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Exploration of Alternative Protein Food and Cyclic Dipeptides that Help Complement Alternative Protein Sources

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Abstract

The global movement towards sustainable food systems has given rise to a growing interest in alternative protein sources, including plant proteins, insect proteins, cultured meats, and microbially derived proteins. This study focuses on the latest trends and future developments in this field, with a particular emphasis on state-of-the-art technologies and methodologies designed to overcome the obstacles associated with alternative proteins, including taste, flavor and texture. A principal objective of this research is to identify proteins that will facilitate the large-scale production of cyclic dipeptides (CDPs), based on the hypothesis that CDPs can leverage their distinctive biochemical attributes to enhance the appeal of alternative protein sources. One of the key challenges in this research is identifying the proteins required for CDP production. Previous studies have shown that lactic acid bacteria, such as *Weissella cibaria*, *Lactobacillus sakei*, *Leuconostoc mesenteroides*, and *Pediococcus pentosaceus* which were isolated from kimchi exhibit strong antimicrobial activity, with CDPs identified among their metabolites. This observation has prompted the utilization of these bacteria in the present study. The findings of this study suggest that CDPs could be an effective solution for developing alternative protein sources and could represent a significant advancement in food technology innovation.

Keywords: Alternative protein, Cyclic dipeptides, Sustainability, Protein sources

Major Classifications: Food Technology, Food biochemistry, Food Science (Food Nutrition)

1. Introduction[12]

In recent years, there has been a notable increase in global interest in the development of sustainable food systems. Food production is a significant contributor to global environmental change, particularly when it is produced on a large scale through factory farming to meet the demand for animal protein. This practice can result in biodiversity loss, climate change (Aiking, 2014), and environmental degradation due to the emission of substantial quantities of greenhouse gases, including carbon dioxide and methane, from ruminant metabolic waste (Hristov et al., 2013)

The investigation and development of alternative protein sources for sustainable food systems is a rapidly evolving field of research, and it is currently a prominent topic in food technology. Changing societal perceptions on religious, ethical, and health issues also represent a significant factor influencing protein choices (Drewnowski, 2018). A market survey of consumers in 12 countries across the Americas, Europe, Asia, and the Pacific revealed that 36% of consumers anticipate utilizing alternative proteins more

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frequently in the next year (Joseph et al., 2020). Alternative sources of animal protein include plant-based alternatives such as soy and gluten (Ismail et al., 2020). identified microbially derived alternatives, such as mycoprotein derived from the fermentation of *Fusarium venenatum*, which is commonly found in soil (Sharif et al., 2021), as a promising avenue for further research. Insect alternatives, including crickets and silkworms (Liceaga et al., 2022), and cultured meat from cell cultures (Chriki & Hocquette, 2020) were also highlighted as potential solutions.

However, the development and commercialization of these alternative protein resources face several challenges, including taste, flavor, texture. In particular, plant-based and insect-based proteins have limitations in mimicking the taste, aroma, and texture of animal-based proteins, and suffer from poor gel-forming ability and allergenicity that need to be addressed (Ma et al., 2022).

2,5-diketopiperazine, also known as cyclic dipeptides, is one of the components of beef flavor and is responsible for its bitter and umami taste. It is not only present in beef, but also in protein-rich foods such as chicken, coffee, and cocaine (Chen et al., 2009). 2,5-diketopiperazines have been demonstrated to elicit a range of chemical effects, including those associated with bitterness and umami, which can contribute to the flavor of a diverse array of foods and beverages. Additionally, they have been demonstrated to possess antibacterial, antifungal, and human protective effects, along with a range of other bioactivities (Borthwick & Da Costa, 2017). The organoleptic evaluation of cyclic dipeptides in beef demonstrated that cis-cyclo(l-Ala-l-Pro) and cis-cyclo(l-Pro-l-Pro) produced beefy and juicy flavors, respectively, while cis-cyclo(l-Pro-l-Ala) and cis-cyclo(l-Phe-l-Pro) resulted in bitter flavors. The addition of Leu-l-Pro and cis-cyclo(l-Phe-l-Pro) resulted in the development of beefy and bitter flavors, respectively. Conversely, the incorporation of cis-cyclo(l-Pro-l-Pro) led to the emergence of juicy flavor. The flavor effects were dependent on the form and concentration of processing (Chen et al., 2009).

Through the analysis of flavor compounds generated during heating and cooking of meat, it is necessary to develop and apply technologies that can be converted into active substances during cooking processing by using encapsulation technology and precursors (Gómez et al., 2018). Nanoencapsulation technology can enhance solubility and bioavailability while shielding unstable compounds from unfavorable conditions during processing, thereby improving quality, stability, and activity (Assadpour & Mahdi Jafari, 2019). On the application of nanoencapsulation technology, (Yadav et al., 2011) states that proteins and peptides can be encapsulated or adsorbed onto nanocarriers through a variety of methods, including emulsion polymerization, interfacial polymerization, solvent evaporation, salt lithography, coacervation, and solvent substitution/solvent diffusion.

