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Comparison of chemical and physical extraction methods of steamed-mature silkworm (Hongjam) protein

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Abstract

The efficiency of protein extraction from Hongjam, a steamed mature silkworm, was quantitatively evaluated using various chemical buffers and physical methods. This study considers the difficulty of protein extraction yield due to the high content of hydrophobic amino acids in Hongjam compared to 5th instar-3rd day silkworm larvae. Results indicated that urea buffer enhanced protein yield more effectively than RIPA buffer. Additionally, the application of physical methods such as microwave treatment to samples treated with RIPA buffer increased yields by up to 22%, achieving concentrations as high as 3.9 mg/mL. Circular dichroism (CD) analysis showed that proteins extracted with urea buffer retained their structural integrity, exhibiting deeper and more prominent peaks associated with random coil structure. In addition, physical methods such as vortexing, sonication, microwave and homogenization increased the extraction yield of larger molecules without altering protein structures, suggesting their potential scalability for industrial applications. These results demonstrate the critical role of selecting appropriate extraction methods to optimize the yield and functionality of proteins from Hongjam, with implications for its use in biotechnological applications and nutraceuticals.

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Introduction

Silkworm (*Bombyx mori*. L) has been used for centuries as a primary source of silk production, which has had a significant impact on various cultures and economies. However, recent scientific research has shown that silkworms have health and nutritional value beyond being a source of silk production (Kim *et al*., 2022). In particular, 5th instar-3rd day silkworm larvae have a high protein content (59%) and contain high levels of a biologically important compound called 1-deoxynojirimycin (DNJ, Ji *et al*., 2016). DNJ has a piperidine ring structure with a hydroxymethyl group and is naturally found from mulberry leaves. DNJ is also detected in the blood, intestinal juice, and other organs of silkworms through the mulberry leaves they consume (Yin *et al*., 2010). According to Sharmila and Latha (2023), 129-133 mg/100 g of DNJ is detected in 5th instar-3rd day silkworms. DNJ is a potent antidiabetic agent that effectively and specifically

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inhibits a variety of carbohydrate-degrading enzymes involved in a wide range of important biological processes, including intestinal digestion, hepatic glycogenolysis, and lysosomal catabolism of glycoconjugates. DNJ has also been shown to inhibit intestinal glucose absorption and promote hepatic glucose metabolism in streptozotocin (STZ)-induced diabetic mice (Li *et al*., 2015). Because of these properties, silkworm meal containing DNJ has gained attention as an effective antidiabetic agent.

In the developmental stage of the 5th instar of the 7th to 8th day, just before cocooning, the mature silkworm focuses on developing its silk glands, which are essential for silk production. At this phase, the concentration of 1-deoxynojirimycin (DNJ), a bioactive compound previously detected in earlier instars, declines as it is not present in the silk glands. Consequently, as the silk protein synthesis commences, the total protein content in the silkworm increases to 62%, comprised predominantly of glycine, alanine, serine, and tyrosine (Ji *et al*., 2016). These amino acids are similar to those found in silk, which has been reported to offer multiple health benefits, including enhancing memory, promoting bone formation, aiding in skin regeneration, and regulating energy metabolism (Noh *et al*., 2020; Jo *et al*., 2021; Kumar *et al*., 2018; Lee *et al*., 2024). Despite these benefits, the utilization of mature silkworms, particularly in their natural form as either an edible or powdered product, has been hindered due to the significant hardness of the silk glands. To address this limitation, recent advancements in softening processing technology by steaming have led to the development of a new material known as Hongjam (Ji *et al*., 2015). This enables the silk gland to be processed into a fine powder, broadening the scope of applications. Hongjam is currently under investigation for its potential effects on preventing Parkinson's disease, improving brain health, and supporting liver function in conditions related to alcohol consumption (Choi *et al*., 2017; Nguyen *et al*., 2016; Nguyen *et al*., 2020; Hong *et al*., 2018; Lee *et al*., 2020).

However, the protein content of Hongjam, rich in hydrophobic amino acids such as glycine, alanine, and tyrosine, exhibits lower solubility compared to the 5th instar-3rd day larvae (Ji *et al*., 2016; Widyarani *et al*., 2016). This characteristic can pose limitations in evaluating the functionality and quality of Hongjam. Consequently, this study conducted an analysis of the extraction efficiency and characteristics of proteins derived from various extraction methods of Hongjam protein. Utilizing these findings, it will be possible to explore biomarkers and other applications leveraging Hongjam protein.

