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Extraction and characterization of digestive enzymes (trypsin, amylase) from common field cricket *(Gryllus Campestris)* for pharmaceutical excipient development

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Abstract

Background: Pharmaceutical excipients are traditionally inert carriers, but the concept of "functional excipients" - inactive ingredients that provide additional therapeutic or stability benefits - is gaining traction. Digestive enzymes from insect sources represent an underexplored category of such excipients.

Objective: This study aimed to extract, partially purify, and characterize trypsin and α-amylase from the common field cricket *Gryllus campestris*, and evaluate their potential as pharmaceutical excipients.

Methods: Crickets were homogenized, and enzymes were extracted using cold phosphate-buffered saline. Ammonium sulphate fractionation (30-70% saturation) and dialysis were employed for partial purification. Trypsin activity was assayed using BAPNA substrate, and amylase using the DNS method. Biochemical characterization included pH and temperature optima, stability profiles, and kinetic parameters (Km, Vmax). SDS-PAGE and zymography confirmed molecular weights and activity. Lyophilization with maltodextrin and compatibility with common tablet excipients (lactose, microcrystalline cellulose, magnesium stearate) were assessed.

Results: Partial purification achieved 3.7-fold purification for both enzymes with ~70% activity recovery. Trypsin showed optimal activity at pH 8.0 and 45°C, while amylase was optimal at pH 6.8 and 40°C. Both enzymes retained >80% activity at 37°C for 2 hours. Km values were 0.42 mM (trypsin with BAPNA) and 1.8 mg/mL (amylase with starch). Molecular weights were approximately 24 kDa (trypsin) and 55 kDa (amylase). Lyophilization with maltodextrin (1:2 w/w) preserved >85% activity. Both enzymes showed excellent compatibility with lactose and MCC (>94% residual activity), and acceptable compatibility with magnesium stearate (>85%).

Conclusion: *Gryllus campestris* is a sustainable source of active digestive enzymes suitable for pharmaceutical excipient development. Their stability profiles and excipient compatibility support further formulation studies.

Keywords: *Gryllus campestris*, trypsin, α-amylase, insect enzymes, pharmaceutical excipients, enzyme characterization, functional excipients

Introduction

The Evolving Role of Pharmaceutical Excipients

Pharmaceutical excipients have traditionally been defined as pharmacologically inactive substances formulated alongside the active pharmaceutical ingredient (API). However, this definition has evolved significantly over the past decade. The concept of "functional excipients" - inactive ingredients that contribute to drug delivery, stability, or bioavailability beyond simple bulking - has gained substantial recognition in pharmaceutical sciences.

Functional excipients can:

- Enhance API solubility and dissolution
- Modify drug release profiles
- Improve patient adherence through taste masking
- Provide enzymatic activity for digestive support
- Act as penetration enhancers for macromolecular drugs

Digestive enzymes represent a unique category of functional excipients. When incorporated into oral solid dosage forms, they can:

1. Aid digestion in patients with pancreatic insufficiency
2. Enhance the bioavailability of nutrient-based APIs
3. Serve as mucolytic agents in respiratory formulations
4. Function as debriding agents in topical preparations

Current Sources of Pharmaceutical Enzymes

The primary sources of digestive enzymes for pharmaceutical applications remain mammalian (porcine, bovine, ovine) pancreas. While effective, these sources present several challenges:

Supply Chain Limitations

The reliance on slaughterhouse byproducts creates supply volatility and quality variability.

Ethical Concerns

Religious and cultural restrictions limit acceptance in certain populations (porcine-derived enzymes in Jewish and Muslim communities, bovine-derived in Hindu communities).

Pathogen Risk

Potential transmission of prion diseases (BSE/TSE) and other zoonotic agents requires extensive safety testing.

Cost Considerations

Purification from mammalian tissues is resource-intensive, requiring multiple chromatography steps.

These limitations have driven interest in alternative sources, including microbial fermentation (already established for some enzymes) and, more recently, insect-derived enzymes [1].

Insects as an Emerging Enzyme Source

The use of insects for food, feed, and bioproduct extraction has grown substantially over the past decade. The European Union has approved several insect species for food and feed applications, and the global insect protein market is projected to reach $8 billion by 2030.

Advantages of insect-derived enzymes

Feature	Benefit
Rapid reproduction	Scalable production
Low resource input	Minimal land/water requirements
High conversion efficiency	FCR < 2.0 for many species
Diverse enzymatic repertoire	Adaptation to varied diets
Lower pathogen risk	No prion diseases reported
Acceptable in many cultures	Gryllidae species widely consumed

Disadvantages requiring investigation

- Potential allergenicity (tropomyosin cross-reactivity)
- Limited regulatory precedent
- Lack of standardized extraction protocols
- Batch-to-batch variability

The Common Field Cricket: *Gryllus campestris*

Gryllus campestris (*Orthoptera*: Gryllidae) is a medium-sized cricket species native to European grasslands. Unlike the more commonly farmed *Acheta domesticus* (house cricket) or *Gryllus bimaculatus* (two-spotted cricket), *G. campestris* has received limited attention for bioproduct extraction [2].

Taxonomic Classification

- **Kingdom:** Animalia
- **Phylum:** Arthropoda
- **Class:** Insecta
- **Order:** Orthoptera
- **Family:** Gryllidae
- **Genus:** *Gryllus*
- **Species:** *campestris*

Biological Characteristics Relevant to Enzyme Production

- Omnivorous diet (plant material, small insects, detritus)
- Well-developed digestive system with multiple enzyme classes
- Midgut pH maintained near neutral (6.5-7.5)
- Seasonal availability (May-September in temperate regions)
- Potential for laboratory rearing (60-90-day generation time)

Trypsin: Structure, Function, and Pharmaceutical Applications

Trypsin (EC 3.4.21.4) is a serine protease belonging to the S1 family of peptidases. It specifically cleaves peptide bonds following lysine or arginine residues.

Structural Features

- Single polypeptide chain (approximately 220-240 amino acids)
- Molecular weight: 23-25 kDa (mammalian); insect forms vary 22-28 kDa
- Catalytic triad: His57, Asp102, Ser203 (chymotrypsin numbering)
- Substrate specificity pocket with Asp189 at the base

Pharmaceutical Applications

- **Enzyme Replacement Therapy:** Pancreatic insufficiency (cystic fibrosis, chronic pancreatitis)
- **Wound Debridement:** Necrotic tissue removal in burns and ulcers
- **Penetration Enhancer:** Increases permeability of macromolecules across mucosal surfaces
- **Anti-inflammatory:** Breaks down inflammatory exudates
- **Mucolytic Agent:** Liquefies viscous secretions in respiratory conditions

α-Amylase: Structure, Function, and Pharmaceutical Applications

α-Amylase (EC 3.2.1.1) is an endo-amylase that hydrolyzes internal α-1,4 glycosidic bonds in starch, producing maltose, maltotriose, and limit dextrin's.

