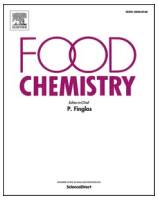
Food Chemistry 464 (2025) 141757

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Buffalo worm (*Alphitobius diaperinus*) proteins: Structural properties, proteomics and nutritional benefits

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ARTICLE INFO

Keywords:
Alphitobius diaperinus
Albumins
Globulins
Prolamins
Glutelins
Protein quality

ABSTRACT

Biophysical methods such as circular dichroism (CD) and differential scanning calorimetry (DSC) have been minimally used to characterize insect-derived proteins. This study examines the insect *Alphitobius diaperinus* as a potential protein source. Techniques such as alkaline solubilization coupled to isoelectric precipitation and Osborne fractionation were used to obtain protein concentrates and fractions (albumins, globulins, prolamins, glutelins). SDS-PAGE results showed dominant protein bands at 78.3, 73.3, 49.3, 34.5, 32.0, and 10.3 kDa. All fractions had over 60 % α-helix and β-sheet structures, indicating stable conformations. Prolamins showed high surface hydrophobicity and thermal stability. Nutritionally, glutelins exhibited the highest concentration of essential amino acids (68.75 g/100 g protein), and demonstrated superior *In vitro* protein-digestibility (84.04 %) as well as the highest *In vitro* protein-digestibility corrected amino acid score (73.11 %). Therefore, this study characterized the structural-function relationship of *A. diaperinus* proteins and collectively assessed their suitability and safety for human consumption.

1. Introduction

The global demand for protein is expected to double by 2050 due to the growing population, making it crucial to identify alternative protein sources that are both nutritionally rich and environmentally sustainable. Edible insects, with their high protein content and high digestibility, present a promising solution to help meet the future protein requirements (Ma, Mondor, Valencia, & Hernández-Álvarez, 2023). Buffalo worm (*Alphitobius diaperinus*; Coleoptera: *Tenebrionidae*) larvae, consists of head capsule, body segments and three pairs of thoracic legs Axtell (1994). The larvae initially emerge with white and turn brown after molting (Francisco & Do Prado, 2001). Compared to other edible insects such as meal worm (*Tenebrio molitor*) and superworm (*Zophobas morio*), which have been extensively studied, *A. diaperinus* has a shorter development cycle, a higher reproduction rate, and a softer exoskeleton (Bjørge et al., 2018). Although, in the past, it has been regarded as a common and difficult to control pest, *A. diaperinus* is now recognized as a potential nutrient source. Since 2017, it has been approved for use as an aquafeed ingredient under EU Regulation (2017/893), afterwards it was approved for human consumption by the European Food Safety Authority (EFSA) in 2022 (Siddiqui et al., 2024). Similar to other edible insects, *A. diaperinus* larvae represent a promising alternative protein source due to their complete amino acid profile and high protein quality, as evidenced by an Essential Amino Acid Index (EAAI) exceeding 70 % (Mastoraki et al., 2022); this makes them a valuable addition to the human diet. Furthermore, based on their amino acid profile, *A. diaperinus* contains a higher level of the limiting amino acid methionine (1.1 g/100 g) compared to soybean (0.429 g/100 g), *T. molitor* (1.01 g/100 g) and *Acheta domesticus* (0.98 g/100 g) (Hong et al., 2020; Kudełka et al., 2021; Leni et al., 2020; Udomsil et al., 2019).

However, a major challenge to the widespread acceptance of insect-based foods is insect neophobia, which acts as a substantial barrier to their consumption (Sánchez-Velázquez et al., 2024). Insect-based protein concentrates or isolates in powdered flour form have been shown to be effective in reducing insect neophobia, particularly in regions where insects are not traditionally consumed, such as North American and European countries where insects consumption is not customary (Melgar-Lalanne et al., 2019). Current methods for extracting protein

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https://doi.org/10.1016/j.foodchem.2024.141757
Received 24 July 2024; Received in revised form 9 October 2024; Accepted 21 October 2024
Available online 22 October 2024

from insects primarily involve wet extraction processes, such as alkaline solubilization, salt solubilization, alkaline solubilization coupled to isoelectric precipitation, and ultrasound-assisted extraction. Other methods, such as ultrafiltration, can also be used for protein recuperation and purification to generate protein concentrates or isolates (Ribeiro et al., 2023). Additionally, dry extraction methods, including fine milling coupled with air classification, are also employed (Ma, Mondor, Valencia, & Hernández-Álvarez, 2023). The method of protein extraction significantly influences the composition and properties of the extracted proteins, which are critical for industrial applications and closely related to protein structure (Benelhadj et al., 2023). Therefore, selecting appropriate extraction methods and studying the structure-function relationships of proteins is essential to gain comprehensive insights into their quality, digestibility, allergenicity, sensory attributes, and techno-functional properties. These factors collectively determine their suitability and safety for human consumption, compatibility with other food ingredients, and the processing requirements necessary for their optimal use in food formulations. The Osborne fractionation sequential procedure classifies proteins based on solubility as water-soluble albumins, salt-soluble globulins, alcohol-soluble prolamins, and alkaline-soluble glutelins (Osborne, 1924). This procedure yields fractions with specific properties such as structure, protein profiles, amino acid composition and protein quality. However, studies on the fractions and characteristics *A. diaperinus* are currently lacking.

The objective of this study is to deepen the understanding of the complex relationship between the extraction methodologies applied to *A. diaperinus* larvae and their effects on the protein's structural and functional properties. By evaluating these factors, this research aims to enhance our knowledge of *A. diaperinus* protein quality, digestibility, and its potential as a viable alternative protein source for various applications. This assessment was undertaken by employing different protein separation methods, including alkaline solubilization coupled to isoelectric precipitation and Osborne fractionation. The structural-related properties such as surface hydrophobicity, thermal stability and isoelectric point were characterized by SDS-PAGE, FTIR, CD, DSC and pI determination. Additionally, this research provides comprehensive nutritional information, particularly the protein quality of *A. diaperinus* protein ingredients. This includes the assessment of the amino acid profile, *in vitro* digestibility (IVPD), the *in vitro* protein digestibility-corrected amino acid score (IVPDCAAS), protein efficiency ratio (PER), amino acid score (AAS), biological value (BV) and essential amino acids (EAA). Furthermore, a proteomics analysis was performed to identify the specific proteins present in *A. diaperinus* protein extracts.

2. Materials and methods

2.1. Materials

Frozen buffalo worms (*A. diaperinus*) were purchased from Kiezebrink Ltd. (Church Farm, Suffolk, UK). These insects were processed through boiling and shock freezing to decrease the microorganism's load and inactivate the enzymes responsible for insect browning and degradation of proteins and lipids. The frozen buffalo worms were freeze-dried (Gamma 1–16; Christ, Newton) and the raw flour (RBW) was obtained by grinding the freeze-dried worms into flour with a cryo-grinder using liquid nitrogen (Pulverisette 11, Fritsch, Germany). The raw flour was defatted using hexane with 1:4 *w/v* ratio, stirring for one hour and centrifuged (5000 rpm, 30 min at 4 °C). The supernatant was discarded, and the pellet was recovered and redissolved with hexane again. Defatting procedure was repeated three times until supernatant became transparent. Residual hexane present in the defatted buffalo worm flour (DBW) was evaporated overnight under the fume cupboard and was recovered.

2.2. Proximate composition

Proximate composition of RBW and DBW was determined. The moisture content was analyzed according to the AACC44–01.01 method by drying RBW and DBW at 105 °C overnight in an oven (UN30, Memmert, Cambridge, United Kingdom). The ash content was determined by ashing sample in a furnace (PS6854, Germany) at 800 °C for 8 h (AACC08–16.01). The lipids were extracted by Soxhlet extraction apparatus and the lipid content was calculated (AACC 30–25.01). Total nitrogen content was determined using the Dumas method (vario MACRO cube, Langenselbold, Germany). For samples with low protein content, rice flour ($N = 1.35 \pm 0.04$) was used as the standard, while EDTA ($N = 9.58 \pm 0.04$) served as the standard for samples with high protein content. Chitin content was assessed using the calcofluor method, as described by Henriques et al. (2020). The protein content (%) was calculated from the total nitrogen content using the following equation:

$$\text{Protein (\%)} = (\text{N}_{\text{total}}{}^{*}\ 6.25) - (\text{Chitin (\%)}{}^{*}\ 0.4287)$$