A notable distinction exists between the gelation properties of vegetables and animal proteins. In particular, vegetable proteins, such as those derived from peas and lentils, tend to form weaker gels than their animal protein counterparts (Banach et al., 2023). Cyclic dipeptides (CDPs) improve gelling properties through specific mechanisms related to their ability to form structured molecular aggregates. These peptides are involved in strong intermolecular hydrogen bonding and non-covalent interactions such as van der Waals forces and π-π stacking. This leads to the formation of stable three-dimensional networks, which is important for enhancing gel properties. These CDPs form fibrous assemblies, which enhance the mechanical properties of the gel (Manchineella & Govindaraju, 2012). Among the cyclic dipeptides, those containing Phenylalanine demonstrated notable gelling capabilities (Hanabusa et al., 1994). Modifying the branched alkyl groups of amino acids not only enhances the gelation ability but also improves the stability of the gel by preventing crystallization (Zhao et al., 2021). It is probable that this will affect protein solubility by modifying the hydrophobicity or charge of the protein molecule, thereby promoting aggregation, which is a prerequisite for gelation.

Cyclic dipeptides have the potential to address an important unmet need in the development of alternative proteins. The aim of this study is to identify proteins that aid in the biosynthesis of CDPs so that CDPs can be used in the development of alternative protein foods. Utilizing naturally occurring peptide substances as complements to alternative proteins is a promising avenue for future research.

Using four lactic acid bacteria isolated from kimchi, we aimed to identify metabolites with potent antimicrobial properties, as CDP is a metabolite with antimicrobial activity. *Pediococcus pentosaceus* has anti-inflammatory and antibacterial properties (Fugaban et al., 2022). Although *P.pentosaceus* is not a strain with proven CDP production capacity, it was compared to Lactobacillus plantarum LBP-K10, which was previously demonstrated to be a predominant producer of CDP (Kwak et al., 2018) and were chosen because of their similar high-performance liquid chromatography (HPLC) fractionation patterns. In addition, strains of *Wessella cibaria*, *Lactobacillus sakei*, and *Leuconostoc mesenteroides* were chosen because, like LBP-k10, they are the main antimicrobial LABs isolated from kimchi, as shown in previous studies (Kwak et al., 2013). To identify the metabolites of antimicrobial activity, or CDPs, and the enzyme proteins that help in their synthesis, each laboratory used high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) to detect the CDPs, and native PAGE and SDS PAGE with cultured cells, followed by liquid chromatography-mass spectrometry (LC-MS) to identify the enzyme proteins.

2. Research Methods and Materials

2.1 The Culture Supernatant

2.1.1 Inoculation

Each LAB was cultured in modified MRS (mMRS medium is without beef extract) with modifications (Son et al., 2024). We used both liquid and solid media. To make the liquid media, we added the following ingredients per 1 L: 5 g of yeast extract, 5 g of sodium acetate trihydrate ($C_2H_3NaO_2{\cdot}3H_2O$), 2 g of ammonium citrate tribasic ($C_6H_{17}N_3O_7$), and 2 g of potassium hydrogen phosphate (K_2HPO_4). Add 1 g of magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$) and 0.05 g of manganese sulfate tetrahydrate ($MnSO_4{\cdot}7H_2O$). 20 grams of D-glucose ($C_6H_{12}O_6$) was added. Four distinct media were employed for the culturing process, including. Medium-1: The medium with the composition mentioned above (non-peptone-containing mMRS). Medium-2: The medium containing 1% peptone in the base medium (peptone/D-glucose containing mMRS). Medium-3: The medium containing 1% peptone, to which D-glucose was added separately and sterilized (peptone containing mMRS with separately and sterilized D-glucose). Medium-4: The medium containing 1% peptone, to which D-glucose was added separately and sterilized, and 2 mM of amino acids (proline, leucine, glycine, phenylalanine, and histidine) (peptone containing mMRS with separately and sterilized D-glucose and amino acid. The reason for using amino acids is that amino acid (AA) metabolism is sufficiently favorable to contribute on a large scale to physiological events such as small peptide and protein biosynthesis, pH regulation, changes in metabolic energy/redox balance, and stress resistance by various types of intermediate metabolites. The most important distinction is whether fermentation takes place in the raw material or in the culture medium (Fernández & Zúñiga, 2006).The seed culture was prepared to 0.1% of the total liquid volume. The seeds were incubated at 30℃ for 72 hours without agitation.

