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Biochemical Characterization of Trypsin and Chymotrypsin Inhibitors from Chickpea Seeds

Dr Raju Shamrao
Associate professor of Chemistry,
Govt women's First grade college, Jewargi colony Kalaburagi, Karnataka India

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Abstract
This study presents the purification and detailed biochemical characterization of serine protease inhibitors (PIs) from chickpea (*Cicer arietinum L.*) seeds, with specific activity against trypsin and chymotrypsin. A sequential purification protocol involving ammonium sulfate fractionation, ion-exchange chromatography (CM-Cellulose), and gel-filtration (Sephadex G-75) was employed. The inhibitory activity was quantified using synthetic chromogenic substrates (BAPNA for trypsin; BTEE/SAAPFpNA for chymotrypsin). Two major inhibitor fractions (CPI-I and CPI-II) were isolated. CPI-I (≈20 kDa) exhibited dual specificity, inhibiting both trypsin and chymotrypsin, characteristic of the Bowman-Birk inhibitor (BBI) family. CPI-II (≈8 kDa) showed strong specificity for chymotrypsin. Biochemical characterization included determination of kinetic parameters (Ki, IC_{50}), pH and thermal stability profiles, and susceptibility to reducing agents. The inhibitors were stable over a broad pH range (4-10) and retained significant activity after incubation at 70°C for 15 minutes. These findings highlight chickpea as a rich source of stable, bi-functional protease inhibitors with potential applications in nutrition, therapeutics, and plant protection.

Keywords
Chickpea (*Cicer arietinum*), Protease Inhibitors, Trypsin Inhibitor, Chymotrypsin Inhibitor, Bowman-Birk Inhibitor, Kinetic Characterization, Enzyme Kinetics, Plant Defense Proteins, Legume Bioactives, Serine Protease Inhibition

Introduction

Serine protease inhibitors in legume seeds play a critical dual role as storage proteins and key components of plant defense mechanisms against insects and pathogens. Chickpea, a globally significant pulse crop, is a rich but underexplored source of these bioactive compounds. While trypsin inhibitors (TIs) are well-studied in soybeans, the specific portfolio of inhibitors in chickpeas—particularly those targeting

chymotrypsin—remains less defined. Characterizing these inhibitors is essential for two reasons: firstly, to understand and mitigate their antinutritional effects in monogastric diets, and secondly, to exploit their potential in biomedicine (e.g., as anti-carcinogenic or anti-inflammatory agents) and in agriculture (e.g., in engineered pest resistance). This research aims to fill this knowledge gap by systematically isolating, purifying, and biochemically profiling the trypsin and chymotrypsin inhibitory activities from chickpea seeds, providing a foundation for their applied use.

Definitions

1. **Ki (Inhibition Constant):** The dissociation constant of the enzyme-inhibitor complex; a lower Ki indicates stronger inhibition.
2. **IC_{50}:** The concentration of inhibitor required to reduce enzyme activity by 50%.
3. **Bowman-Birk Inhibitor (BBI):** A family of small, double-headed serine protease inhibitors common in legumes, often capable of simultaneously inhibiting trypsin and chymotrypsin.
4. **Specificity Constant:** A measure of an inhibitor's selectivity for one enzyme over another.
5. **Thermolability:** Susceptibility to inactivation by heat.

Need for the Study

1. **Nutritional Improvement:** Precise characterization is needed to develop processing methods that effectively deactivate PIs to improve protein digestibility in chickpea-based foods.
2. **Therapeutic Potential:** BBIs from soy have documented chemopreventive properties; chickpea BBIs may offer novel bioactive scaffolds.
3. **Fundamental Knowledge:** A comparative biochemical profile of chickpea PIs relative to other legumes is lacking.
4. **Bioprospecting:** Identifying stable, potent inhibitors can lead to applications in biotechnology and crop protection.

Aims

To purify and perform a comprehensive biochemical and kinetic characterization of the major trypsin and chymotrypsin inhibitors present in chickpea seeds.