2.3. Insect protein extraction

2.3.1. Protein extraction

Alkaline solubilization coupled to isoelectric precipitation (Alk-pI) was carried out according to Zhao et al. (2016) to assess the effect of pH of precipitation on protein content and recovery yield. DWB was mixed with water 1:10 (*w/v*) to solubilize proteins for one hour at pH 10. Then the suspension was centrifuged (14,000 *g*, 30 min, 4 °C), the supernatant was then recovered, and the pH was adjusted to 4.0, 4.25, 4.5, 4.75, 5.0, 5.5 and 6.0 to precipitate the proteins while the solution was allowed to settle for one hour, before being centrifuged again under the same conditions. The pellets obtained after the solubilization phase and the precipitated proteins were weighed and lyophilized (Gamma 1–16; Christ, Newton), and analyzed for nitrogen content. A protein conversion factor of 6.25 was used to convert the nitrogen content into protein content (vario MICRO cube, Elementar, Germany). The precipitated protein (referred as protein concentrate) with the highest protein content and the highest protein recovery yield was selected for the following assays. The protein recovery yield was calculated using the following equation:

$$\text{Protein recovery yield (\%)} = \frac{\text{Weight of precipitated protein*Protein content of precipitated protein}}{\text{Weight of defatted flour*Protein content of defatted flour}} * 100$$

2.3.2. Protein fractionation

The protein fractionation was performed based on Osborne (1924) method. The extraction media was modified to avoid oxidation of the polyphenols according to Giulia (2019). The DBW was mixed with extraction media (1:20 *w/v*) and was solubilized for one hour at 4 °C, then the pellet was recovered and used for sequential extraction procedure. A centrifugation step (3000 *g*, 30 min, 4 °C) was carried out to separate the soluble proteins from the insoluble pellet after each extraction stage. The albumin-rich (ALB), globulin-rich (GLO), prolamin-rich (PRO) and glutelin-rich (GLU) fractions were separated

accordingly. The ALB was extracted using 5 mM sodium ascorbate, 2 mM EDTA, 10 mM Tris-HCl media, then GLO was continuously extracted from the pellet of water-soluble proteins using 0.5 M NaCl, 5 mM sodium ascorbate, and 2 mM EDTA solution. The same procedure was applied to PRO and GLU, the extraction media were 70 % ethanol coupled with 5 mM ascorbic acid, and 0.1 N NaOH, coupled with 5 mM ascorbic acid, respectively. Then the resulting fractions were precipitated by ice-cold acetone (1:4 *v*/v) at −20 °C overnight, and recovered by centrifugation (14,000 *g*, 30 min, 4 °C) before being lyophilized (Gamma 1–16; Christ, Newton).

2.4. Protein characterization

2.4.1. Sodium dodecyl sulphate-polycrylamide gel electrophoresis (SDS-PAGE) analysis

The molecular weight distribution of proteins extracted by Alk-pI and the four fractions was analyzed by SDS-PAGE. Samples were solubilized in 1 mL of Laemmli sample buffer (0.1 M Tris-Tricine, pH 6.8, 2 % SDS, 5 % β-mercaptoethanol and 0.025 % bromophenol blue, 100 mM DTT) (1610737, Bio-Rad, CA, USA), boiled for 5 min, and then centrifuged at 10,000 *g* for 10 min and loaded onto a 12 % Criterion™ XT Bis/Tris gel (20 μg protein per well) and run at 200 V with MES running buffer (Bio-Rad, CA, USA). Gel was stained using Coomassie Brilliant Blue R-250. As a molecular marker, Precision Plus Protein™ standard (10–250 kDa, Bio-Rad Laboratories Inc., CA, USA) was used. The SDS-PAGE gels were photographed using a gel imager system (Gel Doc XR+ system, Bio-Rad, USA). The molecular weight distribution was analyzed by Image Lab (Image Lab 6.1, BIO-RAD, USA).

2.4.2. Protein secondary structure

The secondary structure of proteins extracted by Alk-pI and the four fractions was measured by Fourier Transform Infrared (FTIR) Spectroscopy (ALPHA II, Bruker, Germany) coupled to attenuated total reflectance (ATR). The air background was excluded before measurement between 400 and 4000 cm^{-1}.

A circular dichroism (CD) spectrometer (Chirascan VX, Photophysics, United Kingdom) was also used to generate secondary structure information for the samples in a liquid form. The background noise was first excluded by running the equipment with a blank cuvette, then the standard solution (dissolving media for proteins) was run to avoid the interference of the dissolving media. Afterwards, measurements were carried out with 220 μl solutions (0.2 mg/ml protein) at 20 °C under constant nitrogen purge over 180–260 nm of far-UV. The parameters were set to light bandwidth of 2 nm, step 1.0 and the scan were repeated three times by Pro-Data Chirascan (Version:V4.4.2.0 Applied Photophysics Ltd., Leatherhead, Surrey, UK). The secondary structure compositions were analyzed using CDNN software (Version:2.1.0.223 Applied Photophysics Ltd., Leatherhead, Surrey, UK).

2.4.3. Thermal stability of buffalo worm Osborne protein fractions

The thermal stability of proteins extracted by Alk-pI and the four fractions were assessed by differential scanning calorimetry (DSC) (TA Instruments, New Castle, USA) according to (Makhatadze, Lopez, Richardson, & Thmos, 1998). A sealed aluminum pan (Tzero pan; TA Instruments Ltd., New Castle, USA) with 20 μL water was used as a blank/reference. For the samples, 5 ± 0.1 mg samples and 20 μL water were weighed into aluminum pan, sealed and rehydrated for 21 h. The measurement program was set as: equilibration at 20 °C, heating from 20 °C to 155 °C, and the heat flow was 5 °C/min. Then the denaturation temperature and enthalpy were analyzed using Universal analysis 2000 software.

2.4.4. Isoelectric point determination

The isoelectric point of Osborne protein fractions was determined by analysing the electrophoretic mobility (NANO ZSP Zetasizer, Malvern). In brief, each fraction was dissolved in 0.1 M NaCl (pH ranging from 3 to 6). The diffusion barrier technique was applied for measurement according to the Malvern diffusion barrier technique application note to minimize cell electrodes and protein denaturation (Macovescu, Chelaru, Ignat, Luminita, & Gurau, 2018).

2.4.5. Surface hydrophobicity

The surface hydrophobicity was measured according to (Nakai, 2003). In brief, 2 mL protein solutions in 0.01 M PBS buffer with concentrations of 0.1, 0.08, 0.06, 0.04, 0.02, 0.01 mg/mL were prepared. For each concentration, 1 mL protein solution was applied as sample blank, the remaining 1 mL was mixed with 5 μL 8 mM 8-anilino-1-naphtalenesulfonic acid ammonium salt solution (ANS) in the dark. Then the mixture (200 μL) was transferred into a black 96-well microplate, and the fluorescent intensity of sample blank and sample with ANS were read at excitation 360 nm and emission 460 nm. The protein surface hydrophobicity curve was plotted by subtracting the blank from the samples with ANS, the linear slope was considered as the surface hydrophobicity.

2.5. Protein quality assessment

2.5.1. Amino acid profile

Osborne protein fractions and protein concentrate (2 mg each) were hydrolyzed in 6 N HCl (4 ml) at 110 °C for 24 h in tubes sealed under nitrogen (Alaiz et al., 1992). In brief, after HCl hydrolysis, amino acids were determined by HPLC after derivatization with diethyl ethoxymethylenemalonate, then 20 μl samples were injected into the 300 mm × 3.9 mm i.d. reversed-phase column. The isocratic elution system containing 25 mM sodium acetate, 0.02 % sodium azide (pH 6)/acetonitrile (91:9 *v*/v) were delivered at 0.9 ml/min (Novapack C18, 4 μm; Waters, Milford, MA, USA). And the tryptophan content was determined by HPLC after basic hydrolysis according to Yust et al. (2004).

2.5.2. Protein quality parameters

The Amino acid score (AAS), Biological value (BV), Essential amino acid index (EAAI), and Protein efficiency ratios (PER) were calculated based on amino acid profile ($g/100g_{protein}$) according to Amza et al. (2013), House et al. (2010) and Oser (1959) as follows:

$$\text{AAS}\ (\%) = \frac{\text{mg of amino acids in 1 g of protein}}{\text{mg of amino acids in requirement pattern}} \times 100$$

$$\text{BV} = (1.09^{*}\ \text{EAA}) – 11.73$$

$$\text{EAAI}\ (\%) = \frac{\text{EAA}}{\text{TAA}} \times 100$$

$$\text{PER1} = -0.684 + 0.456^{*}\ \text{Leu} – 0.047^{*}\ \text{Pro}$$

$$\text{PER2} = -0.468 + 0.454^{*}\ \text{Leu} – 0.105^{*}\ \text{Tyr}$$

$$\text{PER3} = -1.816 + 0.435^{*}\ \text{Met} + 0.780^{*}\ \text{Leu} + 0.211^{*}\ \text{His} – 0.944^{*}\ \text{Tyr}$$

$$\text{PER4} = 0.08084^{*}\ (\text{Thr} + \text{Val} + \text{Met} + \text{Ile} + \text{Leu} + \text{Phe} + \text{Lys}) – 0.1094$$

$$\text{PER5} = 0.06320^{*}\ (\text{Thr} + \text{Val} + \text{Met} + \text{Ile} + \text{Leu} + \text{Phe} + \text{Lys} + \text{His} + \text{Arg} + \text{Tyr}) – 0.1539$$

where the % EAA is the total essential amino acids content (g total

amino acid/100 g protein). TAA is the total AAS of standard egg protein (FAO, 1985) (Organization & University, 2007).

