Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Comparative Evaluation of Five Proteases for the Production of Multifunctional Bioactive Peptides from Chickpea (Cicer arietinum L.) Protein Isolate: Optimization of Hydrolysis Conditions, Peptide Characterization, and In Vitro Assessment of ACE-Inhibitory, DPP-IV-Inhibitory, and Antioxidant Activities

Muhammad Moiz Amir

moizamir69@gmail.com

National Institute of Food Science and Technology University of Agriculture

Research Article

Keywords: Cicer arietinum, bioactive peptides, enzymatic hydrolysis, Alcalase, ACE inhibition, DPP-IV inhibition, antioxidant activity, response surface methodology, molecular docking, LC-MS/MS, ultrafiltration

Posted Date: July 28th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10479481/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: The authors declare no competing interests.

Abstract

Background: The enzymatic release of bioactive peptides from plant storage proteins represents a promising strategy for developing natural alternatives to synthetic antihypertensive and antidiabetic drugs. However, the systematic comparative data necessary to guide protease selection and process optimization for multifunctional bioactivity remain conspicuously absent from the literature.

Methods: Chickpea protein isolate (CPI; 88.7 ± 1.2% protein, desi-type var. Punjab-2008) was hydrolyzed using five food-grade proteases—Alcalase, Flavourzyme, papain, trypsin, and Neutrase—under their respective optimal conditions. Alcalase produced the highest degree of hydrolysis (DH = 28.4 ± 1.1%) and the most potent bioactivities, with the unfractionated hydrolysate (CPI-ALC) exhibiting ACE-inhibitory activity (IC_{50} = 0.34 ± 0.02 mg/mL), DPP-IV-inhibitory activity (IC_{50} = 0.82 ± 0.04 mg/mL), DPPH radical scavenging (68.5 ± 2.3% at 1 mg/mL), ABTS radical scavenging (IC_{50} = 0.48 ± 0.03 mg/mL), and ferric reducing antioxidant power (FRAP = 342.6 ± 14.7 μmol Fe^{2+}/g). Response surface methodology (RSM) using a central composite design identified optimal Alcalase hydrolysis conditions as enzyme/substrate ratio of 3.2% (w/w), temperature 52°C, pH 8.0, and hydrolysis time 180 min, yielding an ACE inhibition of 87.3 ± 1.5%. Ultrafiltration fractionation revealed that the <1 kDa fraction possessed the highest bioactivity across all assays, accounting for 42.3% of the total peptide mass. LC-MS/MS analysis of the <1 kDa fraction identified 14 peptide sequences, among which VFPLR, LAFPG, and RQSHF exhibited the strongest predicted ACE-binding affinities (−9.2, −8.8, and −8.5 kcal/mol, respectively) in molecular docking studies, while SPGAG and IAPLP demonstrated favorable DPP-IV docking scores. Enzyme kinetics analysis via Lineweaver-Burk plots indicated that CPI-ALC acts as a mixed-type ACE inhibitor (K_i = 0.18 ± 0.02 mg/mL). These findings establish chickpea protein isolate as a high-potential source of multifunctional bioactive peptides, provide the first standardized comparative dataset to guide protease selection in legume peptide production, and demonstrate the critical importance of integrating peptidomics, molecular docking, and enzyme kinetics into bioactive peptide characterization workflows—an analytical depth that remains rare in the food peptide literature yet is essential for credible translation toward functional food applications.

1. Introduction

Non-communicable diseases (NCDs) are responsible for approximately 74% of global deaths annually, with cardiovascular diseases and diabetes mellitus accounting for the largest share of this burden (World Health Organization, 2024). Hypertension, a primary risk factor for stroke, myocardial infarction, and renal failure, affects an estimated 1.28 billion adults worldwide, with the majority residing in low- and middle-income countries (NCD Risk Factor Collaboration, 2021). Concurrently, the global prevalence of type 2 diabetes mellitus (T2DM) has reached pandemic proportions, affecting approximately 537 million adults and projected to rise to 783 million by 2045 (International Diabetes Federation, 2024). The angiotensin-I-converting enzyme (ACE; EC 3.4.15.1) and dipeptidyl peptidase-IV (DPP-IV; EC 3.4.14.5) are validated therapeutic targets for the management of hypertension and T2DM, respectively (Cushman & Ondetti, 1999; Drucker & Nauck, 2006). However, synthetic ACE inhibitors (e.g., captopril, enalapril) and

DPP-IV inhibitors (e.g., sitagliptin, vildagliptin) are associated with adverse effects including persistent dry cough, angioedema, gastrointestinal disturbances, and pancreatitis, which have motivated the search for safer, food-derived alternatives (Cicero et al., 2017; Singh et al., 2021).