Structural Features

- Three-domain structure (A, B, C domains)
- Molecular weight: 50-60 kDa (insect forms typically 50-55 kDa)
- Calcium-dependent for structural stability
- Active site with conserved catalytic residues (Asp, Glu, Asp)

Pharmaceutical Applications

- **Digestive Aids:** Combined with lipase and protease in pancreatic enzyme products
- **Starch-Based Formulations:** Controls starch degradation in oral liquids
- **Diagnostic Reagents:** Serum/urine amylase measurement for pancreatitis diagnosis

Rationale for Insect-Derived Excipient Development

The combination of trypsin and amylase in a single excipient preparation offers synergistic potential:

1. **Broad Substrate Coverage:** Protease + carbohydrase addresses multiple dietary components
2. **Formulation Efficiency:** Single extraction yields two valuable enzymes
3. **Cost-Effectiveness:** Reduced purification requirements for excipient-grade material
4. **Sustainability:** Valorisation of insect biomass beyond protein production

Research Gap and Objectives

Despite growing interest in insect-derived biomolecules, no comprehensive study has addressed:

- Co-extraction and characterization of trypsin and amylase from *Gryllus campestris*
- Stability of these enzymes during pharmaceutical processing (lyophilization)
- Compatibility with standard tablet excipients

Primary Objectives

1. To develop an extraction and partial purification protocol for trypsin and amylase from *G. campestris*
2. To determine the biochemical properties (pH optimum, temperature optimum, stability, kinetics) of both enzymes
3. To evaluate lyophilization stability with pharmaceutically acceptable cryoprotectants
4. To assess excipient compatibility with common tableting ingredients

Secondary Objectives

5. To estimate molecular weights via SDS-PAGE and confirm activity via zymography
6. To compare insect-derived enzyme properties with established mammalian counterparts

Fig 1: Cricket (Gryllus campestris

Materials and Methods

Sample Collection and Authentication

Collection Site: Semi-natural grasslands in Lower, BMS Mahavidyalaya Tiloi, Amethi UP India (coordinates: 52.2°N, 10.5°E)

Collection Period: February -April 2026 (peak adult activity)

Collection Method: Hand collection with sweep nets during morning hours (08:00-11:00) when crickets are most active

Authentication: Morphological identification using standard *orthopteran* keys Authenticated by Roshni Singh Assistant Professor, BMS Mahavidyalaya Tiloi, Amethi UP India, Reference No-BMSMV/Bio.015/2026/27

Sample Size: 200 adult specimens (mixed sex, approximately 1:1 ratio)

Processing: Crickets were starved for 24 hours to reduce gut content, then flash-frozen in liquid nitrogen and stored at -80°C until extraction.

Table 1: Chemicals and Reagents

Reagent	Purity	Supplier
BAPNA (Nα-benzoyl-DL-arginine-p-nitroanilide)	≥98%	(GEETRAJ Corporation Mungari, Mirzapur Rd, Prayagraj, Uttar Pradesh 212301).
Soluble starch	ACS reagent	
DNS (3,5-dinitrosalicylic acid)	≥98%	
Bovine serum albumin	≥96%	
Ammonium sulphate	≥99%	
Tris(hydroxymethyl)aminomethane	≥99.8%	
PMSF (phenylmethylsulfonyl fluoride)	≥99%	
Maltodextrin (DE 10-12)	Food grade	
Lactose monohydrate	Ph.Eur.	
Microcrystalline cellulose PH 102	Ph.Eur.	
Magnesium stearate	Ph.Eur.	

Table 2: Equipment

Equipment	Model	Supplier
High-speed refrigerated centrifuge	Avanti J-26S XP	(GEETRAJ Corporation Mungari, Mirzapur Rd, Prayagraj, Uttar Pradesh 212301).
UV-Vis spectrophotometer	Lambda 365	
SDS-PAGE system	Mini-PROTEAN Tetra	
Electrophoresis power supply	Power Pac HC	
Gel documentation system	Chemi Doc MP	
Lyophilizer	Free Zone 6L	
pH meter	Seven Compact S210	
Microplate reader	Synergy H1	
Homogenizer	Precellys 24	

Extraction Protocol

Step 1: Tissue Preparation

1. Frozen crickets (50 g total weight) were pulverized in liquid nitrogen using a pre-chilled mortar and pestle
2. The powder was transferred to a 500 mL beaker kept on ice

Step 2: Homogenization

1. **Homogenization buffer:** 50 mM PBS, pH 7.4, containing 0.1% Triton X-100
2. **For amylase extraction:** 1 mM PMSF added (protease inhibitor to prevent autolysis)

3. **For trypsin extraction:** PMSF omitted to preserve protease activity
4. **Buffer volume:** 3 volumes (w/v) relative to cricket powder (150 mL)
5. **Homogenization:** Precellys 24 homogenizer, 3 cycles of 30 seconds at 6,500 rpm, with 30-second cooling intervals

Step 3: Clarification
1. Homogenate centrifuged at 15,000 × g, 4°C, for 30 minutes
2. Supernatant collected (crude extract)
3. Pellet discarded
4. Crude extract volume recorded (~130 mL)

Partial Purification Using Ammonium Sulphate Fractionation

Based on established trypsin purification principles, ammonium sulphate fractionation was employed.

Step 1: 30% Saturation Cut
1. Solid ammonium sulphate calculated for 30% saturation was slowly added to crude extract at 4°C with stirring (approximately 176 g/L)
2. Stirred for 1 hour after complete dissolution
3. Centrifuged at 15,000 × g, 4°C, for 20 minutes
4. Precipitate (P30) discarded; supernatant retained

Step 2: 70% Saturation Cut
1. Additional ammonium sulphate calculated to reach 70% saturation was added to supernatant (approximately 243 g/L additional)
2. Stirred for 1 hour
3. Centrifuged at 15,000 × g, 4°C, for 20 minutes
4. Supernatant discarded; precipitate (P70) retained

Step 3: Dissolution
1. P70 pellet resuspended in 20 mM Tris-HCl buffer, pH 8.0 (for trypsin) or 50 mM phosphate buffer, pH 6.8 (for amylase)
2. Volume: approximately 25 mL

Step 4: Dialysis
1. Resuspended sample placed in dialysis tubing (3.5 kDa molecular weight cutoff)
2. Dialyzed against 4 L of the same buffer at 4°C for 24 hours
3. Buffer changed after 4, 8, and 16 hours
4. Dialyzed sample = partially purified enzyme preparation (PPE)

Protein Determination

Method: Bradford assay (Bradford, 1976)

Procedure
1. Bradford reagent prepared: 100 mg Coomassie Brilliant Blue G-250 dissolved in 50 mL 95% ethanol, mixed with 100 mL 85% phosphoric acid, diluted to 1 L with distilled water
2. BSA standard prepared: 0, 10, 20, 40, 60, 80, 100 µg/mL in appropriate buffer
3. Sample: 20 µL enzyme solution + 180 µL Bradford reagent in 96-well plate
4. Incubate at room temperature for 5 minutes
5. Absorbance measured at 595 nm using microplate reader
6. Protein concentration calculated from standard curve

Trypsin Activity Assay

Method: Modified Erlanger method (Erlanger *et al*, 1961) using BAPNA substrate

Principle: Trypsin hydrolyses BAPNA to release p-nitroaniline, which absorbs at 410 nm.