The *in vitro* protein digestibility (IVPD) was measured by the "pH drop" method according to Hsu et al. (1977). In brief, an amount equivalent to 62.5 mg protein and an enzyme solution containing 3.1 mg/ml chymotrypsin, 1.6 mg/ml trypsin and 1.3 mg/ml protease were prepared for digestion at pH 8.0, 37 °C. The enzyme solution was added at 1:10 *w/v* ratio. This was considered as the 0 min of digestion. The pH variation was recorded every 30 s for 10 min, and the IVPD was calculated as follows:

$$\text{IVPD}\ (\%) = 65.66 + 18.10^{*}\ \Delta\text{pH}_{10min}$$

The *In Vitro* PDCAAS (IVPDCAAS) was estimated as the product of AAS and IVPD (Nosworthy et al., 2017).

2.6. In-gel digestion and LC-MS/MS identification

The in-gel digestion and LC-MS/MS identification of the peptide mixtures were carried out with a Vanquish Neo UHPLC system connected to an Orbitrap Eclipse Tribrid mass spectrometer (Thermo Fisher Scientific, Sunnyvale, CA, USA) according to Ma et al. (2024).

Peak lists in .raw format were imported into Progenesis QI (Version 4.2., Waters) and LC-MS chromatograms aligned. A combined peak list was exported in .mgf format for database searching against the mouse subset of the Tenebrionidae subset of UniProt (86,832 sequences; 37,652,249 residues), appended with common proteomic contaminants (116 sequences; 38,371 residues). Mascot Daemon (version 2.8.0, Matrix Science) was used to submit the search to a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.8.3). Search criteria specified: Enzyme, trypsin; Max missed cleavages, 1; Fixed modifications, Carbamidomethyl (C); Variable modifications, Oxidation (M); Peptide tolerance, 10 ppm; MS/MS tolerance, 0.5 Da; Instrument, ESI-TRAP. Peptide identifications were passed through the percolator algorithm to achieve a 1 % false discovery rate assessed against a reverse database and individual matches filtered to require minimum expect score of 0.05. The Mascot .XML result file was imported into Progenesis QI and peptide identifications associated with precursor peak areas and matched between runs. Accepted protein quantifications were set to require a minimum of two unique peptide sequences. A top 3 method was used to estimate relative protein abundance in each band (ref = DOI: https://doi.org/10.1074/mcp.M500230-MCP200) with the most intense top 3 signal used for calling protein identification in each Coomassie band. All proteomic mass spectrometry data sets and results files are referenced in ProteomeXchange (PXD054184) and available to download from MassIVE (MSV000095430) [doi:10.25345/C5NG4H39N]. Pre-publication access can be obtained with the following link ftp://MSV000095430@massive.ucsd.edu.

2.7. Statistical analysis

All the experiments were carried out in triplicate using the same batch of *A. diaperinus*, and the data are expressed as mean ± standard deviation. Statistical analysis was performed using OriginPro 2021 (Version 9.8.0.200 OriginLab Corporation, Northampton, MA, USA). Significant differences between groups were determined using Tukey's test, with a significant threshold of $p < 0.05$. All graphical representations were plotted using OriginPro 2021.

Table 1
Nutritional composition (dry basis) of *A. diaperinus*.

Sample	Ash %	Lipid %	Protein %	Chitin %
RBW	3.65 ± 0.42[a]	12.24 ± 0.98[a]	57.67 ± 0.012[a]	26.45 ± 0.95[a]
DBW	4.28 ± 0.18[b]	2.71 ± 0.23[b]	57.20 ± 0.49[a]	35.82 ± 2.66[b]

Statistical differences in the same column are indicated by different letters ($p < 0.05$).
RBW: Raw Buffalo Worm, DBW: Defatted Buffalo Worm.

3. Results and discussion

3.1. Proximate composition of A. diaperinus

The proximate composition of raw *A. diaperinus* flour (RBW) and defatted *A. diaperinus* flour (DBW) are presented in Table 1. *A. diaperinus* showed a high amount of protein (57.67 % dry basis), in agreement with the value reported for *A. diaperinus* reared in Czech Republic (Adámková, Kouřimská, Borkovcová, Kulma, & Mlček, 2016). Compared to other common insect species, the protein content of *A. diaperinus* is generally higher than that of *T. molitor* (~45.1 %), *H. illuscens* (~41.1 %) and similar to *A. domesticus* (~55 %) (Rumbos et al., 2019). Therefore, due to its high protein content, *A. diaperinus* has been identified as a promising species for feed and food applications in the EU (Finke, Rojo, Roos, Huis, & &Yen, 2015). Lipids are the second largest component found in edible insects. However, the lipid content of *A. diaperinus* is reported to range between 13.4 % to 29.0 % dry basis (Rumbos et al., 2019). The lipid content of 12.24 % reported in this study is slightly lower, potentially due to variations in diet and life stage (Rumpold & Schlüter, 2013). The lipid content of DBW (2.71 %) is significantly lower than that of RBW (12.24 %), demonstrating the efficiency of the defatting procedure. Despite the defatting step, the protein content did not significantly increase, with DBW (57.20 %) showing no statistical difference compared to RBW (57.67 %).

It is important to note that the chitin content of *A. diaperinus* is high in both RBW and DBW with a value of 26.45 % (dry basis) and 35.82 % (dry basis), respectively. The chitin content in various edible insect species typically ranges from 10 % and 20 %. Specifically, black soldier fly larvae contain chitin levels ranging from 10 to 20 % (dry basis), yellow mealworms from 16 to 17 % (dry basis), waxworms at approximately 14.89 % (dry basis) and silkworms between 3 and 20 % (dry basis) (Rehman et al., 2023). These variations in chitin content can be attributed to differing rearing conditions and stages of the insect life cycle (Ma et al., 2024; Rehman et al., 2023). This is consistent with the observed increase in chitin content from 26.45 % (dry basis) in RBW to 35.82 % (dry basis) after the defatting step. As a result, if the aim is to enhance the protein content of feed or food products, additional steps to remove chitin will be necessary.

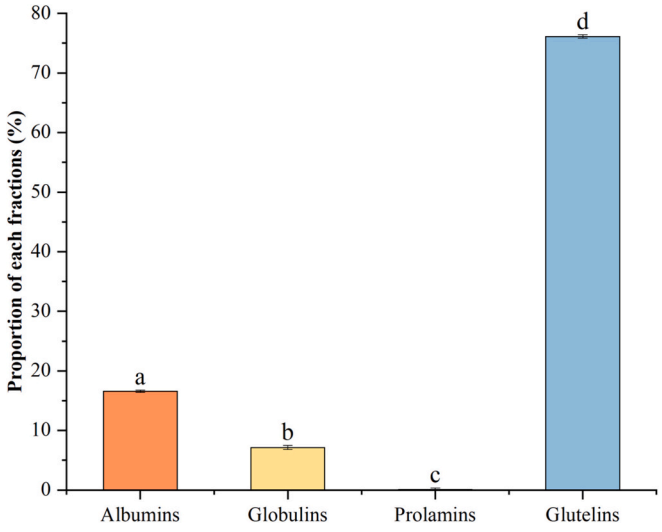

Fig. 1. Proportion of *A. diaperinus* Osborne protein fractions.

3.2. Insect protein extraction

3.2.1. Protein fractionation

To obtain a comprehensive understanding of *A. diaperinus* proteins, protein fractionation is a valuable method for classifying these proteins based on their solubility into four Osborne fractions: water-soluble ALB, salt-soluble GLO, alcohol-soluble PRO and alkaline-soluble GLU (Tenorio et al., 2018). Based on the original protocol, the extraction buffers were modified by adding reagents such as sodium ascorbate, EDTA, and ascorbic acid to avoid protein oxidation during the extraction procedure, due to the presence of phenoloxidase in insects, an enzyme catalyzing browning reactions (Janssen et al., 2017).

The proportions of *A. diaperinus* Osborne fractions are shown in Fig. 1. Among the extractable Osborne fractions, GLU are the most abundant fraction, representing 76.13 %. ALB are the second most dominant proteins at 16.6 %. GLO and PRO are present in lower amount with 7.15 % and 0.12 %, respectively. The proportion of each *A. diaperinus* Osborne fractions shows significant differences compared to other edible insects from the same family. For instance, in *T. molitor* glutelins constitute only 10.9 % of the fractions, making them the least abundant fraction, whereas they are the most abundant fraction in *A. diaperinus's* Osborne fractions. Additionally, *T. molitor*'s has a considerably higher prolamin content (25.8 %) compared to *A. diaperinus*'s (0.12 %) (Stone et al., 2019). Other edible insects, such as *G. bimaculatus* and *G. mellonella* are albumin-dominant, with 32 % and 47.7 %, respectively, while ALB in *A. diaperinus* Osborne fractions account for 16.6 %. Similar to *G. mellonella*, (1.4 %) prolamins are also the least abundant fraction in *A. diaperinus* at 0.12 % (Ma et al., 2024; Stone et al., 2019).

