Journal of Current Science and Technology, April-June 2026 Vol. 16 No. 2, Article 175

ISSN 2630-0656 (Online)

Cite this article: Tanganurat, P., Nanthachai, N., Lichanporn, I., & Kumnongphai, P. (2026). Optimization of extraction parameters and functional evaluation of *Samia ricini* pupae protein for starch-based bioplastic films. *Journal of Current Science and Technology*, *16*(2), Article 175. https://doi.org/10.59796/jcst.V16N2.2026.175

Journal of Current Science and Technology

Journal homepage: https://jcst.rsu.ac.th

Optimization of Extraction Parameters and Functional Evaluation of *Samia ricini* Pupae Protein for Starch-Based Bioplastic Films

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Received 6 October 2025; Revised 1 November 2025; Accepted 11 November 2025; Published online 30 March 2026

Abstract

The escalating global demand for sustainable protein sources and eco-friendly packaging necessitates the valorization of underutilized agricultural by-products. This study systematically optimized the processing of *Samia ricini* (Eri) silkworm pupae, a high-quality sericulture by-product, to maximize protein isolation and evaluate its application in biocomposite edible films. A three-stage optimization process was implemented: 1) steaming pretreatment, 2) ethanol defatting, and 3) alkaline protein extraction. The optimal parameters identified were 6-8 minutes of steaming (for lowest moisture and the highest initial protein content), a 16-hour ethanol defatting duration (achieving 64.19% protein content post-defatting), and a brief 30-minute alkaline extraction (yielding a high-purity protein isolate of 94.94%). The resulting optimal protein isolate was then combined with different starch sources (corn, tapioca, and blend) to produce edible films. Protein incorporation significantly enhanced the film's functional properties, notably reducing the water vapor permeability (WVP) across all formulations ($p \leq 0.05$). The protein–corn–tapioca starch blend demonstrated superior barrier performance with the lowest WVP value of 2.49 ± 0.10 g/h·m^2. Conversely, while the incorporation of protein and different starches did not result in statistically distinct tensile strength values ($p > 0.05$), films made with corn starch exhibited the best qualitative handling properties (uniformity and peelability). These findings demonstrate the potential of *Samia ricini* pupae protein as a bio-derived functional ingredient for developing high-performance, sustainable bioplastic films and supporting the circular utilization of sericulture waste resources.

Keywords: *alternative protein; biodegradable film*; *edible film; pupae powder; silkworm; starch; water vapor permeability*

1. Introduction

The accelerating global demand for sustainable, affordable, and nutritionally balanced food sources has drawn growing attention to alternative proteins as viable complements or replacements for animal-derived proteins. Population growth, climate change, and the substantial environmental burden of conventional livestock production collectively pose pressing challenges to global food security. As a result, research and industrial efforts have intensified around novel protein sources such as plants, algae, fungi, cultured cells, and edible insects (Bryngelsson et al., 2022; Mokaya et al., 2023). These alternative proteins not only provide essential nutrients but also offer the potential to reduce greenhouse gas emissions, water use, and land requirements associated with traditional livestock. Over the past decade, major food companies and investors have increasingly incorporated alternative proteins into mainstream products, demonstrating both scalability and consumer interest (Hartmann & Siegrist, 2018). Among these sources, edible insects have emerged as one of the most promising options, supported by their high protein levels, favorable amino acid composition, micronutrient richness, and lower

ecological footprint than livestock. Protein content in insects typically ranges from 35% to 62%, depending on species, with many species also supplying beneficial lipids, dietary fiber, and bioactive compounds (Pan et al., 2022; Florença et al., 2022). From a sustainability perspective, insect farming requires significantly less land, water, and feed resources while producing far fewer greenhouse gases than conventional livestock (Krongdang et al., 2025). These environmental and nutritional advantages make edible insects attractive within the framework of the circular economy, where waste minimization and resource efficiency are prioritized.

Despite these advantages, consumer acceptance continues to be a major barrier in many parts of the world. While insects have long been consumed as part of traditional diets in Asia, Africa, and Latin America, reluctance remains strong in Western countries due to neophobia and negative perceptions of whole insects (Hartmann & Siegrist, 2018; Florença et al., 2022). To address these barriers, insect proteins are increasingly processed into powders, flours, and isolates, enabling their incorporation into familiar products such as bread, pasta, meat analogues, protein bars, and shakes. Processing not only improves consumer acceptance but also unlocks new techno-functional applications, as insect-derived proteins can contribute solubility, emulsification, foaming, and gelation properties that are competitive with conventional proteins such as soy and whey (Joopawang & Sompongse, 2022; Pan et al., 2022).

Beyond nutritional potential, insect and silkworm pupae proteins have attracted growing interest in the development of biodegradable edible films and coatings as an eco-friendly alternative to petroleum-based plastics. Protein-based films can form cohesive polymer networks through hydrogen bonding and hydrophobic interactions, providing good mechanical strength and gas barrier properties (Mihalca et al., 2021; Zhang et al., 2022). Studies have reported that films derived from insect or silkworm proteins exhibit desirable tensile strength, transparency, and water vapor barrier performance, with biodegradability comparable to that of other biopolymers (Noor et al., 2024). Moreover, composite films combining protein with starch or polysaccharides further enhance flexibility, structural integrity, and moisture resistance. Such blends also improve film homogeneity and reduce brittleness, addressing common limitations of pure protein films.