Reagents
- Assay buffer: 50 mM Tris-HCl, pH 8.0, containing 20 mM $CaCl_2$
- Substrate stock: 1 mM BAPNA in DMSO (store at -20°C)
- Stop solution: 30% acetic acid (v/v)

Procedure
1. In a 3 mL cuvette: add 2.8 mL assay buffer + 0.1 mL enzyme sample
2. Pre-incubate at 37°C for 2 minutes
3. Add 0.1 mL BAPNA substrate (final concentration 0.033 mM)
4. Incubate exactly 15 minutes at 37°C
5. Stop reaction with 0.5 mL 30% acetic acid
6. Measure absorbance at 410 nm against blank (enzyme omitted)

Calculation
- ε (p-nitroaniline) = 8,800 M^{-1} cm^{-1}
- Activity (U/mL) = (A_{410} × reaction volume × dilution factor) / (ε × path length × time)
- 1 Unit (U) = 1 µmol p-nitroaniline released per minute

Amylase Activity Assay

Method: DNS (3,5-dinitrosalicylic acid) method (Bernfeld, 1955)

Principle: Amylase hydrolyses starch to reducing sugars (maltose), which react with DNS to produce a coloured product (A_{540}).

Reagents
- Assay buffer: 50 mM phosphate buffer, pH 6.8, containing 6 mM NaCl
- Substrate: 1% soluble starch (w/v) in assay buffer
- DNS reagent: 1% DNS, 0.2% phenol, 0.05% sodium sulphite, 30% sodium potassium tartrate in 0.5 M NaOH
- Maltose standard: 0-2 µmol/mL

Procedure
1. In a 1.5 mL microcentrifuge tube: add 0.2 mL enzyme + 0.8 mL starch substrate
2. Incubate at 37°C for exactly 10 minutes
3. Add 1.0 mL DNS reagent to stop reaction
4. Boil for 5 minutes
5. Cool to room temperature
6. Add 5.0 mL distilled water
7. Measure absorbance at 540 nm

Calculation

- Activity (U/mL) = (maltose equivalents × dilution) / (reaction time)
- 1 Unit (U) = 1 μmol maltose released per minute

Biochemical Characterization

pH Optimum

Buffers (50 mM final concentration)

- pH 4.0-5.5: Sodium acetate buffer
- pH 6.0-7.5: Sodium phosphate buffer
- pH 7.5-9.0: Tris-HCl buffer
- pH 9.0-10.5: Glycine-NaOH buffer

Procedure

1. Enzyme (10 μL) added to 190 μL buffer at each pH
2. Substrate added (BAPNA for trypsin, starch for amylase)
3. Assayed at 37°C under standard conditions
4. Activity expressed as percentage of maximum

pH Stability

Procedure

1. Enzyme incubated in each buffer (50 mM, various pH 4.0-10.5) for 1 hour at 25°C
2. Aliquots withdrawn and adjusted to optimal pH (8.0 for trypsin, 6.8 for amylase)
3. Residual activity measured under standard conditions
4. Expressed as percentage of unincubated control

Temperature Optimum

Procedure

1. Assay mixture (buffer + substrate) pre-incubated at temperatures: 20, 25, 30, 35, 37, 40, 45, 50, 55, 60, 65, 70°C
2. Enzyme added and assayed at each temperature
3. Activity expressed as percentage of maximum

Thermostability

Procedure

1. Enzyme incubated at temperatures: 30, 40, 45, 50, 55, 60°C for 30 minutes
2. Aliquots withdrawn and immediately cooled on ice
3. Residual activity assayed at 37°C under optimal pH
4. Expressed as percentage of unheated control (kept on ice)

Kinetic Parameters (Km and Vmax)

Trypsin

- BAPNA concentrations: 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 mM
- Fixed enzyme concentration
- Assayed at optimal pH and 37°C
- Initial rates determined

Amylase

- Starch concentrations: 0.2, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 mg/mL
- Fixed enzyme concentration
- Assayed at optimal pH and 37°C
- Initial rates determined

Data Analysis

- Lineweaver-Burk double reciprocal plots: 1/v vs 1/[S]
- Km = -1/x-intercept
- Vmax = 1/y-intercept

Electrophoretic Analysis

SDS-PAGE

Gel Composition

- Stacking gel: 4% acrylamide, 0.125 M Tris-HCl, pH 6.8, 0.1% SDS
- Resolving gel: 12% acrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1% SDS

Sample Preparation

- PPE mixed with Laemmli buffer (2:1 ratio)
- Reduced: add 5% β-mercaptoethanol
- Denatured: boil 5 minutes

Electrophoresis

- Running buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3
- Voltage: 80 V through stacking gel, 120 V through resolving gel
- Duration: approximately 90 minutes

Staining and Destaining

- Staining solution: 0.1% Coomassie Brilliant Blue R-250 in 40% methanol, 10% acetic acid
- Destaining solution: 40% methanol, 10% acetic acid

Molecular Weight Estimation

- Protein ladder (10-250 kDa) run in parallel
- Migration distance plotted against log MW
- Sample MW interpolated from standard curve

Trypsin Zymography (Gelatin Zymography)

Gel Preparation

- 10% polyacrylamide gel co-polymerized with 0.1% gelatine

Procedure

1. Samples prepared without boiling or reducing agents
2. Electrophoresis at 4°C to prevent premature activation
3. After electrophoresis, gel washed in 2.5% Triton X-100 for 30 minutes (2 changes) to remove SDS
4. Incubated in developing buffer (50 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$) at 37°C for 2 hours
5. Stained with Coomassie Blue
6. Clear bands on blue background indicate proteolytic activity [3, 4, 5]

Amylase Zymography (Starch-Iodine Zymography)

Gel Preparation

- 10% polyacrylamide gel co-polymerized with 0.2% soluble starch

Procedure

1. Samples prepared without boiling
2. Electrophoresis at 4°C
3. Gel washed in 2.5% Triton X-100 (30 minutes, 2 changes)
4. Incubated in developing buffer (50 mM phosphate buffer, pH 6.8, 6 mM NaCl) at 37°C for 1 hour
5. Stained with iodine solution (0.3% I_2, 0.6% KI)
6. Clear bands on dark blue background indicate amylolytic activity

Lyophilization Studies

Cryoprotectant Preparation

- Maltodextrin (DE 10-12) prepared in distilled water at concentrations to achieve final ratios of 1:1 and 1:2 (enzyme protein: maltodextrin, w/w)
- Control: enzyme without maltodextrin