2.1.2 Liquid-liquid extraction

The incubated LAB was subjected to centrifugation at 8000 rpm for 15 minutes, resulting in the separation of the culture supernatant and cell pellet. The culture supernatant was then filtered through a 0.22μm nitrocellulose membrane to obtain the culture filtrates (CFs). 4L of CFs was concentrated using a rotary evaporator to 1L. The concentrated culture underwent liquid-liquid extraction using at least four volumes of methylene chloride (MC, dichloromethane, CH_2Cl_2). The mixture is separated through a funnel and the separated solvent portion is evaporated using a rotary evaporator at 55℃.The remaining metabolites were dissolved in 10 ml of TDW.

2.1.3 HPLC fractionation

Preparative-HPLC was performed using a Waters Autopurification system equipped with SunFire C18 OBD preparative column (19 × 250 mm, Waters, USA). The mobile phase was 65% water, 5% acetonitrile, and 30% methanol at a flow rate of 8.53 mL/min. The wavelength was 210 nm, measured with a multi-wavelength diode array detector. To obtain powder samples, each fraction was fractionated, collected, evaporated and lyophilized. The concentrated powder samples were analyzed using gas chromatograph Multi-Omics high resolution mass spectrometry (GC-HRMS) (Thermo Fisher Scientific, USA) method.

2.2 The Identification and Overproduction of Proteins Related to CDPs

2.2.1 Crude protein extraction

The cells obtained after centrifugation of the culture were washed with 50 mM Tris-HCL (pH8.0) buffer. Ultrasonication (KBT, Korea) was used to break the cell wall and cellular proteins were obtained.

2.2.2 DEAE anion exchange chromatography

Cellular proteins were subjected to anion exchange chromatography using a 45 mm x 200 mm glass column filled with DEAE-Sepharose CL-6B resin. For the separation, an elution buffer with a step gradient ranging from 0 to 0.5 M NaCl in ionic strength was applied. The process resulted in 15 distinct fractions, which were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.2.3 Activity staining using ninhydrin

Native PAGE was performed and the resulting gel was exposed to an amino acid reaction at 30℃. We added 5 ml of buffer containing ATP, 0.1% $MnSO_4$, and 0.05% $MgSO_4$. The amino acid buffer was removed and a solution containing 0.1 M ninhydrin and 0.1 M Tris-HCl buffer (pH8.0) was added. The ninhydrin reaction was conducted at 37℃ in the dark for 15 minutes. The ninhydrin colorimetric method was applied to detect amino acids and assess the bioactivity of protein fractions. On the native gel, a colorless band was observed.

2.2.4 Liquid chromatography Multi-Omics High Resolution Mass Spectrometry (LC-HRMS) analysis

Native gels were prepared, and the unstained portion of the gel was excised following the ninhydrin reaction. The resulting material was then subjected to analysis using liquid chromatography multi-omics high-resolution mass spectrometry (LC-HRMS) (Thermo Fisher Scientific, USA).

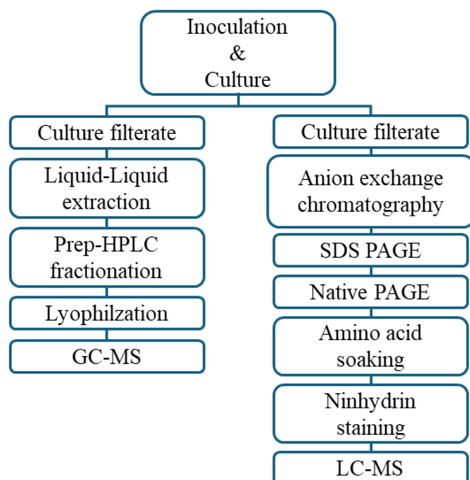

Figure 1: Flow chart of total process and steps

3. Results and Discussion

3.1 Favorable culture conditions for isolating bioactive substances

To enhance the purification of antimicrobial compounds, each lactobacillus was cultivated in four distinct types of mMRS media. Based on the HPLC peaks and the results of the disk diffusion experiments, it was determined that the optimal condition for the purification of the bioactive substance was cultivation of *Leuconostoc mesenteroides* LabMP-A04 in non-peptone-containing mMRS media and peptone-containing mMRS with separately sterilized D-glucose and amino acid media. With regard to medium-4(peptone-containing mMRS with separately sterilized D-glucose and amino acid), the presence of amino acids exerted a more pronounced influence on metabolite production than that of peptone. Consequently, medium-1 (non-peptone containing mMRS) was selected on the basis of its superior capacity to produce metabolites in comparison to the other two media. The remaining strains, *Lactobacillus sakei* LabMP-A02, *Pediococcus pentosaceus* LabMP-A05, and *Weissella cibaria* LabMP-A06, demonstrated robust antimicrobial activity and produced elevated metabolite levels in the peptone/D-glucose-containing mMRS medium. Consequently, we selected medium-2 (peptone/D-glucose-containing mMRS) as the optimal condition for these strains.