3.2.2. Protein extraction by Alk-pI

To determine the optimal precipitation pH for maximizing protein recovery yield, a range of pH values from 4 to 6 was selected and tested (Fig. 2), given that the isoelectric point of most proteins falls between pH 4 and pH 5 (Novák & Havlíček, 2016). Before precipitation, a sample-to-solution ratio of 1:10 (*w/v*) was selected, as this ratio has been demonstrated to achieve lower ionic strength, thereby improving protein solubilization (Torres et al., 2007). Fig. 2 demonstrates that the precipitation pH significantly influences the protein content of the final product. In general, precipitation at pH 4.5 showed the highest protein content (70.81 %). As the pH increased from pH 4.5 to 5.5, gradually moving away from the proteins' isoelectric point, both the protein recovery yield and protein content decreased to their lowest value of 4.00 % and 48.18 %, respectively. Although pH ranging between 5.5 and 6, no significant difference in protein recovery yield was observed for this range. This observation aligns with findings in studies for other (defatted) edible insects, such as *G. bimaculatus* and *T. molitor* (Kurdi et al., 2021; Zhao et al., 2016). Specifically, the precipitated protein content at pH 4.25 for *G. bimaculatus* and *T. molitor* was 73.4 % (dry basis) and 79 % (dry basis), respectively, compared to 71.55 % in this study (Kurdi et al., 2021; Zhao et al., 2016). Regarding the protein recovery yield of ALK-IP, *T. molitor,* which belongs to the same family as *A. diaperinus*, exhibited a higher protein recovery yield (53 %), potentially due to its high content of water-soluble and salt-soluble proteins. Stone et al. (2019) reported that *T. molitor* contains a significant proportion of water-soluble proteins (albumins) and salt-soluble proteins (globulins), constituting 32 % and 31.2 % of its protein content, respectively. In contrast, *A. diaperinus* has a lower total amount of water-soluble and salt-soluble proteins, with 23.75 % in Osborne fractions, as previously discussed. These findings highlight the importance of optimizing the precipitation pH to enhance protein recovery yields, particularly for insect species with varying compositions of water-soluble and salt-soluble proteins.

3.3. Protein characterization

3.3.1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and proteomics analysis

The electrophoretic profiles were determined to analyze the protein distribution of *A. diaperinus* protein concentrates and fractions. Then to identify *A. diaperinus* proteins belonging to the four fractions and protein concentrate, the most intense bands were selected and excised from the SDS-PAGE for LC-MS/MS acquisition. Proteomic analysis of tandem mass spectra requires a proteome database to match the data against and

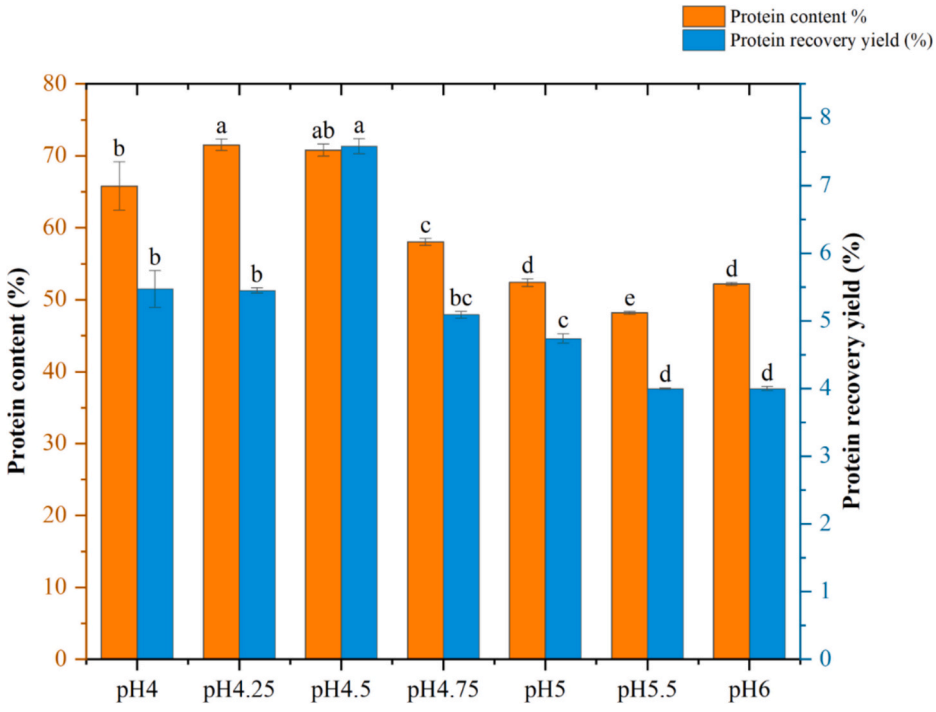

Fig. 2. The pH effectiveness on *A. diaperinus* protein precipitation. The statistical differences within the same parameters are indicated by different letters ($p < 0.05$).

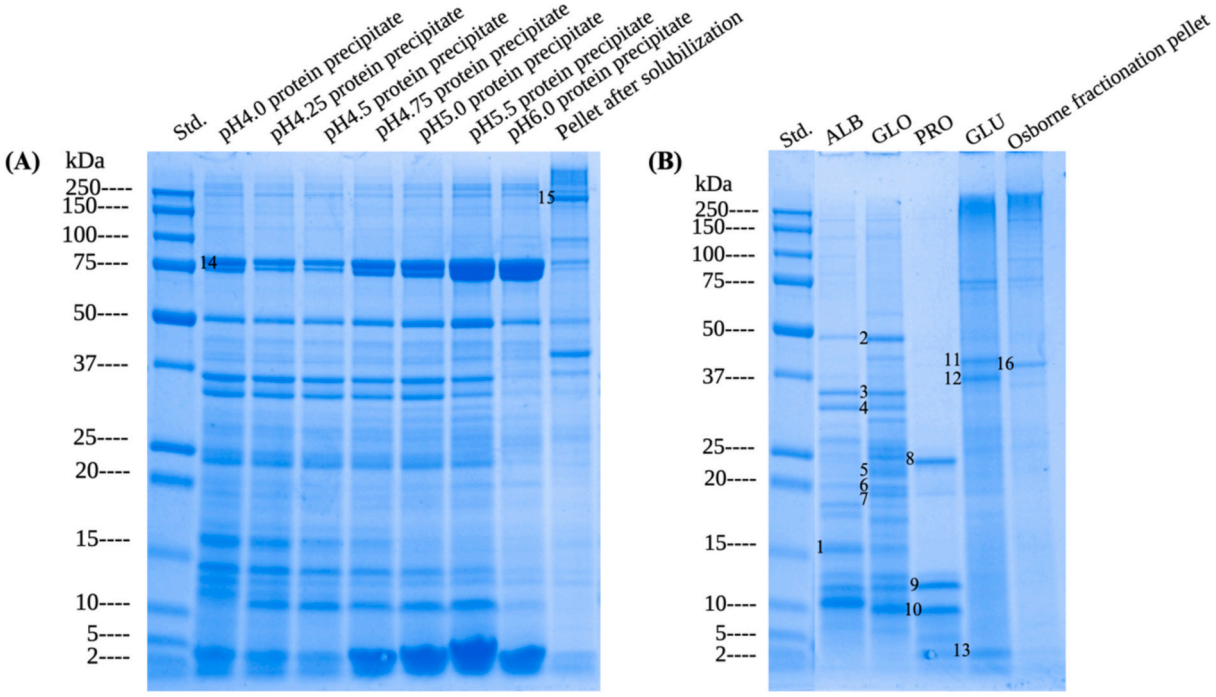

Fig. 3. Protein electrophoretic profiles (A) Molecular weight distribution of *A. diaperinus* protein precipitated at different pHs (by ALK-IP). (B) Molecular weight distribution of *A. diaperinus* Osborne protein fractions. Protein bands (1) 17.13 kDa Myosin light chain alkali (2) 45.58 kDa Troponin T. (3) 40.28 kDa Tropomyosin-1, isoforms 9 A/A/B-like Protein. (4) 32.33 kDa Tropomyosin-1. (5) 21.72 kDa Myosin regulatory light chain 2-like Protein. (6) 20.71 kDa Chitin bind 4 domain containing protein (7) 19.87 kDa Cuticle 3 domain containing protein (Fragment) (8) 24.5 kDa Uncharacterized protein fragments (9) 12.3 kDa Uncharacterized protein fragments (10) 10.2 kDa Uncharacterized protein fragments (11) 42.16 kDa Actin. (12) 36.9 kDa Actin fragments (13) 2.0 kDa Actin fragments (14) 75 kDa Hemocyanin. (15) 180 kDa Vitellogenin-like protein. (16) 42 kDa Arginine kinase.

as the completeness of *A. diaperinus* protein sequence was lacking (just 45 sequences in UniProt), matches were considered to the Tenebrionidae subset of UniProt (87 K Sequences https://www.uniprot.org/uniprotkb?query=Tenebrionidae). Accepted protein matches were ranked by the sum of the three most intense peptide ion peak areas for individual samples, as an estimate of relative abundance and the most intense called as the identification for the band.