Starch-based bioplastic films, in particular, have been extensively studied for their renewability, biodegradability, and low cost. Starch contributes film-forming capability through amylose and amylopectin interactions, and blending it with proteins can enhance elasticity and reduce water sensitivity (Pelissari et al., 2013; Tavares et al., 2019). Among starch sources, corn and tapioca starches are widely used due to their abundance, low cost, and favorable mechanical and barrier characteristics. Corn starch provides better tensile strength and rigidity, while tapioca starch offers higher flexibility and transparency, making their combination ideal for developing biocomposite films with balanced properties (Abotbina et al., 2021; Akhir et al., 2023).

Silkworm pupae, particularly those from *Bombyx mori* (mulberry silkworm) and *Samia ricini* (Eri silkworm), represent a particularly valuable yet underutilized insect protein source. As by-products of the silk industry, pupae are abundantly available in sericulture-producing countries, but large volumes remain underexploited for human consumption or industrial processing (Mokaya et al., 2023; Ferdousi et al., 2023). Nutritionally, silkworm pupae contain approximately 55–62% protein, along with 15–18% fat, essential amino acids, polyunsaturated fatty acids, vitamins A, B, and E, and minerals such as calcium, iron, and zinc. These components not only support human health but also provide opportunities for high-value applications beyond direct consumption. Silkworm pupae oil, for example, is rich in linoleic and linolenic acids and has potential applications in functional foods, cosmetics, and pharmaceuticals (Ferdousi et al., 2023). Meanwhile, defatted pupae protein isolates have demonstrated favorable solubility, emulsification, and foaming capacities, positioning them as functional ingredients in both food and non-food applications (Joopawang & Sompongse, 2022; Noor et al., 2024). The potential of silkworm pupae extends beyond nutrition. In recent years, growing research has explored their use in the development of biodegradable edible films and coatings, which represent a sustainable packaging alternative to petroleum-based plastics. Such films can improve food shelf life while reducing environmental burdens associated with plastic waste. Studies have shown that protein-based films derived from insects such as locusts and silkworm pupae exhibit desirable tensile strength, water vapor barrier properties, and biodegradability (Mihalca et al., 2021; Zhang et al., 2022; Noor et al., 2024). Furthermore, functional enhancements can be achieved by incorporating natural extracts into pupae-based films, leading to improved antioxidant capacity and

antimicrobial properties, which further expand their industrial utility (Noor et al., 2024). These advances align with global sustainability goals, promote circular resource use, and provide added economic value to sericulture by-products.

Eri silkworms (*Samia ricini*) offer additional advantages due to their feeding versatility. Unlike mulberry silkworms, which depend exclusively on mulberry leaves, Eri silkworms thrive on castor and tapioca leaves, enabling stable production across different agricultural systems (Ferdousi et al., 2023). Eri pupae are particularly rich in unsaturated fatty acids and have been reported to contain higher protein and oil levels than soybeans, a major plant-based protein source (Mokaya et al., 2023). This superior nutritional quality highlights their potential role in reducing dependence on conventional protein crops and mitigating competition for arable land. Integrating Eri pupae proteins into food and material applications thus represents a sustainable pathway for diversifying global protein supplies. Despite these promising developments, there remains limited systematic research focusing on optimizing processing parameters for silkworm pupae protein extraction and characterizing its suitability for food and material applications. In particular, little is known about how steaming, ethanol defatting, and protein extraction times influence protein yield and quality, or how these proteins perform in combination with different starches for film formation. Addressing these knowledge gaps is critical for developing standardized methodologies for the valorization of silkworm pupae protein and expanding its applications in sustainable food and material systems. This study therefore aims to provide a systematic evaluation of the extraction, characterization, and application of silkworm pupae protein.

2. Objectives

The primary objectives of this study were to systematically valorize the protein from *Samia ricini* pupae and evaluate its application in sustainable bioplastics. Specifically, the study aimed to:

1) Optimize the defatting and protein extraction process by determining the optimal parameters for steaming time, ethanol extraction duration and alkaline protein extraction time to maximize protein yield from the defatted pupae powder.

2) Characterize the optimal protein isolate by assessing its yield, proximate composition, and physicochemical attributes.

3) Develop and evaluate novel biocomposite films by selecting the optimal starch source (corn, tapioca, or a blend) for use with the optimized silkworm pupae protein isolate, and characterizing the resulting edible films for key properties, including thickness, tensile strength, and water vapor permeability (WVP).

3. Materials and Methods

3.1 Effect of Steaming Time on Pupae

Silkworm pupae (1000 ± 2 g) were subjected to steam treatment at 97 ± 3 °C for durations of 2, 4, 6, and 8 minutes. Following steaming, the samples were dried in a hot air oven at 55 ± 2 °C for 7 hours, or until the moisture content was 5 ± 2%. The dried pupae were then pulverized using a blender and sieved to a particle size of 45 μm. Fat was extracted from the resulting pupae powder using 95% ethanol at a ratio of 1:8 (w/v). The mixture was blended at 6000 rpm for 10 ± 1 minutes, followed by a 24-hour settling period. The solvent was removed by vacuum evaporation at 40 °C (Pan et al., 2022; Ferdousi et al., 2023). The defatted solid residue was dried again at 55 ± 2 °C for 7 hours, or until the moisture content was 5 ± 2%, then finely ground and sieved to a particle size of 125 μm.

A completely randomized design (CRD) with four treatments (steaming times of 2, 4, 6, and 8 minutes) was used, with each treatment replicated three times. The quality of the defatted pupae powder was analyzed for color (using a colorimeter; Model CR-400, Konica Minolta, Japan), moisture, fat, and protein content according to AOAC (2000).