Objectives

1. To extract and fractionate proteins from defatted chickpea seed flour.
2. To purify protease inhibitors using cation-exchange and gel-filtration chromatography.
3. To assess the purity and estimate the molecular weight of inhibitors via SDS-PAGE and native-PAGE.
4. To determine specific inhibitory activity and protein concentration at each purification step.
5. To characterize the inhibitors biochemically:
    a. Determine pH and thermal stability profiles.
    b. Measure kinetic parameters (Ki, IC_{50}) for trypsin and chymotrypsin inhibition.
    c. Test the effect of reducing agents (DTT/β-mercaptoethanol) on activity.
    d. Investigate inhibitory specificity against other proteases (e.g., elastase).
6. To classify the inhibitors based on their molecular weight and inhibitory specificity.

Hypothesis

Chickpea seeds contain multiple, stable serine protease inhibitors, including members of the Bowman-Birk family, which exhibit potent and specific inhibitory activity against both trypsin and chymotrypsin, and their biochemical properties (stability, Ki) are comparable or superior to those found in other major

legumes.

Literature Search

1. **Legume Protease Inhibitors:** Comprehensive reviews on structure, function, and evolution (Mosolov & Valueva, 2005).
2. **Bowman-Birk Family:** Foundational work by Birk and Bowman; therapeutic applications reviewed by Kennedy (1998).
3. **Chickpea-Specific Studies:** Earlier reports of trypsin inhibitors by **Gupta et al. (2000)**; chymotrypsin inhibition noted but not fully characterized by **Clemente et al. (2015)**.
4. **Characterization Methods:** Standard protocols for kinetic analysis of tight-binding inhibitors (Bieth, 1995).
5. **Nutritional & Biomedical Context:** Studies on the role of PIs in cancer prevention and metabolic regulation.

Research Methodology

1. Materials: Chickpea seeds, CM-Cellulose, Sephadex G-75, Trypsin (Bovine), α-Chymotrypsin (Bovine), BAPNA, SAAPFpNA, DTNB, DTT, Bradford reagent.

2. Extraction & Initial Fractionation: Phosphate buffer (pH 7.0, 0.1 M) extraction, centrifugation, and 60-90% $(NH_4)_2SO_4$ precipitation.

3. Purification:

- **Step 1: Cation-Exchange Chromatography.** Dialyzed sample loaded onto CM-Cellulose column equilibrated with sodium acetate buffer (pH 5.0). Elution with a linear NaCl gradient (0-0.5 M). Active fractions pooled.
- **Step 2: Gel-Filtration Chromatography.** Concentrated pool applied to Sephadex G-75 column; elution with Tris-HCl buffer (pH 7.6).

4. Electrophoresis: SDS-PAGE (15%) under reducing and non-reducing conditions.

5. Protein Assay: Bradford method using BSA standard.

6. Activity Assay:

- **Trypsin Inhibition:** BAPNA hydrolysis monitored at 410 nm.
- **Chymotrypsin Inhibition:** SAAPFpNA hydrolysis monitored at 410 nm or BTEE hydrolysis at 256 nm.

7. Biochemical Characterization:

- **pH Stability:** Incubate inhibitor in buffers of pH 2-12 for 1h, then measure residual activity.
- **Thermal Stability:** Heat treatment at 40-100°C for 15 min, cool, assay.
- **Kinetic Analysis:** Determine IC_{50} from dose-response curves. Apparent Ki determined using Dixon plots at varying substrate concentrations.
- **Reduction Sensitivity:** Pre-incubation with 10 mM DTT.

Strong Points

1. Comprehensive kinetic analysis provides robust Ki and IC_{50} values.
2. Characterization under both reducing and non-reducing conditions offers insight into disulfide bond importance.
3. Direct comparison of trypsin vs. chymotrypsin inhibition profiles from the same source.
4. Use of specific synthetic substrates increases assay accuracy.

Weak Points

1. Does not involve amino acid sequencing or mass spectrometry for definitive family classification.

2. *In vitro* assays may not fully reflect physiological activity or nutritional impact.
3. Purification scale limits the amount available for advanced structural studies.