By comparing the protein concentrates precipitated from pH 4 to pH 6 (Fig. 3), it is observed that while the overall intensity of the bands varies, the pattern remains similar. The most intense bands were at 78.3, 73.3, 49.3, 34.5, 32.0 and 10.3 kDa. The intensity of the two bands at 78.3 and 73.3 kDa increases when the precipitation pH is raised to pH 5, corresponding to the two low intensity bands of the same molecular weight in the GLU fraction (Fig. 3B). This observation is also in agreement with the fact that the pI of GLU is around pH 5 (see below, Fig. 4). Similarly, pH 6 protein concentrate lacks bands at 34.5 kDa and 32.0 kDa, which are dominant in both ALB and GLO. A pH value of 6 is far from their pI (see below Fig. 2), resulting in their reduced presence in the electrophoretic profile. On the other hand, water-soluble ALB and salt-soluble GLO showed similar profiles with different band intensities. Similar profiles have been observed for albumins and globulins of *H. illucens*'s (Leni et al., 2020).

The presence of a protein band at 75 kDa is related to hemocyanin (Rose et al., 2023). Hemocyanin is a blue-pigmented oxygen carrier present in arthropods and mollusks, freely dissolving in their hemolymph (Decker et al., 2007). Diverse immune functions have been attributed to hemocyanin (Coates & Nairn, 2014). It is present in the GLU fraction in limited amounts (Fig. 3B; Line GLU, band a). The hemocyanin remains in the pellet after Osborne fractionation (Fig. 3B; Line pellet, band a). Compared to insects belonging to the same family, a set of bands between 32 kDa and 14 kDa likely represent cuticle proteins (Andersen et al., 1995). One known protein within this range is the Cuticle 3 domain containing protein (Fragment), also observed in *T. molitor* (Elpidina et al., 2005). The lower molecular weight proteins, ranging from 13 kDa to 8.5 kDa, are also present in *T. molitor* (Liou et al., 1999).

Protein identifications are summarized in Table 4, including the description of the protein, number of peptides, unique number of peptides, molecular weight (Mw) and the corresponding accessions belonging to each band. Several proteins have been identified in *A. diaperinus* including myosin, troponin, tropomyosin, actin, chitin bind protein and larval serum proteins. Tropomyosin is a common allergen found in insects (López-Pedrouso et al., 2023). In this study, both tropomyosin and its isoforms (band 3 & 4) were identified in *A. diaperinus* proteins. Tropomyosin, a main allergen in crustaceans can cause diarrhea, vomiting, or even life-threatening anaphylaxis (Cheng et al., 2022). Therefore, it is advisable that individuals with known allergies to crustaceans should refrain from consuming insects due to the potential risk of cross-reactivity (Ma, Mondor, Valencia, & Hernández-Álvarez, 2023). As a water-soluble protein (Usui et al., 2013) it is primarily found in water-soluble and salt-soluble fractions, while another allergen arginine kinase cannot be separated by the protein extraction methods applied in this study, as it remains in the pellets. According to current proteomic studies of insect larvae, *A. diaperinus* proteins also contains muscle proteins, such as myosin heavy chain (235 kDa), as reported by Leni et al. (2020) in *H. illucens*. Proteins with similar motor functions were found in *A. diaperinus* with lower molecular weights, including actin (42.16 kDa) and myosin light chain (17.13 kDa).

The proteomics analysis further enriches our understanding by identifying the specific protein components, offering valuable information for future applications in food science and nutrition. This comprehensive approach ensures that *A. diaperinus* proteins are well-characterized, supporting their potential use in developing innovative, sustainable, and nutritious food products. The findings of this study could guide the food industry in harnessing *A. diaperinus* as an alternative protein source, addressing the increasing global demand for

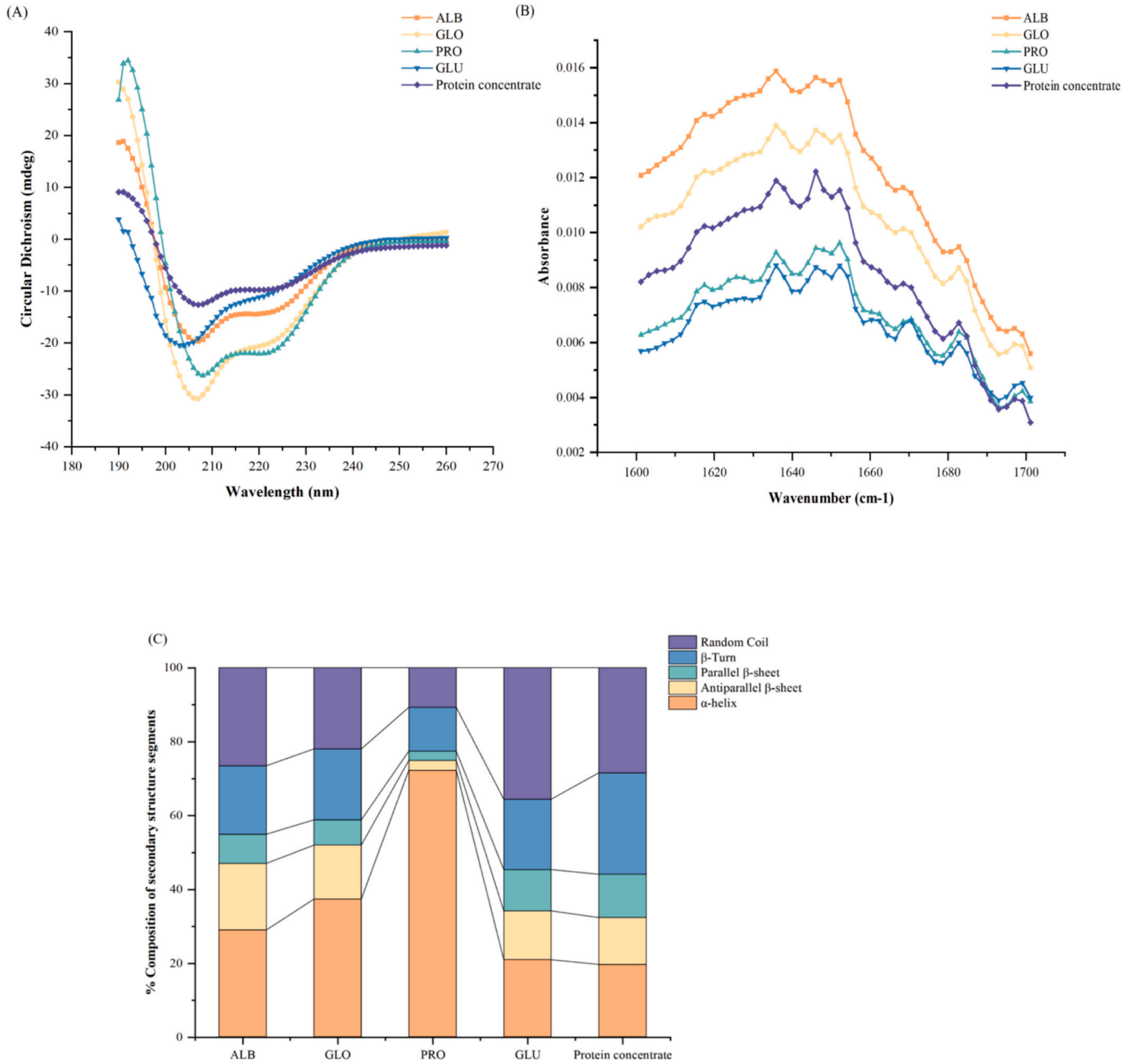

Fig. 4. (A) FTIR spectrum (Amide *I* area 1700–1600 cm^{-1}) of *A. diaperinus* Osborne protein fractions and protein concentrate. (B) CD spectrum of *A. diaperinus* Osborne protein fractions and protein concentrate. (C) Secondary structure composition calculated from CD spectrum.

sustainable protein solutions.