Bioactive peptides, defined as specific protein fragments of 2–20 amino acid residues that exert physiological effects beyond their nutritional value, have emerged as promising natural alternatives for the management of metabolic disorders (Udenigwe & Aluko, 2012; Kadam et al., 2024). These peptides are encrypted within the primary sequences of food proteins and remain latent until released by proteolytic processes—enzymatic hydrolysis, microbial fermentation, or gastrointestinal digestion (Ashaolu & Suttikhana, 2023). The most extensively studied bioactivities include ACE inhibition (antihypertensive), DPP-IV and α-glucosidase inhibition (antidiabetic), and radical-scavenging antioxidant capacity, with structure-activity relationship studies revealing the critical importance of low molecular weight (< 3 kDa), hydrophobic, and proline-rich peptide sequences (Nongonierma & FitzGerald, 2019; Li et al., 2018).

Chickpea (Cicer arietinum L.) is the third most widely cultivated legume globally, with an annual production exceeding 15 million metric tons, and is a dietary staple across South Asia, the Mediterranean, the Middle East, and East Africa (FAOSTAT, 2024). Chickpea seeds contain 20–25% protein with a well-balanced amino acid profile rich in essential amino acids including lysine, leucine, isoleucine, and phenylalanine, making them a nutritionally valuable protein source (Jukanti et al., 2012). The major storage proteins—legumin (11S globulin) and vicilin (7S globulin)—constitute approximately 60–70% of total seed protein and contain numerous encrypted bioactive peptide sequences, as demonstrated by in silico analyses using the BIOPEP-UWM database (Arámburo-Gálvez et al., 2022). Previous studies have documented ACE-inhibitory peptides from chickpea protein hydrolysates including sequences derived from legumin and provicilin (Figueroa-Salcido et al., 2024), DPP-IV-inhibitory peptides such as SPGAGKG from germinated chickpea (Zan et al., 2023), and antioxidant peptides including RQSHFANAQP and NFYHE (Hernandez et al., 2019; Matemu et al., 2021). Despite these advances, a systematic comparative evaluation of multiple food-grade proteases for the simultaneous optimization of ACE-inhibitory, DPP-IV-inhibitory, and antioxidant activities from chickpea protein isolate has not been reported.

The choice of protease is the single most critical determinant of hydrolysate bioactivity, as different enzymes exhibit distinct cleavage specificities that govern the amino acid composition, molecular weight distribution, and terminal residue profiles of released peptides (Gasparre et al., 2024; Mune Mune et al., 2023). Alcalase, an endoprotease from Bacillus licheniformis with broad specificity for hydrophobic residues, has consistently been reported to produce hydrolysates with superior bioactivity from legume proteins (Gharibzahedi et al., 2022). Flavourzyme, an exo/endoprotease complex from Aspergillus oryzae, generates peptides with reduced bitterness through terminal amino acid cleavage (Dent et al., 2023). Papain (from Carica papaya), trypsin (porcine pancreatic), and Neutrase (Bacillus amyloliquefaciens) have been less extensively investigated for chickpea protein but have demonstrated efficacy with other legume substrates (Kusumah et al., 2020; Rezvankhah et al., 2021).

The objectives of the present study were therefore: (i) to compare the hydrolytic efficiency and bioactivity profiles of five food-grade proteases (Alcalase, Flavourzyme, papain, trypsin, and Neutrase) acting on chickpea protein isolate; (ii) to optimize hydrolysis conditions for the most effective protease using response surface methodology with ACE inhibition as the response variable; (iii) to fractionate the optimized hydrolysate by ultrafiltration and characterize the molecular weight-dependent distribution of bioactivity; (iv) to identify peptide sequences in the most active fraction by LC-MS/MS and evaluate their binding interactions with ACE and DPP-IV via molecular docking; and (v) to determine the enzyme inhibition kinetics and mechanism of the optimized hydrolysate.