3.2 Effect of Ethanol Extraction Time on Pupae Powder

Silkworm pupae (1000 ± 2 g) were steamed at 97 ± 3 °C for the optimal time determined in Section 3.1. The steamed pupae were then dried at 55 ± 2 °C for 7 hours (or to a moisture content of 5 ± 2%), pulverized using a blender, and sieved to 45 μm. Fat extraction was performed using 95% ethanol at a 1:8 (w/v) ratio of pupae powder to ethanol, as reported to be effective for lipid removal in insect matrices (Pan et al., 2022). The mixture was blended at 6000 rpm for 10 ± 1 minutes and allowed to settle for 8, 16, and 24 hours. The solvent was removed by vacuum evaporation at 40 °C. The defatted residue was dried at 55 ± 2 °C for 7 hours (or to a moisture content of 5 ± 2%), finely ground, and sieved to 125 μm.

This experiment also followed a CRD with three treatments (ethanol extraction times of 8, 16, and 24 hours), each replicated three times. The quality of

the defatted pupae powder was evaluated for yield, color (using a colorimeter; Model CR-400, Konica Minolta, Japan), fat, and protein content (AOAC, 2000).

3.3 Effect of Protein Extraction Time from Pupae Powder

Defatted pupae powder (prepared as in Sections 3.1 and 3.2) was used for protein extraction. A 500 ± 3 g sample was mixed with distilled water at a 1:6 (w/v) ratio. The pH was adjusted to 9.5 using a 0.25 M NaOH solution, and the mixture was blended at 6000 rpm for 30, 60, and 90 minutes. This alkaline solubilization followed by isoelectric precipitation (pH 4.5 using 0.2 M HCl) is a widely applied method for insect protein recovery (Pan et al., 2022). The precipitate was combined with the solid residue from the initial extraction, dried at 55 ± 2 °C for 7 hours (to 5 ± 2% moisture), finely ground, and sieved to 125 μm to yield the final extracted silkworm pupae protein.

A CRD was used with three treatments (blending times of 30, 60, and 90 minutes), each replicated three times. The protein content of the extracted pupae powder was analyzed (AOAC, 2000).

3.4 Selection of Optimal Starch for Pupae Protein Film Formation

A protein solution (10% w/v) was prepared by dissolving extracted pupae protein in distilled water with continuous stirring. A mixture of flexibility agents comprising xanthan gum (8%), HPMC (1%), and gelatin (0.5% w/v) was incorporated. Film-forming solutions were prepared based on modified methods by García et al. (2000), using starch (corn, tapioca, or corn/tapioca blend). The mixture was combined with 10% glycerol as a plasticizer and heated in a water bath at 90 ± 2 °C for 30 minutes to ensure starch gelatinization, consistent with established solvent-casting approaches for protein-based films (Mihalca et al., 2021; Zhang et al., 2022; Noor et al., 2024). After cooling to 50 ± 2 °C, 30 mL aliquots were cast into 14 cm polystyrene Petri dishes and dried at 50 ± 2 °C for ~24 hours.

A CRD with six treatments (three starch types and three starch–protein combinations) was employed, with each replicated three times. Film quality was assessed based on appearance, film-forming ability, and ease of peeling. Color (using a colorimeter; Model CR-400, Konica Minolta, Japan), film thickness, tensile strength (ASTM D882-18), and water vapor permeability (WVP) were measured using standard methods (Mihalca et al., 2021).

3.5 Proximate Analysis and Film Property Measurement

The quality parameters of the pupae powder and the resulting films were analyzed using established standard methods.

3.5.1 Proximate Analysis of Pupae Powder

Moisture content was determined by oven drying according to the AOAC International Method 934.01. Approximately 2 g of the sample was dried in a hot air oven at 105 °C until a constant weight was achieved.

Fat content was determined using the Soxhlet extraction method (AOAC International Method 920.39). A 2 g sample was extracted with n-hexane for 6 hours. The solvent was removed by rotary evaporation, and the remaining residue was weighed as crude fat.

Protein content was determined using the Kjeldahl method (AOAC International Method 991.20). A 0.5 g sample was digested with concentrated H_2SO_4 and a catalyst tablet. Nitrogen was distilled into boric acid and then titrated with a standardized HCl solution. The total nitrogen content was converted to crude protein content using a nitrogen-to-protein conversion factor of 6.25.

The color values (L*, a*, b*) of the samples were measured using a colorimeter (Model CR-400, Konica Minolta, Japan) based on the CIE L*a*b* color system, reporting the L* (lightness), a* (redness/greenness), and b* (yellowness/blueness) values.

Yield was calculated as the percentage of the final defatted pupae powder weight relative to the initial weight of the raw pupae powder used for the extraction step.

3.5.2 Film Property Measurement

Film thickness was measured at five random points on each film sample using a digital micrometer (precision 0.001 mm), and the average value was used for subsequent calculations.

Tensile strength (TS) was measured using a universal testing machine according to ASTM D882-18 standard. Samples were cut into strips (15 mm × 100 mm) and tested at a crosshead speed of 5 mm/min.

Water vapor permeability (WVP) was measured gravimetrically using the modified cup method

following the standard procedures described by Mihalca et al. (2021). The film was sealed over a test cup containing a saturated salt solution (75% RH), and the cups were placed in a desiccator with controlled relative humidity (30% RH) and temperature (25 °C). The water vapor transmission rate was determined from weight loss/gain over time.