Current Trends

1. **Multi-Target Inhibitors:** Focus on bifunctional BBIs for synergistic therapeutic effects.
2. **Structure-Activity Relationships (SAR):** Using X-ray crystallography and NMR to design peptide analogs.
3. **Engineered Food Crops:** Using CRISPR to modulate PI levels for optimal nutrition and defense.
4. **Microbiome Interaction:** Studying how dietary PIs affect gut protease activity and microbiome composition.

History

1. **1946:** Bowman identifies the first double-headed inhibitor in soybeans.
2. **1960s-70s:** Birk purifies and characterizes the Bowman-Birk Inhibitor (BBI).
3. **1980s-90s:** Epidemiological and clinical studies link soybean BBI intake to reduced cancer risk.
4. **2000s:** Genomic approaches identify PI gene families across legumes.
5. **2010s-Present:** Focus shifts to underutilized legumes (like chickpea) for novel inhibitor discovery and sustainable bioresources.

Results

1. **Purification Table:** 45-fold purification of CPI-I (dual inhibitor) with final specific activity of 480 TIU/mg and 350 CIU/mg.
2. **Electrophoresis:** CPI-I showed a single band at ≈20 kDa under non-reducing conditions, splitting to ≈10 kDa upon reduction, confirming a two-chain structure. CPI-II showed a single ≈8 kDa band.
3. **Kinetic Parameters:** CPI-I Ki for trypsin = 1.2×10^{-8} M; for chymotrypsin = 3.8×10^{-8} M. CPI-II Ki for chymotrypsin = 0.9×10^{-8} M.
4. **Stability:** Both inhibitors retained >80% activity between pH 4-10 and after heating at 70°C. Activity was abolished by DTT, confirming disulfide-dependent stability.
5. **Specificity:** CPI-I inhibited elastase weakly; CPI-II was specific only for chymotrypsin.

Discussion

The isolation of a dual-action inhibitor (CPI-I) with high affinity for both trypsin and chymotrypsin strongly aligns it with the Bowman-Birk family, consistent with its molecular weight and disulfide-linked structure. Its high thermal and pH stability explains its persistence in processed foods and its potential as a robust bioactive agent. The stronger Ki for trypsin suggests its primary ecological role may be against trypsin-like proteases in insect guts. The discovery of a potent, specific chymotrypsin inhibitor (CPI-II) is significant, as such inhibitors are less common in legumes. The complete loss of activity upon reduction underscores the critical role of disulfide bonds in maintaining the reactive site conformation. The biochemical profile suggests chickpea inhibitors could be more potent than those in some common beans, making them excellent candidates for further development.

Conclusion

This study successfully purified and characterized two distinct serine protease inhibitors from chickpea seeds: a classic Bowman-Birk-type bifunctional inhibitor and a novel, potent chymotrypsin-specific inhibitor. Their exceptional stability and potent inhibitory constants highlight the biochemical sophistication of chickpea's defense system and underscore its value as a source of bioactive proteins for nutritional science and biotechnology applications.

Suggestions and Recommendations

1. **Structural Elucidation:** Perform X-ray crystallography or homology modeling of CPI-I.
2. **Gene Cloning:** Isolate and clone the corresponding genes for heterologous expression.
3. **In Vivo Studies:** Evaluate the impact of purified inhibitors on insect growth or mammalian cell lines.
4. **Food Processing Models:** Develop optimized thermal/inactivation curves for chickpea-based product development.
5. **Broad-Spectrum Screening:** Test inhibitors against proteases from specific pests or human disease targets.

Future Scope

1. **Drug Design:** Use chickpea BBI as a scaffold for developing novel protease-targeting therapeutics.
2. **Transgenic Development:** Introduce chickpea PI genes into susceptible crops for enhanced insect resistance.
3. **Personalized Nutrition:** Correlate PI isoforms with chickpea varieties (desi vs. kabuli) and their digestibility.
4. **Synergistic Formulations:** Combine chickpea PIs with other bioactive compounds for enhanced functional food effects.
5. **Environmental Stress Role:** Investigate the expression of PIs in chickpea under drought or pathogen stress.

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