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Protein Extraction Protocol from the Salivary Glands of Hemipterans: Application in *Mahanarva spectabilis*

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We use this protocol and it's working

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Abstract

This protocol describes the collection and protein extraction process from the salivary glands of the spittlebug *Mahanarva spectabilis* (Hemiptera: Cercopidae). The method combines dissection under a stereomicroscope, mechanical and chemical lysis, centrifugation, protein precipitation with TCA/acetone, and resolubilization for proteomic analysis. It includes two workflows:

- **Workflow A** – Extraction of soluble proteins (supernatant)
- **Workflow B** – Extraction of insoluble proteins (pellet)

Guidelines

- Always perform the entire procedure on **ice (0–4 °C)** or in a **refrigerated environment** to prevent protein degradation.
- Prepare all **buffers and reagents fresh** or store them no longer than one week at 4 °C (without PMSF).
- Add **protease inhibitors (PMSF or cocktail)** immediately before use to ensure full protection of proteins.
- **Label all tubes and reagents clearly** to avoid cross-contamination between samples.
- Calibrate and pre-cool all instruments (centrifuge, sonicator, etc.) prior to use.
- Keep the work area clean and organized — dedicate one ice tray and one set of tools per sample batch.
- Use **low-binding plasticware** (e.g., microtubes) for protein samples to minimize sample loss.
- For reproducibility, note the **number of glands** processed per sample and the **approximate weight** of tissue.
- Check pH of buffers before use; ensure that Tris and PBS buffers are within ±0.1 pH unit of the target value.
- Perform all protein-handling steps using **sterile, chilled pipette tips** to avoid contamination.

Materials

- Stereomicroscope
- Fine forceps and scalpel
- Microtubes (1.5 mL, low protein-binding) and Falcon tubes (15/50 mL)
- Manual or mechanical tissue homogenizer
- Probe sonicator (with amplitude control)
- Refrigerated centrifuge
- Orbital shaker
- Ice bath and water bath (37 °C)
- Pipettes and sterile tips
- −80 °C ultrafreezer
- Spectrophotometer (Bradford or BCA assay)
- SDS-PAGE or LC-MS/MS system

Troubleshooting

Problem

Low protein yield, possible cause: PMSF degradation, insufficient tissue

Solution

Prepare PMSF fresh; increase number of glands

Problem

Protein degradation, possible cause: Delay or poor cooling

Solution

Keep on ice at all times; add PMSF promptly

Problem

Pellet difficult to dissolve, possible cause: Over-drying

Solution

Gradually rehydrate or increase buffer volume

Problem

Assay interference, possible cause: Detergent or urea incompatibility

Solution

Dilute sample or use compatible assay kits

Safety warnings

- **Trichloroacetic acid (TCA)** is *corrosive* — handle only under a **fume hood**, wearing gloves and eye protection.
- **Acetone** is *highly flammable* — keep away from open flames, sparks, and heating elements.
- **Phenylmethylsulfonyl fluoride (PMSF)** is *toxic and unstable in aqueous solution* — prepare fresh in **isopropanol** immediately before use, and dispose of properly after use.
- **Urea** decomposes slowly, producing cyanate — do not store buffers containing urea for more than 1 week.
- Use **face protection and hearing protection** during **probe sonication** to prevent aerosol formation and hearing damage.
- Always wear **gloves, lab coat, and protective eyewear** when handling biological material and reagents.
- Dispose of chemical and biological waste following your institution's **biosafety and hazardous waste protocols**.

Before start

Work at **4 °C** or on **ice** at all times.

- Use complete **personal protective equipment (PPE)**: lab coat, nitrile gloves, safety goggles, face shield, and ear protection for sonication.
- **Prepare all buffers and solutions fresh** or store them at 4 °C for no more than one week (without PMSF).
- Add **protease inhibitors (PMSF)** immediately before use.
- **Pre-chill** acetone and TCA/acetone solution to −20 °C for at least 1 h before use.
- Ensure the centrifuge is **pre-cooled to 4 °C**.

Reagents and Preparation

1 **Acidified Water (pH ≈ 3.0)**
Use: Initial storage of dissected glands.
Preparation (100 mL):
- Add **8.3 µL of concentrated HCl (12 M)** to **100 mL Milli-Q water**.
- Mix well and check that pH ≈ 3.0.
- Store at 4 °C for up to one week.

2 **Protein Extraction Buffer (Workflow A)**
Final composition:
- 50 mM Tris-HCl (pH 7.5)
- 150 mM NaCl
- 1 mM EDTA
- 1 % Triton X-100
- 1 mM PMSF (add freshly before use)

Preparation (100 mL):
1. Dissolve **0.605 g Tris base** in 80 mL Milli-Q water.
2. Add **0.876 g NaCl** and **0.037 g EDTA·Na_2**.
3. Add **1 mL Triton X-100** (use wide-bore tip).
4. Adjust pH to **7.5** with 1 M HCl.
5. Bring to 100 mL with Milli-Q water.
6. Filter-sterilize (0.22 µm).
7. Immediately before use, add **PMSF to 1 mM final** from a 100 mM isopropanol stock.
8. Keep on ice during use.