3.6 Statistical Analysis

Experimental data were subjected to analysis of variance (ANOVA). Significant differences among treatments were compared using Duncan's new multiple range test (DMRT) at $p \leq 0.05$. Statistical analyses were performed using SPSS software.

4. Results

4.1 Steaming Time of Pupae

The study on the steaming time of Eri silkworm pupae (2, 4, 6, and 8 minutes) before drying and processing into powder showed that steaming affected various characteristics of the pupae powder. The color analysis of the pupae powder (Figure 1) indicated that all samples had L* values ranging from 29.82 to 32.78, with no statistically significant differences ($p > 0.05$). This suggests that the pupae darkened after the heating and drying processes. No statistically significant differences were found for a* and b* values ($p > 0.05$). The pupae powder had a* values ranging from 13.90 to 15.79 (trending toward red) and b* values from 22.91 to 27.73 (trending toward yellow).

Drying at 55 °C for 7 hours significantly reduced moisture content in all samples ($p \leq 0.05$), as shown in Table 1. The final moisture content after drying was related to steaming duration. The pupae steamed for 2, 4, 6, and 8 minutes had final moisture contents of 10.14 ± 0.05%, 11.08 ± 0.04%, 7.02 ± 0.07%, and 5.80 ± 0.00%, respectively. Longer steaming times (6 and 8 minutes) resulted in lower final moisture content after drying than shorter steaming times (2 and 4 minutes). The analysis of fat and protein content (Table 1) showed that steaming time (2, 4, 6, and 8 minutes) did not have a statistically significant effect on fat content ($p > 0.05$), with values ranging from 32.00 ± 0.57% to 32.82 ± 1.09%. However, the results indicated that steaming time had a statistically significant effect on protein content ($p \leq 0.05$). The highest protein content (on a dry weight basis) was found in the groups steamed for 6 and 8 minutes, with values of 52.11 ± 0.54% and 52.75 ± 0.22%, respectively. These values were higher than those of the groups steamed for 2 and 4 minutes, which had protein contents of 47.68 ± 0.44% and 51.22 ± 0.76%, respectively.

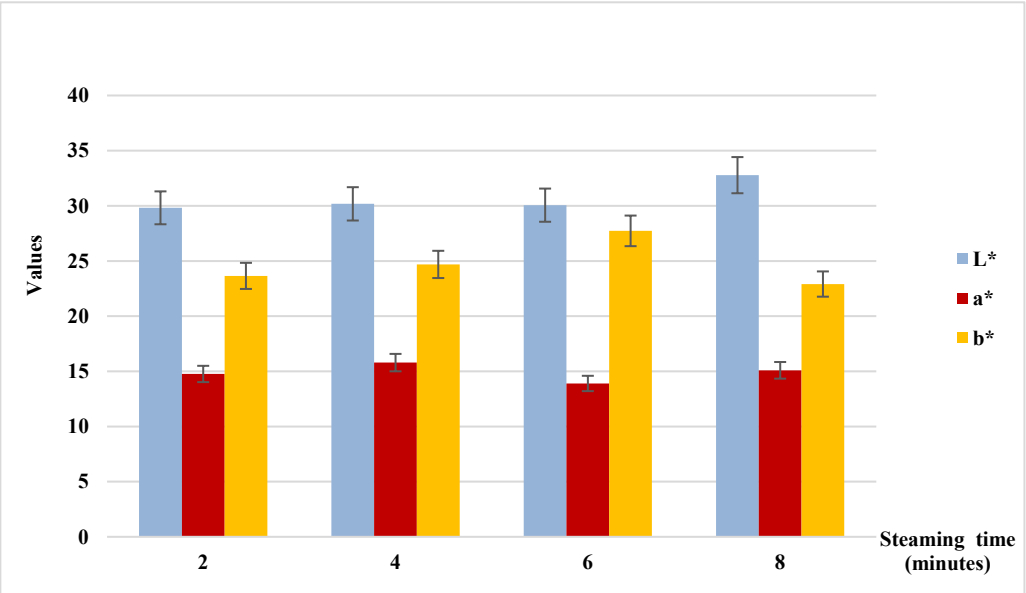

Figure 1 Changes in L*, a*, and b* values of dried silkworm pupae powders at different steaming durations.

Table 1 Moisture, fat, and protein contents of steamed silkworm pupae powders at different steaming durations.

Steaming duration (Minutes)	Moisture (%)	Fat (%) [ns]	Protein (%)
2	10.14 ± 0.05 [b]	32.20 ± 0.73	47.68 ± 0.44 [c]
4	11.08 ± 0.04 [a]	32.82 ± 1.09	51.22 ± 0.76 [b]
6	7.02 ± 0.07 [c]	32.00 ± 0.57	52.11 ± 0.54 [ab]
8	5.80 ± 0.00 [d]	32.68 ± 0.32	52.75 ± 0.22 [a]

[a–d] Different superscript letters within a column indicate significant differences ($p \leq 0.05$).

[ns] Indicates that means within a column are not significantly different ($p > 0.05$).

Table 2 Effect of ethanol extraction time on color parameters, extraction yield, fat content, and protein content of silkworm pupae powder.