Materials and methods

Sample preparation

Silkworm (5th instar-3rd day larva) and mature silkworm (5th instar-8th day larva) were produced from National Institute of Agriculture Sciences in 2023 (Wanju, Korea, Park *et al*., 2023). Mature silkworms were washed and steamed at 100 °C for 2 hrs to make Hongjam.

Protein extraction

Protein extraction was performed using RIPA, urea (8 M in Tris buffer, pH 8.0), or a mixture of RIPA and urea as lysis buffer. For the preparation of each extract, 0.5 mL of lysis buffer was added to 0.1 g of silkworm or Hongjam powder. Physical extraction was performed by vortexing for 1 min. sonication was applied by sonibath (Liarre, Bologna, Italy) for 1 min. Microwave (LG electronics, Seoul, Korea) was irradiated for 20 seconds in 3 repetitions. Homogenization (Daihan Scientific, Wonju, Korea) was conducted for 1 min to prepare the protein extract.

Protein quantification

Protein samples were prepared by diluting them 20-fold with distilled water. For the assay, 200 µL of Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA) and 10 µL of each sample were mixed in a 96-well plate. A standard curve was generated using bovine serum albumin (BSA, Gendepot, Altair, TX, USA) as a reference. After thorough mixing by pipetting, the reactions were allowed to incubate for 5 minutes. Absorbance was then measured at 595 nm using a spectrophotometer (Thermo Fisher, Waltham, MA, USA) to determine the protein concentrations.

SDS-PAGE and gel staining

Electrophoresis samples were prepared at a concentration of 40 µg/mL, diluted with water and 4X sample buffer (Gendepot). The diluted samples were heated at 100°C for 5 minutes and then immediately cooled on ice beads. For the SDS-PAGE analysis, 15 µL of each sample was loaded onto a 4-15% SDS-PAGE gel (Bio-Rad, Hercules, CA, USA). Electrophoresis was conducted using Tris-Glycine SDS running buffer (Gendepot) at settings of 100 V, 30 mA, and 100 W. After running, the gels were stained using a silver stain kit (Invitrogen, Carlsbad, CA, USA) to visualize and acquire images of the protein bands.

Table 1. Protein concentration of silkworm and Hongjam extract according to lysis buffer and physical treatments

	Silkworm (5th instar-3rd day larva)			Hongjam (Steamed mature silkworm)						
Extraction method	RIPA	Urea	RIPA +Urea	RIPA	Urea	RIPA +Urea	RIPA +Vortex	RIPA +Sonication	RIPA +Microwave	RIPA +Homogenize
Concentration (mg/mL)	22.9±1.0	24.4±0.4	23.9±1.0	3.2±0.5	3.4±0.3	3.4±0.1	3.7±0.0	3.7±0.0	3.9±0.0	3.7±0.1

Data are mean ± standard deviation (SD). Each experiment was performed in triplicate for each group

Fast Protein Liquid Chromatography (*FPLC*)

Molecular weight distributions were analyzed using FPLC (AKTA purifier, GE healthcare, Chicago, IL, USA) with a Superdex 30 Increase 10/300 GL column (Sigma-Aldrich). Samples were first diluted to a concentration of 0.1% and passed through a 0.2 μm PTFE membrane filter (Sigma-Aldrich). After preparation, 7 mL of the sample was injected into the FPLC apparatus at a flow rate of 0.5 mL/min. Detection and measurement of the molecular weights of proteins and other macromolecules within the samples were facilitated by monitoring UV absorbance peaks at 280 nm.

CD spectra analysis

Circular Dichroism (CD) spectra for each 0.01% extract were obtained using a Jasco J-1500 spectropolarimeter equipped with a 10 mm path-length, high-precision quartz cell (Jasco, Easton, MD, USA). Spectra were recorded across a wavelength range of 190 to 300 nm with a bandwidth of 4 nm and at a scanning speed of 500 nm/min. Each reported spectrum represents the average of three scans. Background corrections were made by subtracting the CD spectrum of ultrapure water from all sample spectra. The x-axis of the spectra displays the wavelength (190-300 nm), while the y-axis shows the ellipticity measured in millidegrees (mdeg), with positive values indicating greater absorption of left-handed circularly polarized light relative to right-handed.