The novelty of this investigation lies in several specific contributions that address critical deficiencies in the existing literature. First, whereas previous chickpea peptide studies have evaluated individual proteases in isolation—bromelain (Acevedo Martínez & Gonzalez de Mejia, 2021), alcalase alone (Figueroa-Salcido et al., 2024), or simulated gastrointestinal enzymes (Zan et al., 2023)—no study has conducted a head-to-head comparison of five commercially available food-grade proteases under standardized conditions using the same protein substrate. Such a comparison is essential because enzyme selection is arguably the most consequential processing decision in bioactive peptide production, yet practitioners lack systematic comparative data to inform this choice. Second, the present study simultaneously evaluates three distinct bioactivity dimensions (ACE inhibition, DPP-IV inhibition, and antioxidant capacity) on the same hydrolysate panel, enabling an integrated assessment of multifunctional potential that is directly relevant to the clustered pathologies of metabolic syndrome. Prior chickpea studies have typically assessed one or two bioactivities, precluding a holistic evaluation of multifunctionality. Third, the study employs LC-MS/MS-based peptidomics coupled with molecular docking against both ACE and DPP-IV to provide residue-level insights into the structural basis of the observed bioactivities—an integrated analytical workflow that goes beyond the simplistic reporting of IC_{50} values and contributes mechanistic understanding. Fourth, the enzyme kinetics analysis (Lineweaver-Burk and Dixon plots) for both target enzymes provides quantitative parameters (K_m, V_{max}, K_i, inhibition mode) that are essential for predicting in vivo behavior and for benchmarking against pharmaceutical inhibitors, yet are rarely reported in food peptide studies. Finally, by employing a desi-type chickpea variety (Punjab-2008) cultivated in Pakistan—a region with rapidly escalating cardiometabolic disease burden and substantial chickpea production—the study addresses a geographic and agronomic context that has been largely neglected in the predominantly Western-focused plant bioactive peptide literature. This integrated approach provides a comprehensive framework for the rational production of multifunctional chickpea-derived peptide ingredients with potential applications in functional foods targeting cardiometabolic health.

2. Materials and Methods

2.1 Materials and Chemicals

Desi-type chickpea seeds (variety 'Punjab-2008') were procured from the Ayub Agricultural Research Institute, Faisalabad, Pakistan. Alcalase® 2.4 L (≥ 2.4 U/g), Flavourzyme® 500 L (≥ 500 LAPU/g), papain

(≥ 3 U/mg), trypsin (≥ 2500 U/mg), and Neutrase® 0.8 L (≥ 0.8 AU/g) were obtained from Novozymes (Bagsværd, Denmark). Angiotensin-I-converting enzyme (ACE) from rabbit lung (A6778), N-hippuryl-L-histidyl-L-leucine (HHL; H1635), dipeptidyl peptidase-IV (DPP-IV) from porcine kidney (D7052), Gly-Pro-p-nitroanilide hydrochloride (G0513), 2,2-diphenyl-1-picrylhydrazyl (DPPH; D9132), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; A1888), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ; T1253), o-phthaldialdehyde (OPA; P0657), diprotin A (Ile-Pro-Ile; I9759), captopril (C4042), and all other analytical-grade reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrafiltration membranes with molecular weight cut-offs (MWCO) of 1, 3, and 5 kDa were obtained from Millipore (Burlington, MA, USA).

2.2 Preparation of Chickpea Protein Isolate (CPI)

Chickpea seeds were dehulled, ground to flour (60 mesh), and defatted with n-hexane (1:5 w/v, 6 h, 25°C) under continuous stirring. Defatted flour was air-dried overnight and CPI was extracted by alkaline extraction-isoelectric precipitation following the method of Ghribi et al. (2015) with modifications. Briefly, defatted flour was dispersed in deionized water (1:10 w/v), adjusted to pH 9.0 with 1 M NaOH, stirred for 2 h at 40°C, and centrifuged at 10,000 × g for 20 min at 4°C. The supernatant was collected, adjusted to pH 4.3 (isoelectric point of chickpea protein) with 1 M HCl, held at 4°C for 1 h, and centrifuged at 10,000 × g for 20 min. The protein precipitate was washed twice with deionized water (pH 4.3), neutralized to pH 7.0, dialyzed against deionized water (MWCO 12–14 kDa) at 4°C for 24 h, and lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA). The protein content of CPI was determined by the Kjeldahl method (N × 6.25; AOAC 984.13).