Extraction time (hour)	Color value			% Yield	Fat (%)	Protein (%)
	L* [ns]	a* [ns]	b* [ns]			
Control	45.87 ± 3.15	9.80 ± 0.22	24.16 ± 0.47	78.71 ± 1.12 [a]	33.18 ± 1.29 [a]	52.35 ± 0.18 [c]
8	42.45 ± 0.11	8.51 ± 0.49	28.73 ± 0.14	73.79 ± 0.20 [b]	31.86 ± 1.90 [a]	59.22 ± 0.33 [b]
16	43.87 ± 0.51	8.04 ± 0.26	28.84 ± 0.97	71.07 ± 0.45 [c]	26.21 ± 0.66 [b]	64.19 ± 0.29 [a]
24	42.74 ± 2.79	7.78 ± 6.45	20.68 ± 0.72	76.19 ± 1.17 [a]	26.41 ± 2.01 [b]	59.25 ± 1.14 [b]

[a–c] Different superscript letters within a column indicate significant differences ($p \leq 0.05$).

[ns] Indicates that means within a column are not significantly different ($p > 0.05$).

4.2 Ethanol Extraction Time on Pupae Powder

The color values of the pupae powder did not show a statistically significant difference ($p > 0.05$) for L* and a* values across all extraction times, including the control. However, the b* value showed a decreasing trend as extraction time increased, although this change was not statistically significant. The yield percentage and weight loss of the pupae powder were significantly affected by extraction time ($p \leq 0.05$). Longer extraction times resulted in greater weight loss and a corresponding decrease in yield (Table 2). This outcome is expected as the process removes fat and other compounds.

An 8-hour extraction did not significantly reduce fat content compared with the control group ($p > 0.05$). In contrast, both the 16-hour and 24-hour extractions significantly reduced fat content ($p \leq 0.05$) compared with the control and the 8-hour group (Table 2). The remaining fat content was 26.21 ± 0.66% and 26.41 ± 2.01%, respectively, with no statistical difference between the 16 and 24-hour groups ($p > 0.05$). The protein content was also significantly affected by extraction time ($p \leq 0.05$). The control group had the lowest protein content at 52.35 ± 0.18%. The 8-hour and 24-hour extractions increased protein content to 59.22 ± 0.33% and 59.25 ± 1.14%, respectively, with no statistical difference between them. The 16-hour extraction yielded the highest protein content at 64.19 ± 0.29%, which was significantly higher than all other groups (Table 2).

4.3 Protein Extraction Time from Pupae Powder

The 30-minute extraction time provided the highest protein content at 94.94 ± 0.67%, which was statistically higher ($p \leq 0.05$) than both the 60-minute (82.27 ± 0.97%) and 90-minute (81.31 ± 0.50%) extractions. No significant difference ($p > 0.05$) was observed between the 60-minute and 90-minute groups. Therefore, the optimal condition for protein extraction was a 30-minute digestion time, as it demonstrated the highest efficiency.

4.4 The Optimal Starch for Film Formation

This study compared the effectiveness of different starches as film-forming agents in combination with extracted Eri silkworm pupae protein. The appearance and physical characteristics of the films are shown in Figure 2. Based on visual observation and the ease of peeling, the type of starch had a clear impact on film formation and peelability. Films made with corn starch were uniform and easier to peel than those made with tapioca starch or a blended corn/tapioca starch. Other processing factors, such as water content, mixing temperature and time, and drying temperature, were also found to be crucial to the success of the pupae protein film formation.

The colors of the prepared films are presented in Table 3. The incorporation of pupae protein resulted in a statistically significant decrease in lightness (L*) and a significant increase in yellowness (b*) for all corresponding films compared with pure starch films ($p \leq 0.05$). Protein films showed L* values ranging from 36.81 ± 3.52 to 43.06 ± 2.28, significantly lower

than the pure starch films (L* values ranging from 59.15 ± 2.01 to 66.84 ± 1.50). Similarly, protein films exhibited higher b* values (14.24 ± 1.98 to 19.80 ± 2.22) compared with starch-only films (10.41 ± 1.80 to 12.73 ± 4.51). The a* value was highest in the protein–tapioca starch film (3.50 ± 2.35), suggesting that the extracted pupae protein imparted a darker, more yellow-brown color to the final film.

The physical properties of films prepared from protein extracted from Eri silkworm pupae powder blended with different starches are presented in Table 4. The thickness of the films varied significantly depending on the formulation. The protein–corn–tapioca starch blend film exhibited the greatest thickness (0.70 ± 0.07 mm), while the tapioca starch film was the thinnest (0.22 ± 0.07 mm). In general, protein incorporation tended to increase film thickness compared with starch-only films, possibly due to enhanced solid content and stronger intermolecular interactions during film formation. The incorporation of silkworm pupae protein significantly reduced water vapor permeability (WVP) ($p \leq 0.05$) compared with films without protein. Protein-containing films exhibited WVP values ranging from 2.49 ± 0.10 to 3.80 ± 0.38 g/h·m², lower than those of films without protein (4.34 ± 0.50 to 5.16 ± 0.76 g/h·m²). Using starch blends rather than single starches tended to further decrease WVP, particularly in the protein-containing group, which showed the lowest WVP values. The tensile strength (TS) values of the developed films are presented in Table 4. Although no statistically significant differences ($p > 0.05$) were observed among all formulations, notable trends were evident. The protein–tapioca starch film exhibited the highest TS value (22.80 ± 5.60 N), followed by the protein–corn–tapioca starch blend (20.95 ± 6.35 N) and the protein–corn starch film (20.77 ± 7.12 N). Among the starch-only films, the tapioca starch film (19.22 ± 3.54 N) also showed slightly higher TS compared with corn starch (17.53 ± 4.63 N) and the corn–tapioca starch blend (18.45 ± 2.85 N).