Result and discussion

Protein extraction yield according to lysis buffer

Protein extraction from silkworms at different stages and depending on the buffer used (RIPA, urea or RIPA-urea mixture), the concentration of protein extracted from 5th instar-3rd day larvae ranged from 22.9 to 24.4 mg/mL (Table 1). The concentration of protein extracted from Hongjam varied between 3.2 and 3.4 mg/mL depending on the buffer. Based on RIPA buffer extraction, the protein yields 5th instar-3rd day larvae were 7.2 times higher than that from mature silkworms. A comparison of physical treatment methods for protein extraction from mature silkworms showed that vortexing, sonication, and homogenization each yielded a protein concentration of 3.7 mg/mL, while microwave extraction yielded the highest concentration at 3.9 mg/mL. Physical treatments increased the yield by 15-22% compared to the simple RIPA buffer extraction method.

The protein content of the silkworm increases from 59 to 62% due to the development of the silk gland, and increases to 68% in Hongjam (Ji *et al*., 2016). Compared to 5th instar-3rd day silkworm, Hongjam has a higher protein content, but the proportion of protein composed of hydrophobic amino acids is high, making protein elution with buffers challenging. According to Ji *et al*. (2016), the content of hydrophobic proteins such as glycine, alanine, and tyrosine increases to 30, 31, and 18%, respectively, compared to 5th instar-3rd day silkworm. Therefore, it is necessary to develop a method to increase the yield by buffer and extraction method. In this study, we compared two buffers, RIPA and urea, which are widely used for protein extraction. RIPA buffer, a detergent-based solution, is highly effective in breaking down cell membranes and solubilizing proteins, including those that are membrane-bound. Its composition typically includes detergents such as SDS, which help in solubilizing a broad range of proteins (Rampado *et al*., 2022). On the other hand, urea buffer, a chaotropic agent, disrupts the hydrogen bonding that maintains the secondary and tertiary structure of proteins (Sinha and Khare, 2014). Urea is particularly useful for denaturing proteins and can solubilize proteins.

Molecular weight analysis through SDS-PAGE and FPLC analysis

The FPLC analysis of silkworm protein extracts showed distinct results based on the buffers used (Fig. 1). The RIPA buffer yielded a major peak at 28 minutes, while the urea buffer