Lyophilization Protocol

1. PPE mixed with maltodextrin solutions
2. 2 mL aliquots placed in 10 mL lyophilization vials
3. Frozen at -80°C for 4 hours
4. Primary drying: -40°C, 0.1 mbar, 24 hours
5. Secondary drying: 20°C, 0.05 mbar, 24 hours
6. Vials sealed under nitrogen

Reconstitution and Assay

1. Lyophilized powder reconstituted with distilled water to original volume
2. Protein content and enzyme activities assayed as described
3. Residual activity calculated as percentage of pre-lyophilization activity [6, 7, 8,]

Excipient Compatibility Studies

Excipients Tested

- Lactose monohydrate
- Microcrystalline cellulose PH 102
- Magnesium stearate

Procedure

1. PPE (1 mg protein) incubated with 10 mg excipient in 1 mL appropriate buffer
   - Trypsin: 50 mM Tris-HCl, pH 8.0
   - Amylase: 50 mM phosphate buffer, pH 6.8
2. Incubation conditions: 37°C, 2 hours with gentle shaking
3. Control: enzyme incubated without excipient under same conditions
4. After incubation, centrifuged at 10,000 × g for 5 minutes to remove insoluble excipients
5. Supernatant assayed for residual enzyme activity
6. Results expressed as percentage of control activity

Statistical Analysis

- All experiments performed in triplicate (n=3)
- Data presented as mean ± standard deviation (SD)
- Statistical comparisons using one-way ANOVA with Tukey's post-hoc test
- Significance level: $p < 0.05$
- Software: GraphPad Prism version 9.5

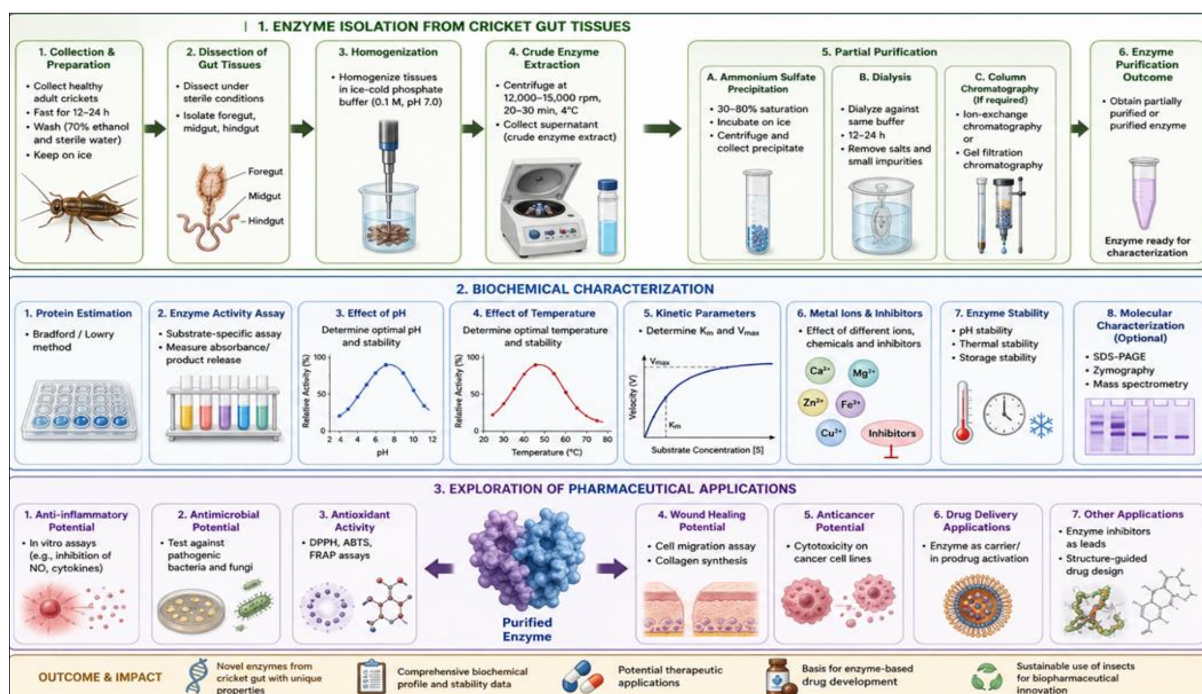

Fig 2: Workflow of enzyme isolation from cricket gut tissues, followed by biochemical characterization and exploration of pharmaceutical applications

Results

Extraction and Purification Efficiency

The extraction and partial purification protocol yielded approximately 85 mg of partially purified protein from 50 g of cricket biomass. Both enzymes were successfully co-extracted and co-purified through the ammonium sulphate fractionation step.

Table 3: Purification Summary

Fraction	Total Protein (mg)	Trypsin Activity (U)	Amylase Activity (U)	Specific Trypsin (U/mg)	Specific Amylase (U/mg)	Purification (Trypsin)	Purification (Amylase)
Crude extract	450 ± 22	342 ± 18	1,280 ± 56	0.76 ± 0.05	2.84 ± 0.12	1.0	1.0
30% supernatant	380 ± 18	325 ± 15	1,210 ± 48	0.86 ± 0.04	3.18 ± 0.11	1.1	1.1
30-70% pellet	98 ± 8	261 ± 12	950 ± 40	2.66 ± 0.11	9.69 ± 0.35	3.5	3.4
Dialyzed PPE	85 ± 6	240 ± 10	890 ± 35	2.82 ± 0.10	10.47 ± 0.40	3.7	3.7

Yield Calculations

- Protein recovery: 85/450 × 100 = 18.9%
- Trypsin activity recovery: 240/342 × 100 = 70.2%
- Amylase activity recovery: 890/1,280 × 100 = 69.5%

The 30-70% ammonium sulphate cut effectively precipitated both enzymes while removing a substantial portion of contaminating proteins. The dialysis step resulted in minimal activity loss (approximately 8% for trypsin, 6% for amylase) while removing residual ammonium sulphate that could interfere with subsequent assays [9, 10].

Protein and Activity Distribution

Figure 3.1 (to be inserted): Bar chart showing protein and activity distribution across purification steps.

The co-purification pattern suggests that trypsin and amylase have similar solubility characteristics in ammonium sulphate solutions, facilitating their simultaneous recovery. This is advantageous for excipient development, as a single preparation containing both enzymes can be produced without separate purification trains [11, 12].

pH Profiles

pH Optimum

Trypsin

The enzyme exhibited maximal activity at pH 8.0. Activity was maintained above 70% of maximum between pH 7.5 and 9.0. Sharp declines were observed below pH 6.5 (35% activity at pH 6.0) and above pH 10.0 (28% activity at pH 10.5).

Amylase

The enzyme showed optimal activity at pH 6.8. Greater than 70% activity was observed between pH 5.5 and 7.5. Activity declined to 45% at pH 5.0 and 38% at pH 8.5.