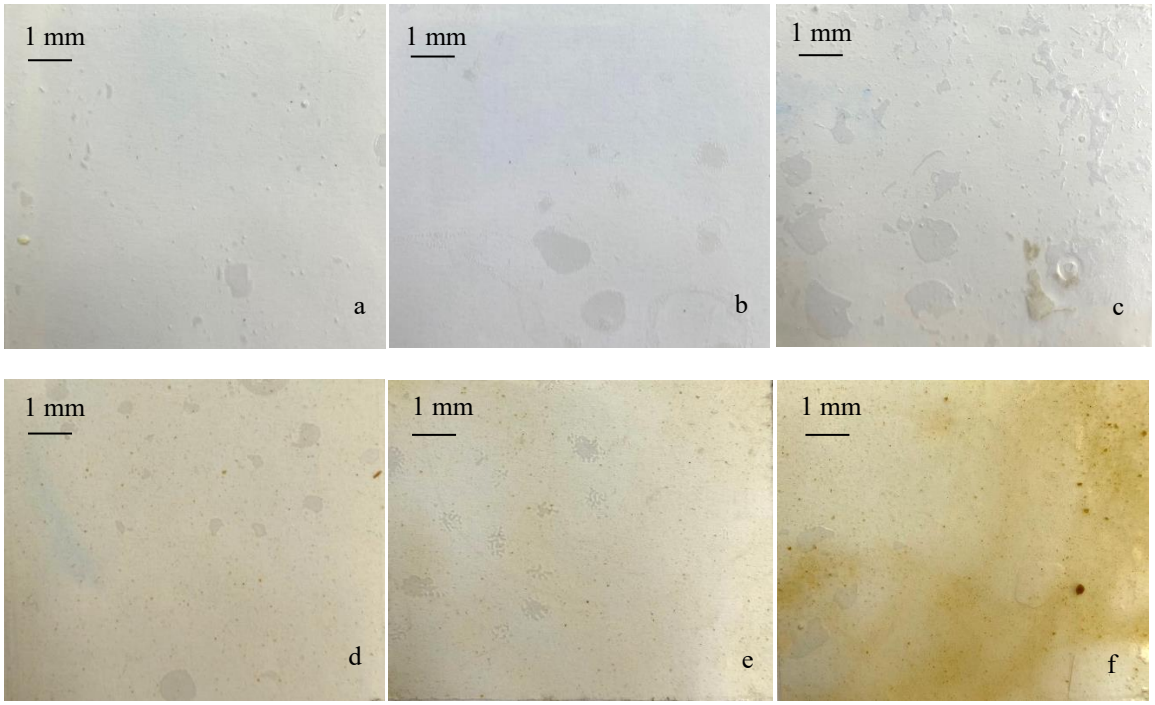

Figure 2 Films prepared with starch solutions: (a) corn starch, (b) tapioca starch, (c) corn–tapioca starch blend, (d) protein–corn starch, (e) protein–tapioca starch, and (f) protein–corn–tapioca starch blend.

Table 3 Color parameters (L*, a*, and b*) of Eri silkworm pupae protein films

Film type	L*	a*	b*
Corn starch	65.63±1.52 [a]	1.12±3.15 [c]	10.41±1.80 [d]
Tapioca starch	59.15±2.01 [b]	1.55±2.20 [c]	10.75±1.97 [d]
Corn–tapioca starch blend	66.84±1.50 [a]	1.34±1.53 [c]	12.73±4.51 [c]
Protein–corn starch	43.06±2.28 [c]	3.20±1.25 [b]	14.24±1.98 [b]
Protein–tapioca starch	39.12±1.80 [d]	3.50±2.35 [a]	19.80±2.23 [a]
Protein–corn–tapioca starch blend	36.81±3.52 [d]	3.15±1.13 [b]	17.66±3.12 [b]

[a-d] Different superscript letters within a column indicate significant differences ($p \leq 0.05$).

Table 4 Thickness, water vapor permeability and tensile strength of Eri silkworm pupae protein films

Film type	Thickness (mm)	WVP (g/h·m²)	Tensile strength (N)[ns]
Corn starch	0.47±0.02[b]	4.34±0.50[abc]	17.53±4.63
Tapioca starch	0.22±0.07[d]	5.16±0.76[a]	19.22±3.54
Corn–tapioca starch blend	0.44±0.02[b]	4.40±0.36[ab]	18.45±2.85
Protein–corn starch	0.34±0.01[c]	3.80±0.38[bc]	20.77±7.12
Protein–tapioca starch	0.33±0.02[c]	3.37±0.75[cd]	22.80±5.60
Protein–corn–tapioca starch blend	0.70±0.07[a]	2.49±0.10[d]	20.95±6.35

[a-d] Different superscript letters within a column indicate significant differences ($p \leq 0.05$).

