep was evaluated using the microdilution method, and the minimum inhibitory concentration (MIC) was determined against *Micrococcus luteus*, *Bacillus subtilis*, and *Escherichia coli*. The MIC values were 100 µg/mL for *M. luteus*, 100 µg/mL for *B. subtilis*, and 200 µg/mL for *E. coli*. These results demonstrated that recombinant CtHep exhibits significant inhibitory effects on both Gram-positive and Gram-negative bacteria, with higher activity observed against Gram-positive strains. A summary of these MIC values is provided in Table 1.

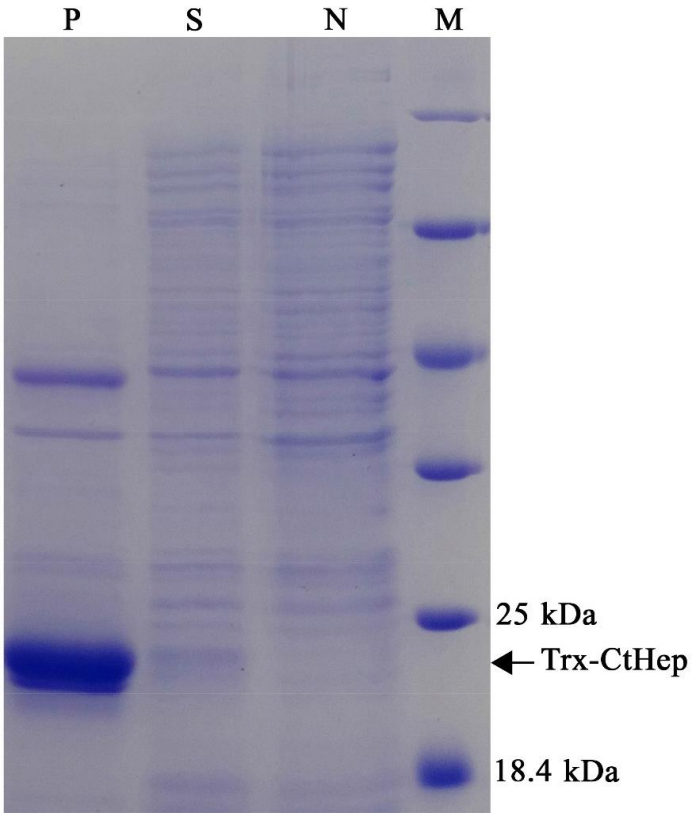

Figure 3. 12% SDS-PAGE analysis of protein expression in E. coli BL21 transformed with pET32c(+)-CtHep. The gel was stained with Coomassie Brilliant Blue R-250. Lane M: Broad-range protein marker; N: Total cell protein of BL21 cells before IPTG induction; S: Soluble fraction of IPTG-induced BL21 cells; P: Insoluble fraction (inclusion bodies) of IPTG-induced BL21 cells. The recombinant Trx-CtHep fusion protein is indicated by an arrow.