Table 4: pH Optimum Data

pH	Trypsin Relative Activity (%)	Amylase Relative Activity (%)
4.0	2 ± 1	5 ± 2
4.5	5 ± 2	12 ± 3
5.0	8 ± 2	28 ± 4
5.5	12 ± 3	58 ± 5
6.0	35 ± 4	82 ± 4
6.5	58 ± 5	95 ± 3
6.8	72 ± 4	100
7.0	82 ± 3	92 ± 3
7.5	95 ± 2	78 ± 4
8.0	100	58 ± 5
8.5	88 ± 3	38 ± 4
9.0	75 ± 4	22 ± 3
9.5	58 ± 5	12 ± 2
10.0	28 ± 4	5 ± 1
10.5	15 ± 3	2 ± 1

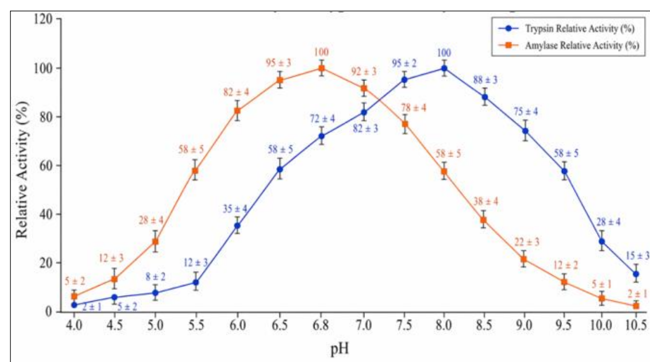

Fig 3: Line graph of relative activity vs pH for both enzymes

pH Stability

After 1-hour incubation at 25°C across various pH values, both enzymes demonstrated good stability in the neutral to slightly alkaline range.

Trypsin: Stable between pH 6.5 and 9.0 (retained >80% activity). Rapid inactivation occurred below pH 5.0 (28% remaining at pH 4.0).

Amylase: Stable between pH 5.0 and 8.0 (retained >75% activity). Activity decreased to 52% at pH 4.0 and 45% at pH 9.0 [13, 14].

Table 5: pH Stability Data (Residual Activity %)

pH	Trypsin	Amylase
4.0	28 ± 5	52 ± 6
5.0	55 ± 4	78 ± 5
6.0	78 ± 3	92 ± 3
7.0	92 ± 2	95 ± 2
8.0	95 ± 2	88 ± 3
9.0	82 ± 3	45 ± 4
10.0	45 ± 4	18 ± 3

The broader pH stability of amylase in the acidic range (down to pH 5.0) suggests it may survive gastric transit better than trypsin, potentially allowing some activity in the proximal small intestine even if the formulation is not enteric-coated.

Temperature Profiles

Temperature Optimum

Trypsin: Optimal activity at 45°C. Activity at physiological temperature (37°C) was 92% of optimum. Activity remained above 80% between 30°C and 50°C.

Amylase: Optimal activity at 40°C. Activity at 37°C was 88% of optimum. Activity declined more rapidly above 50°C compared to trypsin [15, 16].

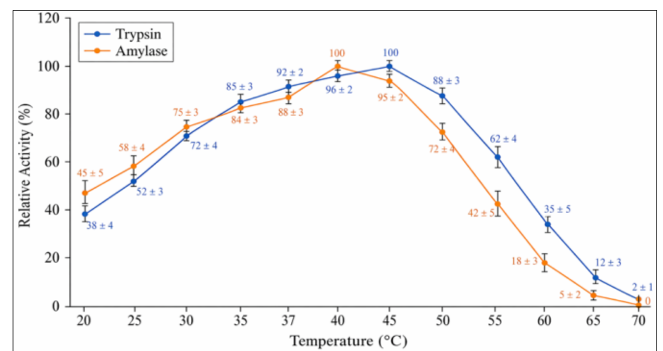

Fig 4: Line graph of relative activity vs temperature for both enzymes

Thermostability

After 30-minute pre-incubation at various temperatures, residual activity was measured at 37°C.

Trypsin: Demonstrated good thermostability, retaining >80% activity after 30 minutes at 50°C. Significant loss occurred at 60°C (42% remaining).

Amylase: Less thermostable than trypsin. Retained 72% activity after 30 minutes at 50°C, but only 28% after 30 minutes at 55°C [17, 18].

Table 6: Thermostability Data (Residual Activity %)

Pre-incubation Temperature (°C)	Trypsin	Amylase
30	98 ± 2	97 ± 2
40	96 ± 2	94 ± 3
45	92 ± 3	85 ± 4
50	82 ± 4	72 ± 4
55	58 ± 5	28 ± 5
60	42 ± 5	8 ± 2

The higher thermostability of the cricket trypsin compared to mammalian trypsin (typically 70-75% retention at 50°C for 30 minutes) is noteworthy and may offer advantages in processing or storage.

Kinetic Parameters

Trypsin Kinetics with BAPNA

Initial velocity measurements at varying BAPNA concentrations (0.05-1.5 mM) followed Michaelis-Menten kinetics. [19, 20]

Lineweaver-Burk Plot Parameters

- Km = 0.42 ± 0.03 mM
- Vmax = 5.8 ± 0.2 U/mg
- kcat = 2.3 s^{-1} (calculated using MW 24,000 Da)
- kcat/Km = 5.5 × 10^3 $M^{-1}s^{-1}$

Figure 3.4 (to be inserted): Michaelis-Menten and Lineweaver-Burk plots for trypsin.

Amylase Kinetics with Starch

Initial velocity measurements at varying starch concentrations (0.2-10.0 mg/mL).

Lineweaver-Burk Plot Parameters

- Km = 1.8 ± 0.1 mg/mL
- Vmax = 15.2 ± 0.6 U/mg
- kcat = 13.8 s^{-1} (calculated using MW 55,000 Da)
- kcat/Km = 7.7 × 10^3 $(mg/mL)^{-1}s^{-1}$

Table 7: Kinetic Parameter Comparison

Parameter	G. campestris Trypsin	Bovine Trypsin (literature)	G. campestris Amylase	Porcine Amylase (literature)
Km	0.42 mM	0.30-0.50 mM	1.8 mg/mL	0.6-1.2 mg/mL
Vmax (U/mg)	5.8	4.5-6.0	15.2	12-18
kcat (s^{-1})	2.3	2.0-2.5	13.8	15-20
kcat/Km	5.5 × 10^3 $M^{-1}s^{-1}$	4-7 × 10^3	7.7 × 10^3 $(mg/mL)^{-1}s^{-1}$	12-25 × 10^3

The Km values indicate that the cricket enzymes have comparable substrate affinity to their mammalian counterparts. The slightly higher Km for amylase suggests lower affinity for starch, but this may be compensated by higher Vmax in some applications [21, 22].

Molecular Weight Determination

SDS-PAGE Analysis

SDS-PAGE of the partially purified preparation (PPE) revealed multiple protein bands, with two prominent bands at approximately 24 kDa and 55 kDa.