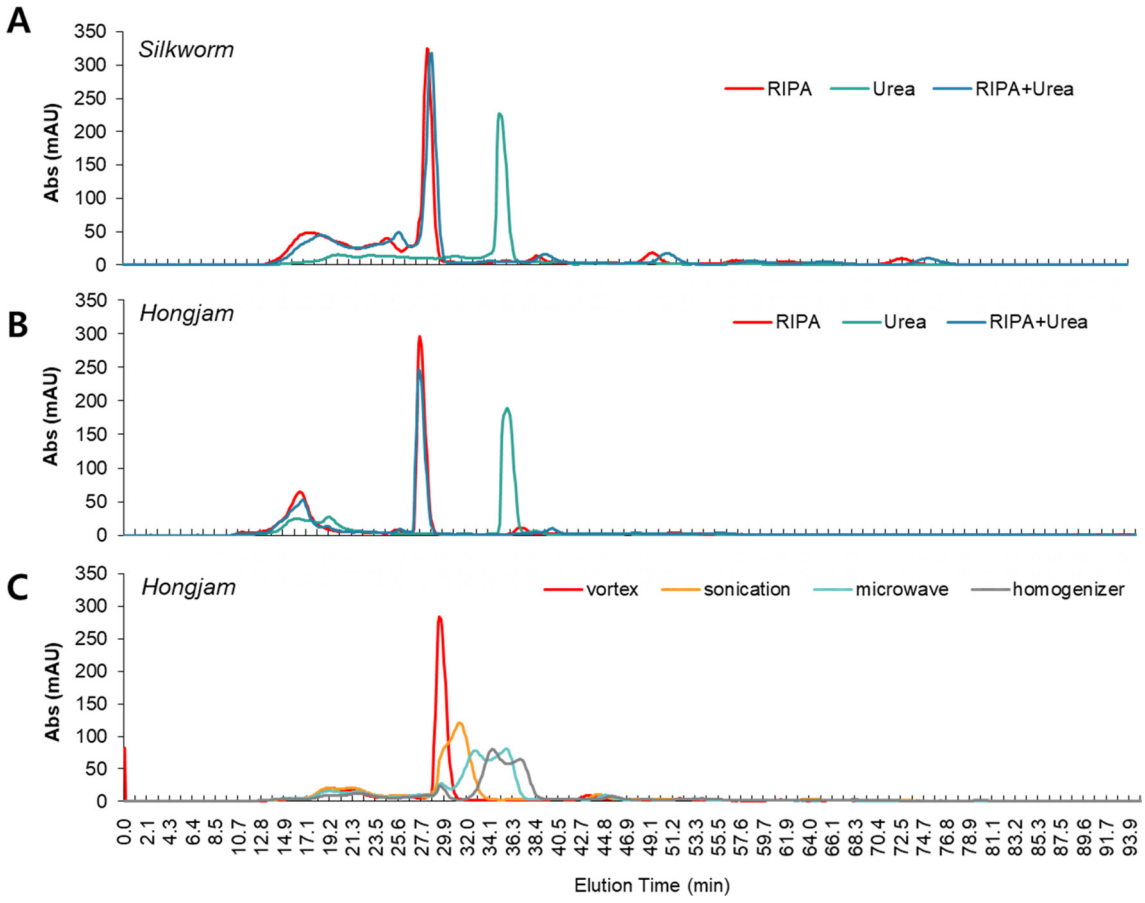

Fig. 1. Analysis of molecular weight distribution of protein extracts by FPLC
FPLC chromatograms showing the separation of protein extracts from silkworm and Hongjam using RIPA buffer, urea buffer, and a combination of both. The chromatograms display the retention times of protein elution from a Superdex 30 Increase 10/300 GL column. (A) Comparison of proteins extracted from 5th instar-3rd day silkworms using different lysis buffers. (B) Comparison of proteins extracted from Hongjam using different lysis buffers. (C) Comparison of protein extracts from Hongjam using different physical extraction methods.

resulted in a peak at 35 minutes. The use of a combined RIPA-urea buffer mirrored the retention time observed with RIPA buffer alone. Similarly, protein extracts from Hongjam also exhibited peaks at 27 minutes with the RIPA buffer and 35 minutes with the urea buffer. The combined use of RIPA and urea buffers in this case similarly paralleled the retention profile seen with RIPA buffer alone. Different physical extraction methods influenced the retention times and peak profiles observed. Vortexing, which slightly delayed the peak to 29 minutes, maintained a peak shape similar to that of RIPA buffer alone. However, more intense physical treatments like sonication, microwave, and homogenization displayed broader peak ranges spanning 30-31 minutes, 33-36 minutes, and 34-37 minutes, respectively. The retention time in FPLC is proportional to molecular weight, indicating that the urea buffer is more effective for extracting higher molecular weight proteins compared to RIPA buffer (Muindi *et al*., 2022). Physical treatment methods also broadened the range of protein molecular weights extracted. SDS-PAGE results reinforced these findings, with silkworm proteins extracted using urea buffer staining strongly in the range above 70 kDa (Fig. 2). When RIPA and urea were mixed, the results were similar to those obtained with RIPA buffer alone. The SDS-PAGE results for Hongjam proteins also showed similar trends when RIPA and urea were used in combination compared to using RIPA alone.

Secondary structure analysis through CD spectra analysis

Circular Dichroism (CD) analysis was conducted to examine the secondary structures of protein extracts from silkworm and Hongjam (Fig. 3). The CD spectrum of silkworm showed a negative peak at 210 nm, while Hongjam displayed a negative