5. Discussion

Steaming is a critical heat treatment process used before further processing or storage to inactivate enzymes and reduce microbial load (Fellows, 2017). The color analysis of the pupae powder showed that L* values ranged from 29.82 to 32.78 across all steaming durations, with no statistically significant differences ($p > 0.05$), indicating that the heating and drying processes produced consistent, darkened powder regardless of steaming time. This change is likely due to non-enzymatic browning reactions, such as the Maillard reaction between reducing sugars and amino acids, or caramelization of sugars (Martins et al., 2000). Although steaming may help inhibit browning enzymes, the heat applied, particularly during the 7-hour drying process at 55 °C, was sufficient to trigger these chemical reactions. The lack of significant change in the a* and b* values ($p > 0.05$) confirms that the primary effect was a reduction in lightness rather than a shift in hue. Regarding moisture and component concentration, our results revealed a clear structural alteration: longer steaming times (6 and 8 minutes) resulted in significantly lower final moisture content (7.02 ± 0.07% and 5.80 ± 0.00%) after the identical 7-hour drying period, compared to shorter steaming times (e.g., 2 minutes: 10.14 ± 0.05%). This result suggests that extended steaming alters the pupal microstructure, reducing its capacity to retain water during subsequent drying (Maskan, 2001; Baek et al., 2023). In contrast, the protein content was significantly higher in the 6- and 8-minute groups (52.11 ± 0.54% and 52.75 ± 0.22%, respectively) compared to 2 minutes (47.68 ± 0.44%). This apparent increase does not reflect a true gain in absolute protein mass; rather, it is attributable to the concentration effect — the loss of non-protein components, primarily moisture and volatile or water-soluble substances, during extended steaming and drying, which increased the protein's proportion relative to total dry weight. This loss of mass concentrated the protein, causing its proportion relative to the total dry weight to increase in the longer-steamed groups. This aligns with the greater weight and moisture loss observed in the 6- and 8-minute samples (Kalpana Devi et al., 2013).

The study confirmed the kinetics of fat extraction using a polar solvent (ethanol). The 8-hour extraction was insufficient to significantly reduce fat content compared to the un-extracted sample ($p > 0.05$). However, increasing the duration to 16 and 24 hours significantly reduced fat content (to 26.21 ± 0.66% and 26.41 ± 2.01%), confirming that longer durations facilitate greater mass transfer. Nevertheless, fat content did not differ significantly between the 16- and 24-hour extractions. This suggests that the system may have approached equilibrium, or the extraction rate had slowed down considerably during this period (Sovová, 2005). The resulting final fat content of approximately 26% remains high, which is inherent to the use of ethanol, a polar solvent, which is a poor solvent for the nonpolar triglycerides that constitute the majority of pupae fat, compared with nonpolar solvents such as hexane (Sovová, 2005). This is because ethanol is a polar solvent, making it less effective at dissolving nonpolar fat, although it can still extract some fat

given enough time. The increase in protein content after fat extraction (from 52.35% in the control to 59-64% in the extracted groups) is a direct result of the removal of fat and other ethanol-soluble compounds. The 16-hour extraction produced the highest protein level (64.19%), suggesting that it achieved an optimal balance between fat removal and the preservation of ethanol-insoluble dry mass. At 24 hours, extended solvent exposure likely caused the loss of additional ethanol-soluble components (e.g., pigments, sugars, minerals), slightly lowering relative protein content despite comparable fat removal. These results indicate that 16-hour of ethanol extraction maximizes protein recovery efficiency without excessive co-extraction of desirable solids.

The results establish 30 minutes as the optimal digestion time for protein extraction, yielding the highest protein content (94.94%) compared with 60 minutes (82.27%) and 90 minutes (81.31%) ($p \leq 0.05$). This finding confirms that the alkaline solubilization (pH 9.5) method effectively concentrates pupae protein (Bußler et al., 2016; Niveditha et al., 2020). The rapid achievement of maximum yield at 30 minutes, followed by a significant decline with prolonged exposure, is a key phenomenon. During the initial 30 minutes, the alkaline condition enhances protein solubility by disrupting structure and increasing electrostatic repulsion (Kristinsson & Rasco, 2000). However, extended exposure (60–90 minutes) is detrimental, as it likely triggers excessive peptide bond hydrolysis and unfavorable side reactions, such as lysinoalanine formation (Friedman, 1999; Whitaker et al., 1983). These degradation processes generate smaller, unquantifiable peptides and reduce the amount of measurable protein. Thus, a 30-min extraction represents the optimal compromise between solubilization and degradation, supporting efficient recovery of functional proteins (Niveditha et al., 2020; Bußler et al., 2016; Ma et al., 2010).

The superior peelability and uniformity of corn starch films, as visually confirmed, can be attributed to its higher amylose content, which promotes stronger retrogradation-driven structural networks (Miles et al., 1985). Films made with corn starch were uniform and easier to peel than those made with tapioca starch or a blended corn/tapioca starch. Other processing factors, such as water content, mixing temperature and time, and drying temperature, were also found to be crucial to the success of the pupae protein film formation. The superior performance of corn starch can be attributed to its fundamental properties. Corn starch generally has a higher amylose content than tapioca starch. Amylose's linear structure tends to align and form strong hydrogen bonds as it cools (a process called retrogradation). This creates a more robust structural network, giving the film greater structural integrity, reduced stickiness, and better shape stability, which makes it easier to peel (Miles et al., 1985; Rindlav-Westling et al., 2002).

Furthermore, the interaction between the pupae protein molecules and the amylose/amylopectin structure of corn starch may be more conducive to forming a uniform composite film with a better balance between strength and adhesion compared with tapioca starch (Zhang et al., 2025). The inferior peelability of the starch-only corn–tapioca blend film during preparation may be attributed to the differing gelatinization temperatures and retrogradation behaviors of the two starches, which can lead to phase separation at the microscopic level and result in an inconsistent film structure with localized weak points (Bourtoom, 2008). However, this processing limitation did not translate to poor barrier performance. On the contrary, when protein was incorporated into the corn–tapioca blend, the resulting composite film achieved the lowest WVP value of all formulations (2.49 ± 0.10 g/h·m², Table 4), suggesting that the pupae protein effectively bridges the incompatibility between the two starch phases — filling structural gaps, reinforcing intermolecular interactions, and creating a denser, more cohesive polymer network that maximizes moisture barrier performance.