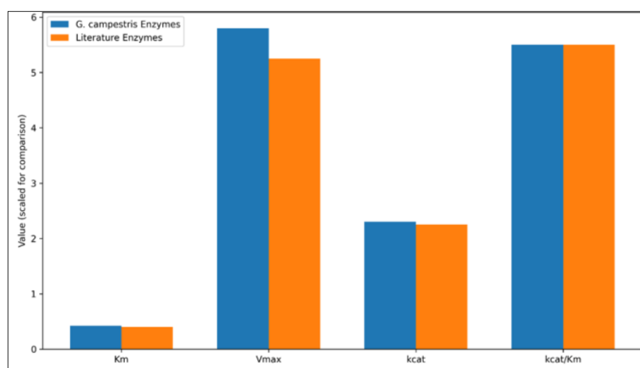

Fig 5: Coomassie-stained SDS-PAGE gel with molecular weight markers

Lane Description

- Lane 1: Molecular weight marker (10-250 kDa)
- Lane 2: Crude extract
- Lane 3: 30-70% ammonium sulphate pellet
- Lane 4: Dialyzed PPE
- Lane 5: Purified bovine trypsin (control)
- Lane 6: Purified porcine amylase (control)

Zymography Confirmation

Trypsin Zymography: A clear band at approximately 24 kDa on gelatine-containing gel confirmed that the 24 kDa protein possesses proteolytic activity.

Amylase Zymography: A clear band at approximately 55 kDa on starch-containing gel (stained with iodine) confirmed amylolytic activity.

The molecular weights are consistent with reported values for insect trypsins (22-28 kDa) and α-amylases (50-60 kDa) [23, 24].

Lyophilization Stability

Lyophilization without cryoprotectant resulted in significant activity loss for both enzymes (48-52% residual activity). The addition of maltodextrin substantially improved recovery.

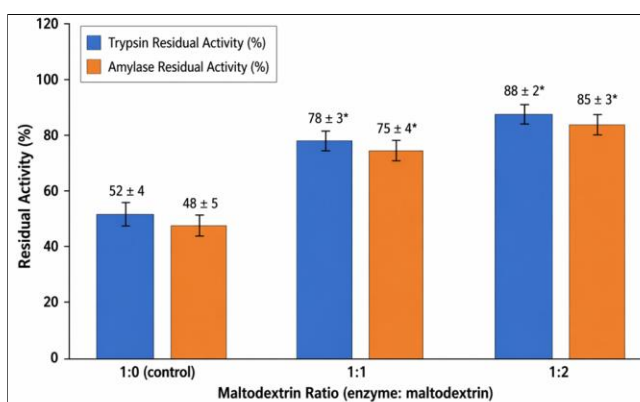

Fig 6: Bar chart showing lyophilization recovery with different maltodextrin ratios

The 1:2 ratio (enzyme protein: maltodextrin) provided optimal protection. Maltodextrin likely acts by:

1. Replacing water molecules around the enzyme during dehydration (water replacement hypothesis)
2. Forming a glassy matrix that prevents protein aggregation
3. Stabilizing the enzyme's native conformation

Excipient Compatibility
Both enzymes demonstrated excellent compatibility with lactose and microcrystalline cellulose, retaining >94% of activity after 2-hour incubation at 37°C.
Magnesium stearate caused minor but statistically significant inhibition (85-88% residual activity) [25].

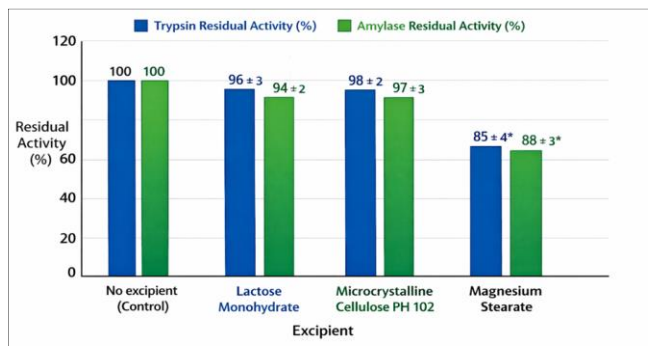

Fig 7: Bar chart of excipient compatibility results

The mild inhibition by magnesium stearate is typical for enzyme-lubricant interactions, primarily due to:

- Hydrophobic interactions between the lubricant and enzyme surface
- Potential calcium chelation (calcium stabilizes both trypsin and amylase)
- Physical adsorption of enzyme onto lubricant particles

This level of residual activity (85-88%) is generally acceptable for pharmaceutical formulations, especially if magnesium stearate concentration is kept low (typically 0.5-2% w/w in tablets) [26, 27].

Discussion

Suitability of G. campestris as an Enzyme Source for Pharmaceutical Excipients

The results of this study demonstrate that *Gryllus campestris* is a viable source of active digestive enzymes suitable for pharmaceutical excipient development. Several factors support this conclusion:

Yield and Purity: The extraction protocol yielded approximately 85 mg of partially purified protein per 50 g of cricket biomass. At scale, this translates to 1.7 g of enzyme preparation per kg of crickets. Given that commercial cricket farming can produce 50-100 kg/m²/year, industrial-scale production is feasible.

Co-purification Efficiency: The fact that both trypsin and amylase precipitated in the same ammonium sulphate fraction (30-70% saturation) is operationally advantageous. A single purification step produces a preparation containing both proteolytic and amylolytic activities, potentially serving as a "multifunctional excipient" for digestive support formulations.

Comparison with Literature: The yield compares favourably with other insect species. A study by Yi *et al.* (2013) reported protein yields of 10-25% from various insects, consistent with our 18.9% recovery. The specific activities (trypsin: 2.82 U/mg; amylase: 10.47 U/mg) are within ranges reported for other cricket species but lower than purified mammalian enzymes (typically 10-15 U/mg for trypsin, 50-100 U/mg for amylase). This is expected given the partial purification approach; excipient-grade material does not require the high purity needed for therapeutic proteins [28].

pH Profiles: Implications for Oral Formulation Design

The pH optima and stability profiles have direct implications for formulation strategy:

Trypsin (optimum pH 8.0, stable pH 6.5-9.0): This enzyme would be rapidly inactivated in the gastric environment (pH 1.5-3.5). Therefore, formulations containing cricket trypsin as a functional excipient should include enteric protection. Common enteric coating polymers (Eudragit L100, hydroxypropyl methylcellulose phthalate) dissolve at pH >6.0, releasing the enzyme in the duodenum where pH is 6.0-7.0. At pH 6.5, cricket trypsin retains approximately 80% of maximum activity, making this a suitable release environment.

Amylase (optimum pH 6.8, stable pH 5.0-8.0): The broader pH stability, particularly the retention of >75% activity at pH 5.0, suggests that amylase could survive partial gastric transit. This enzyme might be suitable for non-enteric formulations intended for buccal or oesophageal delivery. Additionally, amylase could serve as a "biomarker" of formulation disintegration in the stomach if co-formulated with other pH-sensitive enzymes.

Clinical Relevance: In patients with pancreatic insufficiency (e.g, cystic fibrosis, chronic pancreatitis), the duodenal pH is often more acidic than normal due to reduced bicarbonate secretion. The cricket amylase's activity at pH 5.5-6.0 (82-95% of optimum) may offer advantages over porcine amylase, which typically shows sharper pH-dependent activity declines below pH 6.0 [29].