3.3.2. *Protein secondary structure*

Fourier transform infrared spectroscopy (FTIR) was applied to measure the secondary structure of *A. diaperinus* fractions and protein concentrate, with additional analysis conducted using circular dichroism (CD) (Fig. 4). The amide I area between 1600 and 1700 cm^{-1} of the FTIR spectrum, attributed to the C═O stretching vibrations of peptide linkages, indicates the conformation of protein's secondary structure. This region includes β-sheet (1613–1637 cm^{-1}; 1682–1696 cm^{-1}), α-helix (1645–1662 cm^{-1}), β-turns (1662–1682 cm^{-1}; 1630 cm^{-1}), and unordered structures (1637–1645 cm^{-1}) (Vanga et al., 2016). In far-UV CD spectrum, the α-helix shows two shoulders between 208 and 210 nm and 222 nm, while the β-sheet presents a characteristic low peak at 216 nm (Woody, 1996). The relative secondary structure compositions, shown in Fig. 4C, revealed that α-helix and β-sheet (parallel and antiparallel) are the major components of ALB (29.2 % α-helix; 26.2 % β-sheet; 18.7 % β-turn), GLO (34.9 % α-helix; 20.1 % β-sheet; 18.0 % β-turn), GLU (21.8 % α-helix; 25.5 % β-sheet; 11.7 % β-turn), as well as the protein concentrate (21.2 % α-helix; 26.4 % β-sheet; 29.7 % β-turn). The significant proportion of β-turns in the protein concentrate indicates the protein unfolding and dissociation during isoelectric precipitation (Xu et al., 2017). Besides, random coils are also present in small proportion, since the dominance of α-helix, β-sheet and β-turn structures (collectively exceeding 60 %) suggests that these proteins possess ordered and stable conformations (Choi & Ma, 2007). Similar protein structures with α-helix and β-sheet as the main components have been observed in protein concentrates of *A. cordifera* and *B. mellifica* (Baigts-Allende et al., 2021). Prolamins, however, shows significant differences from other fractions, with α-helix being the predominant segment (71.2

Table 2
Onset Temperature (T_0), Denaturation Enthalpy ($\triangle H$) and Denaturation Temperature (T_d) of protein concentrate and Osborne protein fractions.

Protein sample	T_0 (°C)	$\triangle H$ (J/g)	T_d (°C)
ALB	40.35 ± 1.67^{c}	2417.51 ± 7.22^{a}	81.90 ± 1.41^{a}
GLO	88.94 ± 0.01^{b}	2473.65 ± 1.30^{a}	100.87 ± 0.13^{b}
PRO	122.14 ± 12.73^{a}	873.26 ± 11.40^{c}	131.25 ± 0.18^{c}
GLU	97.69 ± 2.36^{b}	682.69 ± 1.43^{d}	106.05 ± 0.02^{d}
Protein concentrate	82.10 ± 0.02^{b}	2275.37 ± 1.78^{b}	86.93 ± 2.02^{a}

Values with different letters within column are significantly different ($P < 0.05$).

%). This finding is in agreement with the hydrophobic nature of prolamins. Wang et al. (2014) observed that the hydrophobicity of soy protein isolates increased with the α-helix content. A large number of studies have documented the high proportion of α-helix and hydrophobic residues in prolamins (Argos et al., 1982; Kretschmer, 1957). Similarly, α-helix dominance in prolamins was also observed in the CD spectrum of sorghum prolamins (Wu et al., 1971). These findings enhance our understanding of the structural characteristics of *A. diaperinus* proteins and suggest potential applications based on their ordered and stable secondary structures.

3.3.3. Thermal stability

The thermal parameters including onset temperature (T_0), denaturation enthalpy ($\triangle$H), and denaturation temperature ($\triangle T_d$) of proteins/water suspension are shown in Table 2. Water was added to the samples prior to the denaturation measurement due to its crucial role in affecting protein conformations in food systems (Arntfield et al., 1990). The differences observed in denaturation temperatures among the samples indicate different thermal stabilities of the proteins/fractions. However, all the fractions and protein concentrate showed characteristic endotherms. The endotherm peaks showed that both Alk-pI extraction and Osborne fractionation are mild methods for protein separation and they do not denature the proteins. The endotherm peaks were also found in black soldier fly proteins prepared by ALK-pI method at 150 and 200 °C, and in waxworm proteins prepared using the same method at 107 °C (Ma et al., 2024; Queiroz et al., 2021). Similar denaturation temperatures were observed in wasp larvae protein concentrates at 76 °C, jumil protein concentrates at 82 °C (Baigts-Allende et al., 2021), and *A. diaperinus* protein concentrates at 82.10 °C. The highest denaturation temperature (131.25 °C) was observed in PRO, which also required the greatest amount of energy (873.26 J/g) for thermal transition. This result can be attributed to their highest percentage of ordered structure compared to other fractions (Masson & Lushchekina, 2022). Similar denaturation temperature and enthalpies were observed for ALB and protein concentrate, consistent with their similar protein electrophoretic profiles. GLO had a higher denaturation temperature (100.87 °C) than ALB. Tang et al. (2019) similarly reported that rice bran globulins (81.3 °C) have a higher denaturation temperature than albumins (70.1 °C). This may due to the globular nature of GLO, which are stabilized by intramolecular hydrogen bonds (Myers & Pace, 1996). Therefore, GLO may contain more hydrogen bonds than the protein concentrate, requiring more energy to disrupt their conformational stability.

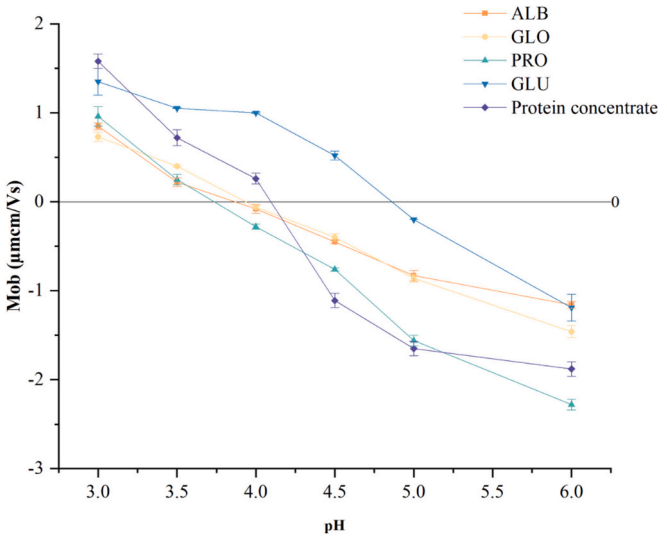

Fig. 5. Isoelectric point of *A. diaperinus*'s Osborne protein fractions.

3.3.4. Isoelectric point determination

The isoelectric points (pI) of *A. diaperinus*'s Osborne fractions and protein concentrate are shown in Fig. 5. The pI value is a crucial characteristic of proteins, providing practical guidance in protein separation. According to the theory of Yao et al. (1994), the mobility of protein yields a pI value when plotting protein mobility against pH; the intercept of zero mobility corresponds to the pI value. Results indicated that both ALB and GLO have the same isoelectric point (pI = 3.97). This is also observed in the electrophoretic profile (Fig. 3B), where ALB and GLO share bands with similar molecular weights, except for several bands from GLO, such as 43.9, 30.8, and 17.9 kDa, which do not affect the pI. In contrast, PRO and GLU showed distinct pI values, with PRO having the lowest pI (3.77) and GLU having the highest pI (4.82). The difference aligns with their electrophoretic profiles (Fig. 3). The three most intense bands of PRO are primarily low molecular weight proteins (24.0, 12.2 and 10.1 kDa), while GLU contain proteins with higher molecular weight profiles, with the two main bands at 40.8 and 36.4 kDa. The pI of the protein concentrate is 4.36, which is close to pH 4.5 (mentioned in the previous section) which resulted in the highest protein recovery yield as well as the highest protein content. These different profiles contribute to the isoelectric point differences between PRO and GLU. As previously shown in the SDS-PAGE profiles, a possible source of GLU is actin, which has an isoelectric point of 5.8 (Sonobe et al., 1986). This could explain the higher isoelectric point observed for GLU. Similar results were reported in *Oedaleus australis* (Australian locust), with a 40 kDa protein exhibiting a pI around 5.6 (Stadler & Hales, 2002). Additionally, actins in spider thoracic muscle, crab claw muscle, and crayfish claw muscles have been found to have isoelectric points ranging from 5.5 to 5.7 (Huang et al., 1984). Specifically, the pI values for cicada thoracic actins, leg actins, and sound organ actins are 5.65, 5.68 and 5.57, respectively (Huang et al., 1984).