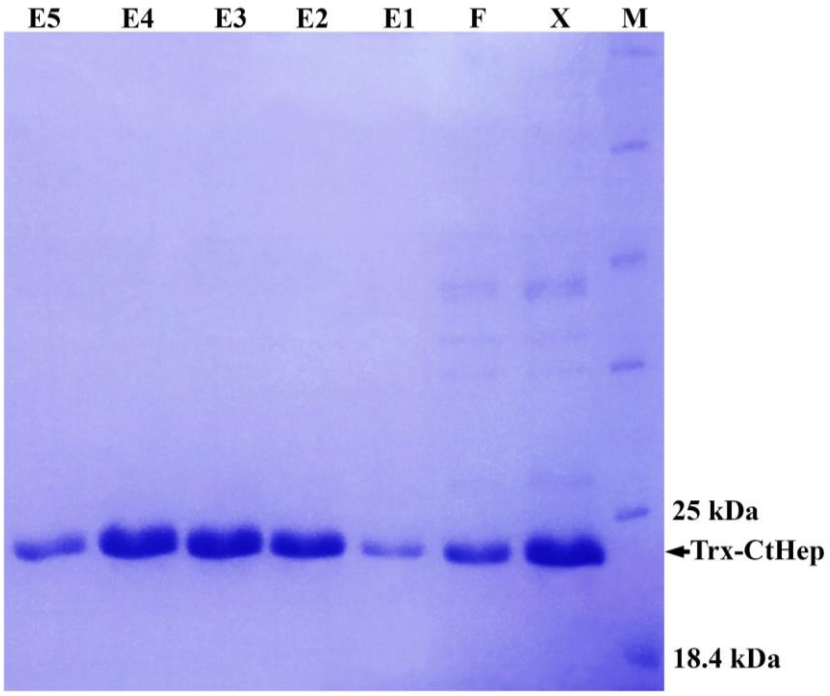

Figure 4. 12% SDS-PAGE analysis of the purified recombinant Trx-CtHep fusion protein using a HisTALON™ gravity column. Lane M: Broad-range protein marker; X: Solubilized inclusion bodies; F: Flow-through (proteins not bound to the His-tag); E: Protein fractions eluted from the column; E1–E5: Sequential elution fractions. The location of the recombinant Trx-CtHep fusion protein is indicated by an arrow.

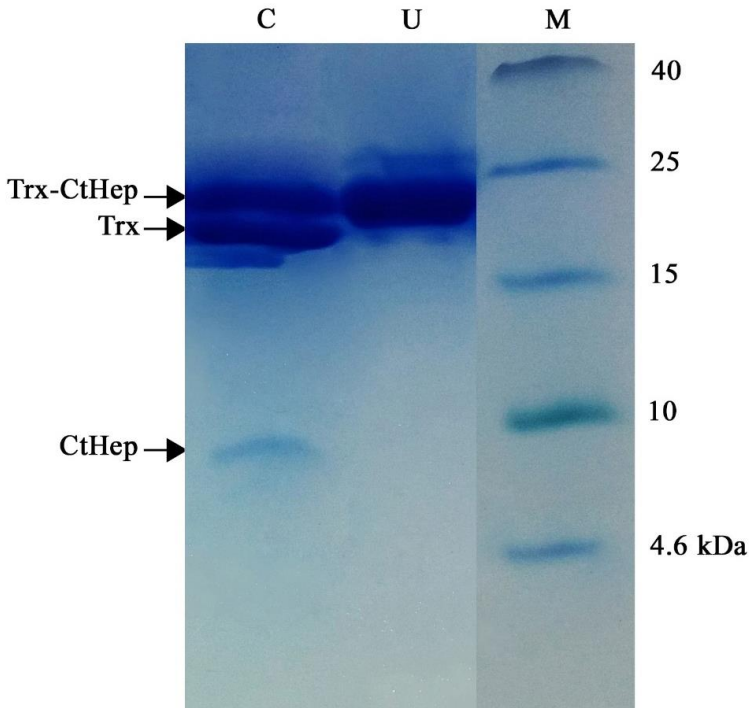

Figure 5. Tricine-SDS-PAGE (16%) analysis of the cleavage of recombinant Trx-CtHep fusion protein using TEV protease. The gel was stained using the "Blue-Silver" Coomassie staining method. Lane M: Low-range protein marker; U: Purified recombinant Trx-CtHep fusion protein (uncleaved); C: Cleavage reaction mixture containing uncleaved Trx-CtHep fusion protein, cleaved Trx fusion partner, and cleaved CtHep.

Discussion

Recombinant antimicrobial peptides, including hepcidins, are promising candidates for use in aquaculture and animal husbandry due to their broad-spectrum antimicrobial activity and ease of large-scale production. They can be utilized as dietary supplements to enhance disease resistance in farmed animals. However, the development of cost-effective production methods is critical for their industrial application (Gong et al., 2022).

This study demonstrated that recombinant Caspian trout hepcidin (CtHep) exhibited significant antimicrobial activity against *B. subtilis*, *M. luteus* and *E. coli*. The MIC values of CtHep were lower for Gram-positive bacteria, consistent with previous findings for other fish hepcidins (Ke et al., 2015; Shirdel et al., 2019; Wang et al., 2009; Zhang et al., 2009). Studies have shown that recombinant hepcidins from various fish species exhibit significant antimicrobial activity and protective benefits. For instance, recombinant *Labeo rohita* inhibited the growth of *Edwardsiella tarda* and *A. hydrophila* in vitro (Mohapatra et al., 2019), while recombinant grass carp hepcidin (CiHep) improved survival rates in *Ctenopharyngodon idellus* infected with *F. columnare* through dietary supplementation or injection (Wei et al., 2018). Similarly, Yellow River carp hepcidin, expressed in *Pichia pastoris X-33*, displayed strong antibacterial activity against *A. hydrophila*, *E. coli*, *V. anguillarum* and *B. subtilis*, highlighting its potential as a dietary supplement (Qiao et al., 2023). Additionally, recombinant hepcidin from Japanese seabass exhibited broad-spectrum activity against Gram-positive and Gram-negative bacteria, as well as fungi (Gong et al., 2022). These findings suggest that recombinant hepcidin has promising potential for future applications as a novel antimicrobial agent in aquaculture and medicine.