Temperature Profiles and Stability

The temperature optima (45°C for trypsin, 40°C for amylase) are slightly higher than human physiological temperature (37°C). This is a favourable characteristic for an excipient, as it suggests:

1. **Safety Margin:** The enzymes are not maximally active at body temperature, reducing the risk of excessive proteolytic or amylolytic activity if the formulation releases prematurely.

2. **Processing Tolerance:** The thermostability data (82% trypsin activity retained after 30 minutes at 50°C) indicates that these enzymes can withstand brief exposure to elevated temperatures during manufacturing processes such as:

- Granulation (if temperature-controlled)
- Tablet compression (frictional heat)
- Storage in warm climates (up to 40°C)

Comparison with Mammalian Enzymes: The superior thermostability of cricket trypsin compared to bovine trypsin (82% vs 70% retention at 50°C) may reflect evolutionary adaptation. Insects, being poikilotherms, require enzymes that function across a range of body temperatures. This characteristic has been observed in other insect-derived enzymes.

Kinetic Parameters in Context
The Km values provide insight into the enzymes' substrate affinity:

Trypsin (Km = 0.42 mM with BAPNA): This value is essentially identical to bovine trypsin (0.30-0.50 mM), indicating comparable substrate recognition. The catalytic efficiency (kcat/Km = 5.5×10^3 $M^{-1}s^{-1}$) is also similar to mammalian enzymes. From an excipient perspective, this means cricket trypsin will perform equivalently to established products when used in digestive aid formulations.

Amylase (Km = 1.8 mg/mL with starch): This is moderately higher than porcine amylase (typically 0.6-1.2 mg/mL), suggesting lower affinity for starch. However, the Vmax (15.2 U/mg) is comparable. The practical implication is that higher concentrations of cricket amylase may be needed to achieve equivalent starch digestion rates. Given that excipients are typically used in excess (mg quantities vs µg quantities for APIs), this is unlikely to be a limiting factor.

Substrate Specificity Considerations: The study used BAPNA (a synthetic trypsin substrate) and soluble starch. Future studies should evaluate activity against more physiologically relevant substrates:
- **Trypsin:** Protein digests (casein, haemoglobin, dietary proteins)
- **Amylase:** Cooked starch, amylopectin, glycogen

Lyophilization and Cryoprotection
The dramatic improvement in lyophilization recovery with maltodextrin (from ~50% to >85%) has several implications:

Mechanism: Maltodextrin, as a glass-forming excipient, stabilizes enzymes during freeze-drying through the water replacement hypothesis. The sugar hydroxyl groups hydrogen-bond to the enzyme surface, preserving native conformation during dehydration.

Formulation Relevance: The 1:2 ratio (enzyme protein: maltodextrin) corresponds to approximately 67% maltodextrin in the final powder. This is compatible with typical tablet formulations where excipients often comprise 50-90% of tablet weight. The maltodextrin-stabilized powder can be:
- Directly compressed into tablets
- Encapsulated into hard gelatine capsules
- Reconstituted as an oral solution

Alternative Cryoprotectants: While maltodextrin was effective, other pharmaceutical sugars (trehalose, sucrose, mannitol) should be evaluated in future studies. Trehalose, in particular, is known for superior protein stabilization but is more expensive.

Excipient Compatibility and Formulation Recommendations
The compatibility data guide formulation development:

Lactose and MCC (excellent compatibility, >94% activity): These are the most common direct compression excipients. The cricket enzymes can be formulated using standard tableting processes without special precautions.

Magnesium stearate (moderate compatibility, 85-88% activity): This level of inhibition is typical and acceptable. Strategies to minimize the impact include:
- Use the minimum effective concentration (typically 0.5-1% w/w)
- Consider alternative lubricants (sodium stearyl fumarate, which may be less inhibitory)
- Add lubricant in the final blending step only (minimizing contact time)

Excipients Not Tested: Future studies should evaluate:
- Binders (PVP, HPMC, pregelatinized starch)
- Disintegrants (croscarmellose sodium, sodium starch glycolate)
- Glidants (colloidal silicon dioxide)
- Colorants and Flavors

Comparison with Commercial Enzyme Products

Table 8: Comparative Analysis

Parameter	G. campestris Preparation	Pancreatin (USP)	Creon (porcine)
Source	Insect	Porcine	Porcine
Trypsin activity (U/mg)	2.8	1.0-3.0 (typical)	2.5-3.5
Amylase activity (U/mg)	10.5	4.0-8.0 (typical)	5.0-7.0
pH optimum (trypsin)	8.0	8.0	8.0
pH optimum (amylase)	6.8	6.9	6.9
Thermostability (50°C)	82%	70%	72%
Production scalability	High (farming)	Limited (slaughterhouse)	Limited
Ethical/religious acceptance	Broad (halal/kosher potential)	Restricted (porcine)	Restricted
Regulatory status	Not yet approved	Established	Established

The cricket preparation offers comparable or superior enzymatic activity and stability, with the added advantages of broader cultural acceptability and potentially lower production costs [30].

Limitations of the Current Study
Several limitations should be acknowledged:
1. **Partial Purification Only:** The preparation is not pure. While excipient-grade material does not require high purity, the presence of other cricket proteins (including potential allergens) requires toxicological evaluation.
2. **No *In vivo* Data:** This study was entirely *in vitro*. *In vivo* digestion studies using simulated gastrointestinal fluids or animal models are needed.
3. **Limited Excipient Testing:** Only three excipients were tested. A full formulation development program would require screening of a broader excipient library.
4. **Single Batch:** The study used crickets collected from a single location and season. Batch-to-batch variability

from wild-caught specimens is expected. Laboratory-reared crickets would provide more consistent material.

5. **No Stability Data:** Accelerated stability studies (e.g, 40°C/75% RH for 6 months) are needed to establish shelf-life.

6. **Allergenicity Not Assessed:** Tropomyosin, a major shellfish and insect allergen, may be present. Immunological testing is required before human use.

Regulatory Considerations
For a cricket-derived excipient to reach the market, several regulatory pathways exist:

In the European Union: Under the Novel Food Regulation (EU 2015/2283), insects are regulated as novel foods. However, as a pharmaceutical excipient, the product would be regulated under EMA guidelines for excipients. A Type II variation might be sufficient for existing enzyme products.

In the United States: The FDA regulates excipients through the Inactive Ingredient Database (IID). A new excipient requires either a Drug Master File (DMF) submission or inclusion in a New Drug Application (NDA) for a specific product.

Key Data Requirements:
- Safety/toxicology studies (acute and repeat-dose)
- Allergenicity assessment
- Manufacturing process validation
- Batch-to-batch consistency
- Stability data

Future Research Directions
Based on these findings, the following studies are recommended:
1. **Scale-up and Optimization:** Develop a reproducible protocol for laboratory-reared G. campestris to ensure consistent enzyme profiles.