Protein incorporation significantly altered film color and barrier properties. The addition of pupae protein decreased L* values and increased b* values ($p \leq 0.05$), yielding darker, yellowish films. This change likely stems from the inherent pigmentation of the protein and mild browning during processing. The most significant finding regarding film properties was that the incorporation of pupae protein significantly reduced the water vapor permeability (WVP) across all formulations ($p \leq 0.05$). Specifically, the pure starch films had WVP values ranging from 4.34 to 5.16 g/h·m^2, while the protein-containing films ranged from 2.49 to 3.80 g/h·m^2. This improvement suggests that protein–starch interactions create a denser microstructure with reduced free volume and more tortuous diffusion paths for water molecules (Gennadios et al., 1994; Pelissari et al., 2013). The lowest WVP (2.49 ± 0.10 g/h·m²) in the protein–corn–tapioca blend indicates a synergistic effect between the rigid amylose network of corn starch and the flexible amylopectin matrix of tapioca, both stabilized

by the protein component. This enhanced molecular density minimizes the free volume within the film, thereby maximizing the moisture barrier effect.

Although film thickness can influence the water vapor permeability (WVP) by increasing the diffusion path length, the observed reduction in WVP across the protein-containing films cannot be solely attributed to thickness. For instance, the protein–corn starch and protein–tapioca starch films exhibited moderate thickness (0.33–0.34 mm) but showed markedly lower WVP values compared with pure starch films of similar or even greater thickness. This indicates that the presence of protein played a more dominant role in reducing WVP, likely by forming a denser and more cohesive polymer network that limited water molecule diffusion. Therefore, the decreased WVP observed in the composite films resulted from a combined effect of both increased structural density due to protein–starch interactions and, to a lesser extent, film thickness differences.

The incorporation of protein generally increased the TS of all films, suggesting that the formation of protein–starch interactions contributed to enhanced network strength and film cohesion. Proteins can form intermolecular hydrogen bonds and hydrophobic interactions with amylose and amylopectin chains, thereby improving stress transfer within the polymer matrix (Mihalca et al., 2021; Noor et al., 2024). The trend of higher TS in protein–tapioca starch films may be attributed to the high amylopectin content of tapioca starch, which promotes flexible and cohesive molecular packing, reducing crack propagation during tensile deformation. In contrast, corn starch, which contains a higher amylose fraction, tends to form more rigid and brittle matrices, potentially limiting extensibility despite strong intermolecular bonding (Tavares et al., 2019). Moreover, the protein–corn–tapioca starch blend displayed an intermediate TS, indicating a synergistic balance between the rigidity of corn starch and the flexibility of tapioca starch. This result aligns with previous reports suggesting that blending starches with different amylose/amylopectin ratios can yield biocomposite films with improved mechanical uniformity and durability (Abotbina et al., 2021; Akhir et al., 2023). The slightly higher TS in all protein-containing films also implies that the protein isolate may act as a reinforcing biopolymer, facilitating denser cross-linking within the matrix. Although statistical differences were not significant, the observed patterns provide meaningful insight into the structural interactions among proteins and starches. Mechanical reinforcement likely depends not only on the type of starch but also on the distribution and compatibility of the protein within the starch matrix, which affect chain entanglement and the film's capacity to dissipate stress under tension. Overall, the results indicate that protein addition contributes to improved tensile behavior, particularly when incorporated with tapioca starch, supporting the hypothesis that starch composition and protein–starch interactions play a key role in determining the mechanical integrity of biocomposite films.

6. Conclusion

This research successfully established and optimized a sequential three-stage processing methodology to maximize the quality of Eri silkworm pupae protein isolate. The findings identify 6–8 minutes of steaming, followed by a 16-hour ethanol defatting duration, and a brief 30-minute alkaline protein extraction as the optimal sequence for obtaining a protein isolate of exceptional purity (94.94%). Critically, the study demonstrated that high-quality pupae protein is an effective functional ingredient, particularly for enhancing the moisture barrier properties of biocomposite films. The protein–starch blended film, specifically the protein–corn–tapioca blend, showed the most substantial reduction in water vapor permeability, confirming the utility of this insect by-product for developing superior, sustainable food packaging materials. These optimized protocols and application results directly address the knowledge gap regarding standardized industrial valorization of silkworm pupae protein, paving the way for its commercial adoption in both food and materials science sectors.

7. Acknowledgements

This research was supported by the National Science, Research and Innovation Fund, Thailand Science Research and Innovation (TSRI), through Rajamangala University of Technology Thanyaburi (FRB67E0205) (Grant no. FR670027/0168).

8. Abbreviations

Abbreviation	Full Term
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
ASTM	American Society for Testing and Materials

Abbreviation	Full Term
CIE	International Commission on Illumination (*Commission Internationale de l'Éclairage*)
CRD	Completely Randomized Design
DMRT	Duncan's New Multiple Range Test
HCl	Hydrochloric Acid
HPMC	Hydroxypropyl Methylcellulose
NaOH	Sodium Hydroxide
RH	Relative Humidity
TS	Tensile Strength
WVP	Water Vapor Permeability

9. CRediT Statement

Palida Tanganurat: Investigation, Methodology, Formal analysis, Data curation, Visualization, Writing – original draft.
Nunchanok Nanthachai: Conceptualization, Supervision, Writing – review & editing, Project administration.
Intira Lichanporn: Resources, Validation, Data curation.
Pradit Kumnongphai: Resources, Supervision.

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