3.3.5. Surface hydrophobicity

The surface hydrophobicity (H_0) reflects the exposure of hydrophobic clusters on the protein surface and is related to the interfacial activity (Liu et al., 2018). As a structure-related function, surface hydrophobicity depends on the size and shape of protein molecules, as well as the amino acid composition, particularly the hydrophobic amino acid content and sequence (Jiang et al., 2015). Fig. 6 shows the H_0 value and the hydrophobic amino acid content of Osborne fractions and protein concentrate. The H_0 of the protein concentrate (133.24) and ALB (232.70) are significantly lower ($p < 0.05$) than those values observed for GLO (438.13) and GLU (387.63). This observation is consistent with the findings of Papalamprou et al. (2009) and Wojciechowski (2022), who reported that albumins have a lower surface hydrophobicity than globulins. Although both the protein concentrate and ALB are water-soluble proteins, the isoelectric precipitation process can result in the co-precipitation of various Osborne fractions. For instance, the 75 kDa hemocyanin precipitate contributes to the diverse protein composition in the protein concentrate and explains its distinct surface hydrophobicity value. When compared to the H_0 value of 102.5 for the alkaline-soluble proteins (obtained at pH 10) from *Tenebrio molitor* flour (Azagoh et al., 2016), the H_0 value of the protein concentrate in this study (133.24) is observed to be comparable. The slight difference may

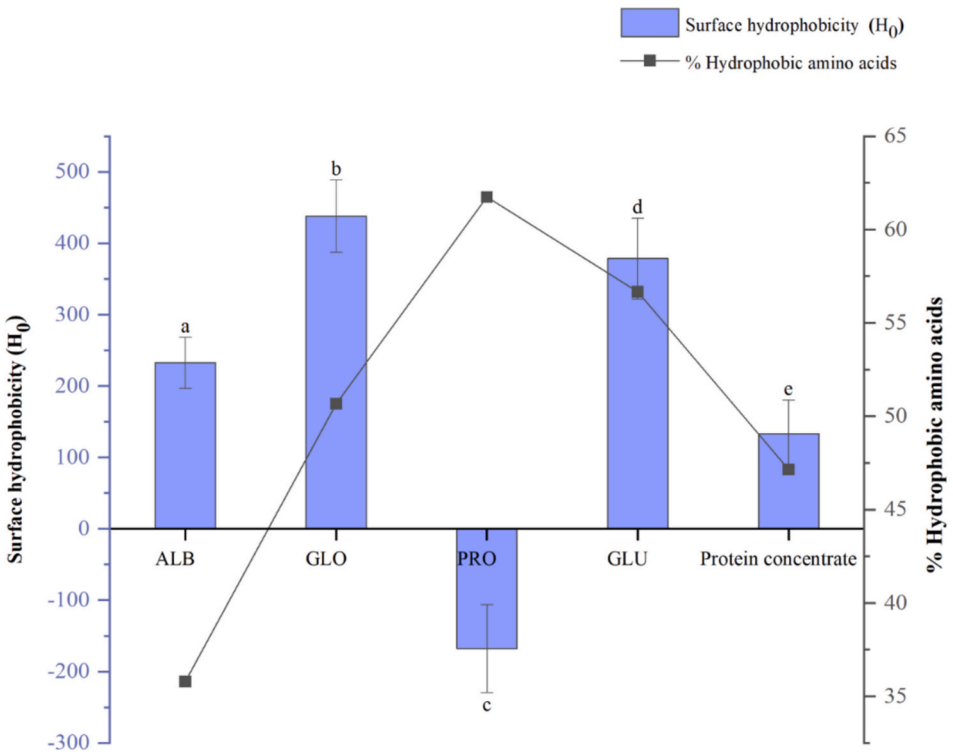

Fig. 6. Surface hydrophobicity (H_0) and % hydrophobic amino acids of *A. diaperinus*'s Osborne protein fractions and protein concentrate. Different letters in the same column indicate statistical differences by Tukey test ($p < 0.05$).

be attributed to the precipitation step used in this study, which can increase protein aggregation and consequently lead to higher hydrophobicity (Wagner et al., 2000).

A lower H_0 indicates that the protein surface has fewer hydrophobic groups, and this characteristic leads to greater solubility (Liu et al., 2018). GLO shows the highest H_0, followed by GLU, due to their high proportions of hydrophobic amino acids (Fig. 6). The elevated H_0 value observed for the GLU may result from the alkaline extraction conditions, which break trimer, tetramer or hexamer-type proteins into subunits, exposing buried hydrophobicity side-chains and increasing

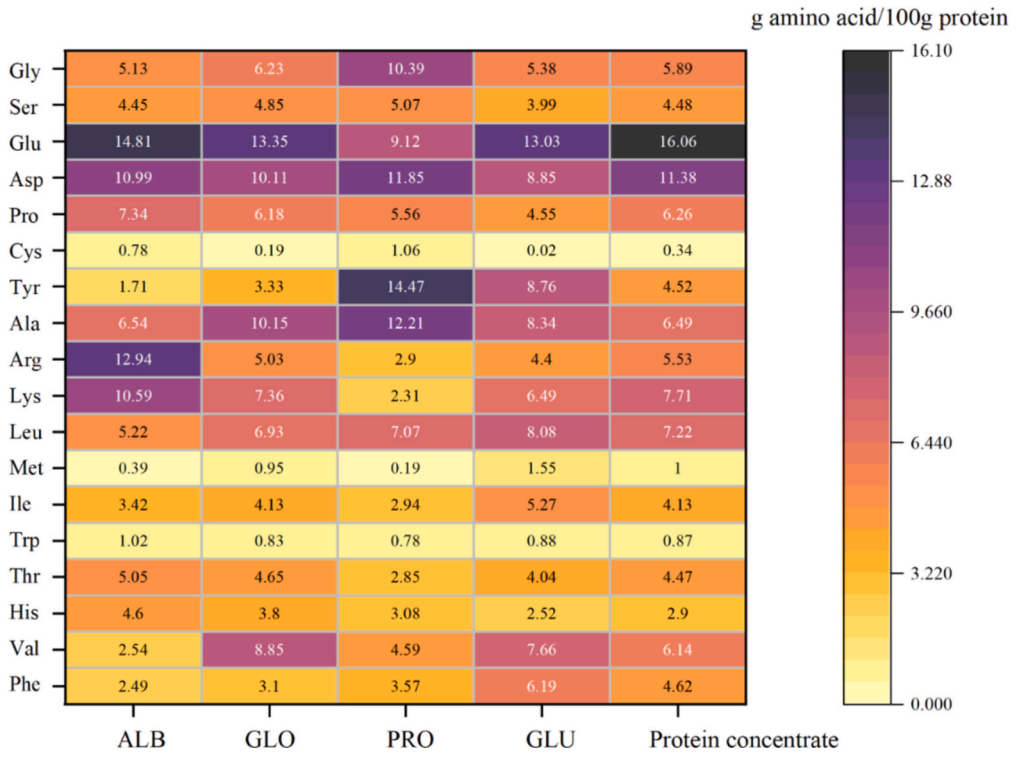

Fig. 7. Amino acid profile (g/100 g protein) of *A. diaperinus* Osborne protein fractions and protein concentrate.

hydrophobicity. However, due to the limited proteomics knowledge of *A. diaperinus*, identifying and listing these polymeric proteins remain challenging. Proteins with high H_0 values generally demonstrate better surfactant properties, which can enhance foaming and emulsifying capacities. Given that GLU has a high H_0 value and it constitutes the largest fraction among the four Osborne fractions in *A. diaperinus*, they hold potential for foaming and emulsifying applications. However, the H_0 value for PRO was negative, differing from other fractions. This is due to the protein aggregation within PRO. As an insoluble fraction, the hydrophobic zones are entrapped within the protein structure, resulting in a fewer hydrophobic sites exposed on the surface (Xing et al., 2023). This observation is in agreement with the theory that a high surface hydrophobicity leads to reduced solubility (Wagner et al., 2000).

3.4. Protein quality assessment

3.4.1. Amino acid profile

The amino acid profile of *A. diaperinus* Osborne fractions and protein concentrate, expressed as total amino acid g/100 g protein, is shown in Fig. 7. Essential amino acids (EAAs) are listed from lysine to phenylalanine, while non-essential amino acids (NEAAs) are listed from glycine to arginine. Generally, in each fraction and the protein concentrate, NEAAs, particularly glutamine and asparagine, are present in higher concentrations compared to EAAs. Similar findings have been reported in other insects belonging to the *Tenebrionidae* family, such as *T. molitor*, which also shows high levels of glutamine (123.9 mg/g protein) and glycine (53.8 mg/g protein) (Finke, 2007). Similarly, EAAs such as threonine, tryptophan, cysteine and methionine are found in lower amounts in *T. molitor*, *Z. morio*, *R. phoenicis*, *S. acupunctatus* larvae (Bukkens, 1997; Elemo et al., 2011; Finke, 2002, 2007). This suggests that insects within the same family may share similar amino acids profile.

The GLU contains the highest amount of EAAs (68.75 g/100 g protein), whereas the protein concentrate contains the lowest amount (62.20 g/100 g protein). PRO, GLO, and ALB have comparable EAAs contents (63.56 g/100 g protein, 65.46 g/100 g protein, and 64.61 g/100 g protein, respectively), which is higher than the EAA content reported for beef rib (46.67 g/100 g protein) (Holló et al., 2001). In contrast, when compared to the amino acid requirements for children aged 2 to 5 years as established by the FAO/WHO (1991) (Joint, and Organization, W. H, 2007; Milt-Ward et al., 1991), cysteine and methionine are considered deficient amino acids in *A. diaperinus* protein. However, the cysteine content in GLO, GLU, and protein concentrate is lower than the recommended requirement, the levels of other EAAs (His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val) are significantly higher than the recommended values. Therefore, *A. diaperinus* protein concentrate, GLO and GLU (the largest fraction) have the potential to be alternative protein sources. Conversely, other fractions, such as ALB, which contains low amount of leucine and valine, and PRO, which are deficient in isoleucine and lysine, may not meet the EAA requirements as effectively.