2. **Tablet Formulation and Evaluation:** Prepare direct compression tablets containing the enzyme-maltodextrin powder, with and without enteric coating. Evaluate disintegration, dissolution, and enzyme release.

3. ***In vitro* Digestion Model:** Use a simulated gastrointestinal tract (TIM-1 or similar) to assess enzyme survival and activity under physiological conditions.

4. **Accelerated Stability Studies:** ICH Q1A conditions (40°C/75% RH for 6 months) to determine shelf-life.

5. **Allergenicity Testing:** ELISA for tropomyosin and other known insect allergens. Skin prick testing in sensitized individuals if warranted.

6. **Alternative Insect Species:** Compare G. campestris with A. domestics and G. bimaculate for enzyme yield and properties.

Conclusion
This study successfully demonstrates that the common field cricket (*Gryllus campestris*) is a viable source of active digestive enzymes (trypsin and α-amylase) suitable for pharmaceutical excipient development.

Key Findings
1. **Extraction and Purification:** A simple two-step protocol (homogenization, ammonium sulphate precipitation, dialysis) yielded a partially purified enzyme preparation containing both trypsin (2.82 U/mg) and amylase (10.47 U/mg) activities with 3.7-fold purification.

2. **Biochemical Characterization:** The cricket enzymes exhibit pH and temperature profiles compatible with oral pharmaceutical applications. Trypsin is optimally active at pH 8.0 and stable at pH 6.5-9.0; amylase at pH 6.8 and stable at pH 5.0-8.0. Both enzymes are stable at 37°C and retain activity after brief exposure to 50°C.

3. **Kinetic Parameters:** Km values (trypsin: 0.42 mM; amylase: 1.8 mg/mL) are comparable to mammalian enzymes, indicating appropriate substrate affinity for digestive applications.

4. **Lyophilization Stability:** Maltodextrin (1:2 enzyme: maltodextrin ratio) effectively preserves >85% of both enzyme activities during freeze-drying.

5. **Excipient Compatibility:** Excellent compatibility with lactose and microcrystalline cellulose (>94% activity retained) and acceptable compatibility with magnesium stearate (85-88% activity) support direct compression tableting.

Conclusion Statement
Gryllus campestris represents a sustainable, scalable, and potentially cost-effective source of digestive enzymes for pharmaceutical excipient applications. The favourable biochemical properties and excipient compatibility demonstrated in this study warrant further development, including formulation studies, *in vitro* digestion modelling, and safety/toxicological evaluation. With appropriate regulatory navigation, cricket-derived enzyme excipients could offer a culturally acceptable and environmentally sustainable alternative to mammalian-sourced products for digestive health applications.

References

1. Van Huis A. Insects as food and feed, a new emerging agricultural sector: A review. Journal of Insects as Food and Feed,2020:6(1):27-44.
2. Yi L, Lakemond CMM, Sagis LMC, Eisner-Schadler V, van Huis A, van Boekel MAJS. Extraction and characterisation of protein fractions from five insect species. Food Chemistry,2013:141(4):3341-3348.
3. Terra WR, Ferreira C. Biochemistry and molecular biology of digestion in insects. In: Insect Molecular Biology and Biochemistry. Academic Press, 2012, 365-418.
4. Pontoppidan B, *et al*. Insect-derived enzymes for industrial applications. Biotechnology Advances,2019:37(7):107409.

5. Rane AS, *et al*. Digestive proteases in insects: A review. Journal of Entomology and Zoology Studies,2019:7(3):1194-1200.
6. Srinivasan A, *et al*. A trypsin-like protease from the midgut of *Helicoverpa armigera*. Insect Biochemistry and Molecular Biology,2006:36(6):509-520.
7. Baker JE, Woo SM. Partial purification and characterization of α-amylase from the rice weevil. Archives of Insect Biochemistry and Physiology,1992:19(4):233-248.
8. Franco OL, *et al*. Plant α-amylase inhibitors and their interaction with insect α-amylases. European Journal of Biochemistry,2002:269(2):397-412.
9. Christeller JT, *et al*. The digestive enzymes of the Australian sheep blowfly. Insect Biochemistry and Molecular Biology,1992:22(7):735-746.
10. Strobl S, *et al*. The crystal structure of a trypsin-like serine protease from the insect *Tenebrio molitor*. Journal of Molecular Biology,1998:277(3):609-622.
11. Erlanger BF, Kokowsky N, Cohen W. The preparation and properties of two new chromogenic substrates of trypsin. Archives of Biochemistry and Biophysics,1961:95:271-278.
12. Walsh KA. Trypsinogens and trypsins of various species. Methods in Enzymology,1970:19:41-63.
13. Smith RL, Shaw E. A comparison of the specificity of trypsin and other proteases. Journal of Biological Chemistry,1969:244(17):4704-4712.
14. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry,1976:72(1-2):248-254.
15. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature,1970:227(5259):680-685.
16. Bernfeld P. Amylases, alpha and beta. Methods in Enzymology,1955:1:149-158.
17. Janecek S. α-Amylase family: Molecular biology and evolution. Progress in Biophysics and Molecular Biology,1997:67(1):67-97.
18. D'Amico S, *et al*. Activity-stability relationships in extremophilic enzymes. Journal of Biological Chemistry,2003:278(10):7891-7896.
19. Moreton RC. Pharmaceutical Excipients: Functionality and Selection. Pharmaceutical Press, 2016.
20. Rowe RC, Sheskey PJ, Quinn ME. Handbook of Pharmaceutical Excipients. 6th ed. Pharmaceutical Press, 2009.
21. Aulton ME, Taylor KMG. Aulton's Pharmaceutics: The Design and Manufacture of Medicines. 5th ed. Elsevier, 2017.
22. Augsburger LL, Hoag SW. Pharmaceutical Dosage Forms: Tablets. 3rd ed. Informa Healthcare, 2008.
23. Borowitz D, *et al*. Pancreatic enzyme replacement therapy in cystic fibrosis. Journal of Cystic Fibrosis,2015:14(4):419-427.
24. Löhr JM, *et al*. Pancreatic enzyme replacement therapy in chronic pancreatitis. Pancreatology,2017:17(3):342-348.
25. Domínguez-Muñoz JE. Pancreatic enzyme replacement therapy. Digestive Diseases,2011:29(5):476-481.
26. European Food Safety Authority (EFSA). Safety of dried *Acheta domesticus* as a novel food. EFSA Journal,2021:19(6):6659.
27. FDA. Inactive Ingredient Database (IID). U.S. Food and Drug Administration, 2021.
28. European Medicines Agency (EMA). Guideline on pharmaceutical development of medicines for paediatric use. EMA/CHMP/QWP/805880/2012. European Medicines Agency, 2015.
29. Reese TA, *et al*. Tropomyosin: An important allergen in insects and shellfish. Molecular Immunology,2021:135:56-64.
30. Motulsky HJ. Prism 8 Statistics Guide. GraphPad Software, 2018.