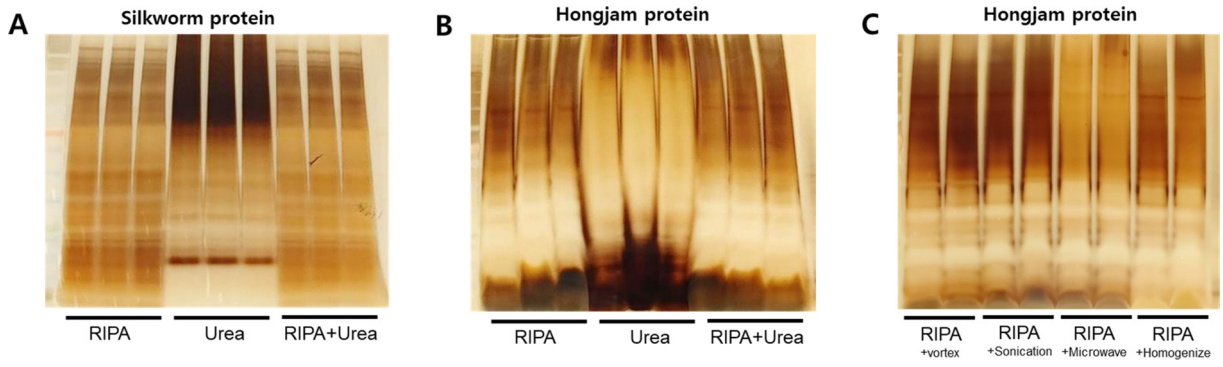

Fig. 2. Analysis of molecular weight distribution of protein extracts by SDS-PATE
Gel electrophoresis of protein extracts analyzed on a 4-15% SDS-PAGE gel. Samples were treated with RIPA buffer, urea buffer, and a combination of both, showing the separation of proteins according to their molecular weights. (A) Comparison of proteins extracted from 5th instar-3rd day silkworms using different lysis buffers. (B) Comparison of proteins extracted from Hongjam using different lysis buffers. (C) Comparison of protein extracts from Hongjam using different physical extraction methods.

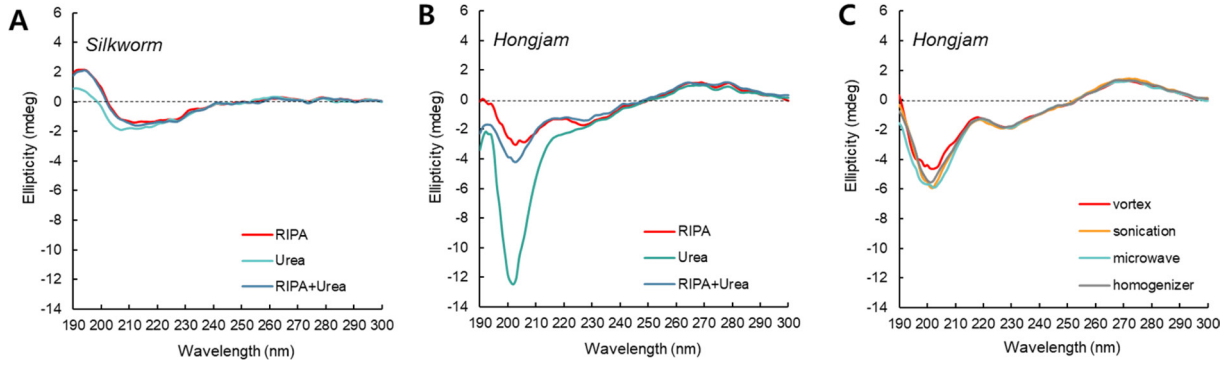

Fig. 3. Structural analysis of protein extracts by CD
CD spectra of protein extracts from silkworm and Hongjam. The spectra were recorded using a Jasco J-1500 spectropolarimeter, illustrating the secondary structure profiles of proteins extracted with different buffers. (A) Comparison of proteins extracted from 5th instar-3rd day silkworms using different lysis buffers. (B) Comparison of proteins extracted from Hongjam using different lysis buffers. (C) Comparison of protein extracts from Hongjam using different physical extraction methods.

peak at 202 nm. When comparing buffers, Hongjam samples extracted with urea buffer exhibited deeper peaks compared to those extracted with RIPA buffer. Physical extraction methods did not cause any changes in the CD peak profiles.

The 202 nm peak observed in the CD spectrum of Hongjam is similar to that of silk extracts, suggesting that silk is a major component of Hongjam (Muindi *et al*., 2022). The negative peak at 195-202 nm indicates a random coil structure, while the positive peak at 185-200 nm and the broad negative peak around 217 nm are characteristic of a β-sheet structure (Kang *et al*., 2018). Therefore, the protein extracts from Hongjam are predominantly random coil, while those from silkworm are predominantly beta-sheet. Despite deeper peaks at 202 nm due to physical extraction methods in Hongjam, no change in peak shape was observed, indicating increased extraction yield without structural change.

Summary

This study compared protein extraction methods from Hongjam, a steamed mature silkworm, using various protein lysis buffers and physical techniques. Hongjam, rich in hydrophobic amino acids, showed lower protein yields compared to silkworm larvae (5th instar-3rd day). However, the use of urea buffer increased protein yield and the extraction of high molecular weight proteins compared to RIPA buffer. Proteins extracted with urea buffer exhibited more pronounced random coil structure-

related peaks in CD analysis, indicating structural preservation. Physical treatments improved the extraction of larger molecules without altering protein structures, enhancing overall protein solubility. These results suggest that selecting the appropriate buffer and physical methods can optimize protein extraction from Hongjam for applications such as immunoblotting.

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