3.2. Bioactive Proteins

Table 1: Results from LC-MS analysis of purified CDPs biosynthesis protein

Accession	Description	Score	AAs	MW[kDa]
KAF0506407.1	Universal stress protein [*Pediococcus pentosaceus*]	255.93	151	16.7
GEP16201.1	Xaa-Pro aminopeptidase [*Leuconostoc mesenteroides*]	2.1	365	39.9
CAI54339.1	Similar to universal stress protein, UspA family [*Lactobacillus sakei* subsp. *Sakei* 23K]	27.66-171.48	144	17.9
RGO80327.1	Proline tRNA ligase [*Weissella cibaria*]	370.73	573	63.9

The separation of each cell crude extract was conducted using DEAE Sepharose CL-6B anion exchange chromatography, followed by native-PAGE analysis. The distinct bands observed following amino acid soaking and ninhydrin staining were subjected to multi-omics high-resolution mass spectrometry analysis. The results of protein identification are shown in Table 1. These results do not represent a complete proteomic analysis, but rather a selection of enzyme protein candidates for each strain that are most likely to be involved in CDPs biosynthesis: we chose universal stress proteins in *P. pentosaceus*, Xaa-Pro aminopeptidases similar to universal stress proteins in *L. mesenteroides*, the UspA family in *L. sakei*, and proline tRNA ligases in *W. cibaria*. Universal stress proteins function to protect organisms by overexpressing when exposed to stressful situations. Additionally, they exhibit structural diversity, functioning as cellular signal transducers and metabolic regulators (Chi et al., 2019; Udawat et al., 2016). It is anticipated that they will play a role in intracellular CDP synthesis. Xaa-pro aminopeptidase is responsible for cleaving amino acids adjacent to the N-terminus of proline residues (Matsushita-Morita et al., 2010). We expected that the special features of this enzyme, such as its stability and low molecular weight, would affect CDP biosynthesis (Nandan & Nampoothiri, 2014). Similar to universal stress proteins, the UspA family was chosen because it is believed that it can help organisms survive in unfavorable stress environments when synthesizing bioactive compounds (Kvint et al., 2003). Proline tRNA ligases, also called prolyl-tRNA synthetases, are enzymes of the aminoacyl-tRNA synthetase family. Like aminoacyl-tRNA synthetases, they are expected to play an important

role in the biosynthesis of cyclic dipeptides (CDPs) by supplying aminoacylate tRNA, which is the substrate for cyclodipeptide synthases (CDPs) (Giessen & Marahiel, 2014; Skinnider et al., 2018).

Overproduction of the recombinant protein involved in the biosynthesis of CDP will soon be required for mass production of CDP. Further work will include IPTG induction to determine the optimal induction time and temperature, SDS-PAGE, and protein expression under these conditions. CDPs will then be extracted by MC extraction and preliminary HPLC analysis. This suggests that the biosynthetic mechanism of CDP can be explored using the recombinant protein and the results will allow us to assess the feasibility of large-scale production.

The research and development of alternative protein sources for a sustainable food system is a rapidly evolving area of research and development. However, there are still a number of limitations in the development of alternative protein sources, including taste, aroma, and texture(Ma et al., 2022).

2,5-Diketopiperazine, also known as cyclic dipeptide, is responsible for bitter and umami flavors (Chen et al., 2009). They also improve gelling properties through specific mechanisms related to their ability to form structured molecular aggregates (Hanabusa et al., 1994; Manchineella & Govindaraju, 2012). However, there is still a lack of research on the use of cyclic dipeptides as additives in alternative protein food sources. We aimed to identify enzymatic proteins that help biosynthesis cyclic dipeptides, which have potential applications in the field of alternative proteins. This study identified the activity of CDP synthases from *Pediococcus pentosaceus*, *Wessella cibaria*, *Lactobacillus sakei*, and *Leuconostoc mesenteroides,* suggesting the possibility of their synthesis. Antimicrobial activity was confirmed using disc diffusion experiments, which were partly based on previous work by our team (data not shown). Identification of proteins reacting with specific amino acids was performed using AEC, native-PAGE and ninhydrin reactions. LC-HRMS was used to analyze the active proteins. The exploration of the biosynthetic mechanism of CDP suggests that it can be applied as an alternative protein food additive through a novel mass synthesis method.

While cyclic dipeptides have great potential as food additives due to their antimicrobial and antioxidant properties, their safety needs to be thoroughly evaluated through toxicological studies, allergenic potential assessments, and impact on human gut microbiota.

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Availability of Data and Materials

All supporting information including table of results and detailed methods is available upon request.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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