3 **10 % (w/v) TCA in Acetone (Chilled)**
Use: Protein precipitation.
Preparation (100 mL):
1. Dissolve **10 g trichloroacetic acid (TCA)** in **100 mL anhydrous acetone**.
2. Mix until fully dissolved (use fume hood).
3. Store at **−20 °C** in an amber bottle for up to one month.

4 **100 % Acetone (Chilled)**
Use: Washing of protein pellets.
- Keep acetone at **−20 °C** prior to use.
- Always use chilled acetone for washes.

5 **Solubilization Buffer (Workflow B)**
Final composition:
- 8 M urea
- 2 % CHAPS
- 50 mM Tris-HCl (pH 8.0)

- 1 mM EDTA
- 1 mM PMSF (fresh)

Preparation (100 mL):

1. Dissolve **48 g urea** in 60 mL Milli-Q water (gentle stirring).
2. Add **2 g CHAPS**, mix until dissolved.
3. Add **0.605 g Tris base** and **0.037 g EDTA·Na_2**.
4. Adjust pH to **8.0** with 1 M HCl.
5. Bring to 100 mL final volume.
6. Store at 4 °C for up to 7 days.
7. Add **PMSF to 1 mM** immediately before use.

6 **PBS 1× (Phosphate-Buffered Saline)**

Use: Washing step for pellets.

Final composition:

- 137 mM NaCl
- 2.7 mM KCl
- 10 mM Na_2HPO_4
- 1.8 mM KH_2PO_4
- pH 7.4

Preparation (1 L):

1. Dissolve **8.0 g NaCl**, **0.2 g KCl**, **1.44 g Na_2HPO_4**, and **0.24 g KH_2PO_4** in 800 mL Milli-Q water.
2. Adjust pH to **7.4** with 1 M HCl.
3. Bring to 1 L with water.
4. Store at 4 °C for up to one month.

7 **Safety Notes**

- **TCA** is **corrosive** → handle in fume hood with gloves and goggles.
- **Acetone** is **flammable** → keep away from ignition sources.
- **PMSF** is **toxic and unstable in water** → prepare only under a fume hood and discard properly.
- **Urea** decomposes to cyanate → prepare fresh when possible.
- Dispose of all waste according to institutional chemical-safety regulations.

Workflow A — Soluble Protein Fraction (Supernatant)

8 Workflow A — Soluble Protein Fraction (Supernatant)**Gland Collection**

- Dissect salivary glands from adult *M. spectabilis* under a stereomicroscope.
- Place immediately into microtubes containing acidified water (pH 3).

Keep on ice or store at −80 °C until extraction.

9 **Buffer Addition**
- Transfer glands into 1.5 mL microcentrifuge tubes.
- Add **500 µL of extraction buffer**. Keep on ice.

10 **Homogenization and Lysis**
- Homogenize manually or mechanically until uniform suspension forms.
- Sonicate (probe): **5 cycles of 30–60 s at 20–30 % amplitude**, with **1 min on ice between cycles**.

11 **Cold Incubation**
- Incubate the homogenate on ice for **30 min** for optimal solubilization.

12 **Centrifugation (Clarification)**
- Spin at **12 000 × g for 10 min at 4 °C**.
- Transfer the supernatant (soluble proteins) to a new tube.
- Store pellet at −80 °C for Workflow B.

13 **Protein Precipitation**
- Add **an equal volume** of chilled **10 % TCA/acetone** (1:1).
- Mix gently and incubate on ice for **30 min**.

14 **Post-Precipitation Centrifugation**
- Centrifuge at **12 000 × g for 10 min at 4 °C**.
- Carefully discard the supernatant.

15 **Pellet Washing**
- Wash pellet **three times** with **1 mL chilled acetone (100 %)**.
- Each wash: mix gently, spin 12 000 × g for 5 min at 4 °C.
- Air-dry the pellet for 3–5 min (do not overdry).

16 **Resuspension and Storage**
- Resuspend pellet in a suitable buffer (e.g., **8 M urea + 2 % CHAPS**).
- Vortex or pipette until fully dissolved.
- Store at −20 °C or −80 °C.
- Quantify protein concentration (Bradford or BCA) before SDS-PAGE or MS analysis.

Workflow B — Insoluble Protein Fraction (Pellet)

17 **Pellet Washing**
- Add **1 mL cold PBS 1×**, vortex gently, and spin **12 000 × g for 5 min at 4 °C**.
- Discard supernatant.

18 **Pellet Solubilization**
- Add **500 µL solubilization buffer** (8 M urea + 2 % CHAPS + PMSF 1 mM).
- Homogenize by pipetting or gentle vortexing.

19 **Incubation for Solubilization**
- Shake at room temperature for **1–2 h** or incubate at **37 °C for 30 min** to assist solubilization.

20 **Centrifugation**
- Spin **12 000 × g for 10 min at 4 °C**.
- Transfer the supernatant (solubilized proteins) to a new tube.

21 **Preparation for Analysis**
- Quantify protein concentration (Bradford or BCA).
- For SDS-PAGE: add sample buffer containing SDS + β-mercaptoethanol and heat to **95 °C for 5 min**.
- For proteomics: perform enzymatic digestion (e.g., trypsin) followed by LC-MS/MS.

22 **Storage**
- Store samples at **−20 °C or −80 °C** in aliquots to avoid freeze–thaw cycles.

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