3.4.2. Protein quality parameters

The protein quality values are summarized in Table 3. EAAI serves as a screening method for evaluating alternative proteins. It is calculated based on the essential amino acid profile of novel proteins in comparison to standard egg proteins (Oser, 1959; Peñaflorida, 1989). Unlike EAAI, AAS provides information about the overall quality of the protein. IVPDCAAS and BV are both used to assess protein quality. IVPDCAAS is calculated based on IVPD measurement and focuses on the potential of proteins to meet amino acid requirement, while BV represents the proportion of absorbed food-derived amino acids that become incorporated into the proteins of the organism's body (Kumar et al., 2022). PDCAAS has now largely replaced BV as a rapid and routine assay for protein quality assessment (Schaafsma, 2005).

The EAAI, AAS, BV, IVPD and IVPDCAAS values for PRO are lower compared to other fractions and protein concentrate. This is due to the PROs' deficiency in essential amino acids (Fig. 7). The low IVPD and IVPDCAAS values are attributed to the structure and hydrophobicity of PRO. As discussed earlier, PRO contains the highest proportion of α-helix structures, which are difficult to break down during digestion. Wang et al. (2014) indicated that the degradation α-helix structures lead to an increase in protein digestibility. Besides, the high surface hydrophobicity of PRO further reduces their digestibility. This feature hinders the access of digestive enzymes, resulting in lower digestibility. For instance, the binding of hydrophobic amino acids such as phenylalanine, which are targets for digestive enzymes, is more stable, making digestion more challenging (Liu et al., 2021). Compared to the EAAI of black soldier fly protein concentrate (150.28) (Huang et al., 2019), the largest fractions in *A. diaperinus*, GLU, in this study shows a significantly higher value of 674.76. Another fraction, GLO, also exhibits a higher EAAI value (296.43) compared to black soldier fly. The GLU demonstrates the highest IVPD at 84.04 %, AAS (0.87 Met + Cys) and IVPDCAAS (73.11 %). The IVPDCAAS of *A. diaperinus* GLU is comparable to that of waxworm ALB (42 %), but lower than waxworm GLO (84 %) (Ma et al., 2024). The AAS values of other edible insects, such as *H. pomatia* (0.75 Met + Cys) and L. *littoria* (0.48 Met + Cys) are lower than those reported for each of the *A. diaperinus* fractions and protein concentrates (Igwe, 2015). However, the AAS values for each *A. diaperinus* fraction and protein concentrate are lower than those for *R. phoenicis* (1.16 Met + Cys), and *Z. variegatus* (1.07 Met + Cys) (Igwe, 2015).

Table. 3
Protein quality parameters of *A. diaperinus* Osborne protein fractions and protein concentrate.

Protein sample	IVPD %[a]	EAAI %[b]	AAS[c]	BV[d]	PER_1[e]	PER_2[f]	PER_3[g]	PER_4[h]	PER_5[i]	IVPDCASS %[j]
ALB	71.38 ± 0.13[a]	40.38	0.90 (Met + Cys)	46.09	1.35	1.72	1.77	2.29	2.94	62.24
GLO	81.07 ± 0.06[b]	296.43	0.73 (Met + Cys)	35.08	2.18	2.33	1.66	2.80	2.89	59.18
PRO	70.07 ± 0.29[a]	3.68	0.80 (Lys)	22.31	2.28	1.22	−9.23	1.79	2.62	56.05
GLU	84.04 ± 0.04[b]	674.76	0.87 (Met + Cys)	36.19	2.79	2.28	−2.58	3.07	3.32	73.11
Protein concentrate	83.60 ± 0.00[b]	273.24	0.75 (Met + Cys)	37	2.31	2.33	0.59	2.74	2.89	62.70

Note: EAAI%, AAS%, BV, PER_1, PER_2, PER_3, PER_4, PER_5 and IVPDCAAS are calculated values, no standard deviation is available. Numbers in parentheses indicate SD where applicable.

[a] *In vitro* protein digestibility.
[b] Essential amino acid index.
[c] Amino acids score.
[d] Biological value.
[e] Protein efficient ratio.
[f] Protein efficient ratio.
[g] Protein efficient ratio.
[h] Protein efficient ratio.
[i] Protein efficient ratio.
[j] *In vitro* protein-digestibility corrected amino acid score.

Table. 4
Proteomics analysis of *A. diaperinus* Osborne protein fractions and protein concentrate.

Bands No.	Description	Peptides	Unique peptides	Mw (kDa)	Accession
1	Myosin light chain alkali	2	2	17.13	D6W9T6; A0AA38J150
2	Troponin T	11	2	45.58	A0AA38J8K5; A0A482W1B3
3	Tropomyosin-1, isoforms 9 A/A/ B-like Protein	7	5	40.28	A0A139WAL9; A0A8J6HQ45; A0AA38M370; A0A4P8D332; A0AA38MG45
4	Tropomyosin-1	17	2	32.33	A0A482V7C0; A0AA38HP64
5	Myosin regulatory light chain 2-like Protein	4	2	21.72	D6WZU7
6	Chitin bind 4 domain containing protein	2	2	20.71	A0A482W0R2
7	Cuticle 3 domain containing protein (Fragment)	2	2	19.87	A0A482W3L2; A0A482VQ12
8	Uncharacterized protein fragments	2	2	94.80	A0A8J6L419; A0A482VCK2
9	Uncharacterized protein fragments	2	2	28.78	A0A8J6H7P3
10	Uncharacterized protein fragments	2	2	94.80	A0A8J6L419; A0A482VCK2
11	Actin	11	2	42.16	A0A8J6HUM0; A0A482WAL1; B5A8W7; A0A482W8M0; A0AA38HGX0; A0A482W1C6
12	Actin fragments	11	2	42.16	A0A8J6HUM0; A0A482WAL1; B5A8W7; A0A482W8M0; A0AA38HGX0; A0A482W1C6
13	Actin fragments	11	2	2.16	A0A8J6HUM0; A0A482WAL1; B5A8W7; A0A482W8M0; A0AA38HGX0; A0A482W1C6
14	Larval serum protein 2-like Protein fragments	8	3	91.04	D6WUQ7; D6WUQ8; A0A482VNR1

4. Conclusion

This study provides the first comprehensive analysis of the proteins and structure-related properties of *A. diaperinus* larvae. The Osborne fractionation method revealed that GLU (76.13 %) and ALB (16.6 %) are the dominant protein fractions. Notably, the allergen tropomyosin was detected in both the ALB and GLO. All Osborne fractions exhibited stable structures, with α-helix and β-conformations accounting for over 60 % of their content. Among the four Osborne fractions, PRO demonstrated the highest α-helix content at 71.2 %.

The secondary structure (α-helix, β-sheet, and β-turn) significantly influenced the properties, including thermal stability, surface hydrophobicity, and digestibility. The PRO showed the lowest digestibility (70.07 %) and the highest denaturation temperature (131.25 °C), likely due to its highly ordered structure. The GLO exhibited a higher denaturation temperature than ALB due to its globular nature. In terms of surface hydrophobicity, GLO had the highest H_0 value, followed by GLU, due to their higher proportion of hydrophobic amino acids, demonstrating better surfactant properties, which can enhance foaming and emulsifying capacities.

Isoelectric points were closely linked to protein type, with GLU mainly composed of actin, as identified through proteomic analysis, explaining its higher pI value (pH 4.82). From a nutritional perspective, GLU emerged as the most promising fraction, exhibiting the highest EAAI (674.76 %), IVPD (84.04 %), AAS (0.87 for Met + Cys), and IVPDCAAS (73.11 %).

CRediT authorship contribution statement

Zidan Ma: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Martin Mondor:** Writing – review & editing, Validation, Supervision, Methodology, Investigation. **Adam A. Dowle:** Writing – review & editing, Validation, Software, Data curation. **Francisco M. Goycoolea:** Data curation, Formal analysis, Writing – review & editing. **Alan Javier Hernández-Álvarez:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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.

Data availability

No data was used for the research described in the article.

Acknowledgements

This work is supported by the University of Leeds, School of Food Science and Nutrition, United Kingdom (Grant number: 95522790). This work was funded by the UK National Alternative Proteins Innovation Centre (NAPIC), which is an Innovation and Knowledge Centre funded by the Biotechnology and Biological Sciences Research Council and Innovate UK (Grant Ref: BB/Z516119/1).

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