Although thioredoxin was part of the mixture used in this study, previous studies have reported that thioredoxin alone does not exhibit antimicrobial properties, and several studies have utilized thioredoxin in combination with antimicrobial peptides, either as a mixture or a fusion, to perform antimicrobial assays (Herbel et al., 2015; Wang et al., 2012; Xia et al., 2013; Yu et al., 2016). This supports the conclusion that the

Table 1. Antibacterial activity of the mixture of recombinant Trx and CtHep

Bacteria	Gram +/-	MIC (µg/ml)
Bacillus subtilis	+	100
Micrococcus luteus	+	100
Escherichia coli	-	200

observed activity was primarily due to CtHep. Moreover, our previous study demonstrated that the synthetic CtHep exhibited antimicrobial activity against *B. subtilis*, *M. luteus* and *E. coli* (Shirdel et al., 2019). Nevertheless, the use of the CtHep-thioredoxin fusion protein introduces potential limitations. Although thioredoxin did not interfere with activity in previous studies, its presence in this work may have influenced CtHep's interactions with bacterial membranes or affected the MIC values. Further optimization of the purification protocols, such as incorporating size exclusion chromatography, could improve CtHep recovery and allow a more detailed characterization of its properties.

The antimicrobial activity of CtHep may involve mechanisms observed in other hepcidins, such as disrupting bacterial membranes or interacting with bacterial DNA. For example, rainbow trout hepcidin has been shown to penetrate membranes and hydrolyze bacterial gDNA (Alvarez et al., 2014), while flounder hepcidin binds DNA and disrupts bacterial membranes (Li et al., 2023). Additionally, brown trout hepcidin disrupts bacterial outer membranes (Huang et al., 2019). While these studies provide insight, the exact mechanism of CtHep remains speculative. Alternative pathways, such as targeting intracellular processes or membrane-associated components, could also play a role (Ghodsi et al., 2020). Direct evidence, such as assays measuring membrane disruption or DNA binding, is needed to confirm CtHep's mechanism of action.

The predicted size of recombinant CtHep is 2.8 kDa, but SDS-PAGE analysis showed an apparent molecular weight of 7-8 kDa. This discrepancy is likely due to the cationic nature of CtHep. SDS may not have completely neutralized the positive charge of the peptide, resulting in its slower migration on the gel and, consequently, its positioning at a higher apparent molecular weight than expected. A similar observation was reported by Herbel et al. (2015) for Snakin-2, a cationic antimicrobial peptide from tomato. To confirm the actual molecular weight of CtHep, techniques such as mass spectrometry could be used in future studies to provide definitive validation.

Conclusion

The antimicrobial peptide hepcidin from Caspian trout (CtHep) was successfully produced, extracted, and refolded in *E. coli*, demonstrating significant antimicrobial efficacy against both Gram-positive and Gram-negative bacteria. This highlights its potential as a therapeutic agent for controlling fish diseases in aquaculture, where antibiotic resistance is a growing concern. The optimized cultivation conditions and production methodology developed in this study provide a valuable framework for future research on recombinant antimicrobial peptides. However, challenges in purifying CtHep from its thioredoxin fusion partner and the reliance on inclusion body formation highlight limitations that need to be addressed. Overcoming these hurdles, such as by optimizing purification techniques or exploring alternative expression strategies, would enhance the feasibility of scaling up CtHep production for practical applications. Despite these challenges, the promising efficacy of recombinant CtHep reinforces its potential as a novel antimicrobial agent for aquaculture, contributing to sustainable disease management strategies.

Ethical Statement

All process and experimental protocols have been approved by Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Germany.

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Author Contribution

First Author: Conceptualization, Supervision, Writing -review and editing; Second Author: Data Curation, Formal Analysis, Investigation, Methodology, Visualization and Writing -original draft; Third Author: Funding Acquisition, Project Administration, Resources, Writing -review and editing.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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