2.3 Enzymatic Hydrolysis

CPI was dissolved in deionized water to a concentration of 5% (w/v, protein basis). Hydrolysis was conducted using each of the five proteases under their respective optimal pH and temperature conditions: Alcalase (pH 8.0, 55°C), Flavourzyme (pH 7.0, 50°C), papain (pH 6.5, 55°C), trypsin (pH 7.5, 37°C), and Neutrase (pH 7.0, 50°C). The enzyme/substrate (E/S) ratio was standardized at 2% (w/w, enzyme protein/substrate protein) for the comparative study. Hydrolysis was conducted in a thermostated water bath with continuous pH adjustment using a pH-stat titrator (Titrando 902, Metrohm, Herisau, Switzerland). After 180 min, the reaction was terminated by heating at 85°C for 15 min, followed by rapid cooling in an ice bath. Hydrolysates were centrifuged at 12,000 × g for 15 min at 4°C, and supernatants were lyophilized and stored at − 20°C until analysis. Hydrolysates were designated as CPI-ALC (Alcalase), CPI-FLA (Flavourzyme), CPI-PAP (papain), CPI-TRY (trypsin), and CPI-NEU (Neutrase). All hydrolyses were performed in triplicate.

2.4 Degree of Hydrolysis

The degree of hydrolysis (DH) was determined using the OPA method as described by Nielsen et al. (2001) with minor modifications. The OPA reagent was prepared fresh daily by dissolving 40 mg of OPA in 1 mL of methanol, followed by the addition of 25 mL of 0.1 M sodium tetraborate, 100 mg of sodium dodecyl sulfate (SDS), and 100 µL of β-mercaptoethanol, made up to 50 mL with deionized water.

Aliquots of hydrolysate (50 µL) were mixed with 2 mL of OPA reagent, incubated at 25°C for 2 min, and the absorbance was measured at 340 nm using a UV-Vis spectrophotometer (UV-2600i, Shimadzu, Kyoto, Japan). L-Serine (0.1–1.0 mM) was used for calibration. The total number of peptide bonds was determined by complete acid hydrolysis (6 M HCl, 110°C, 24 h), and DH was expressed as the percentage of cleaved peptide bonds relative to the total number of peptide bonds.

2.5 Optimization of Hydrolysis Conditions by Response Surface Methodology

Based on the results of the comparative screening, Alcalase was selected for hydrolysis optimization. A three-factor, three-level central composite design (CCD) with five center points was employed using Design-Expert software version 13.0 (Stat-Ease Inc., Minneapolis, MN, USA). The independent variables were E/S ratio (X_1: 1.0–5.0% w/w), temperature (X_2: 40–60°C), and hydrolysis time (X_3: 60–300 min), with ACE inhibition (%) as the response variable. A total of 20 experimental runs including 6 axial points (α = 1.682) and 5 replicates at the center point were conducted in randomized order. The quadratic polynomial model was: $Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\Sigma\beta_{ij} X_i X_j$, where Y is the predicted response and β values are regression coefficients. Model adequacy was evaluated by analysis of variance (ANOVA), lack-of-fit test, coefficient of determination (R^2), and diagnostic plots.

2.6 Ultrafiltration Fractionation

The optimized CPI-ALC hydrolysate was fractionated using a stirred ultrafiltration cell (Amicon 8400, Millipore) with sequential MWCO membranes of 5, 3, and 1 kDa under nitrogen pressure (50 psi) at 4°C. Four fractions were collected: F1 (> 5 kDa), F2 (3–5 kDa), F3 (1–3 kDa), and F4 (< 1 kDa). The permeate from each filtration step served as the feed for the subsequent membrane. All fractions were lyophilized and stored at − 20°C.

2.7 ACE Inhibitory Activity Assay

ACE inhibitory activity was measured using the spectrophotometric method of Cushman and Cheung (1971) as modified by Jimsheena and Gowda (2009). Briefly, 50 µL of hydrolysate solution (0.1–2.0 mg/mL in 0.1 M sodium borate buffer, pH 8.3, containing 0.3 M NaCl) was pre-incubated with 50 µL of ACE solution (25 mU/mL) at 37°C for 10 min. The reaction was initiated by adding 150 µL of 5 mM HHL substrate in the same buffer and incubated at 37°C for 60 min. The reaction was terminated with 250 µL of 1 M HCl. The liberated hippuric acid was extracted with 1.5 mL ethyl acetate, centrifuged at 3,000 × g for 10 min, and 1 mL of the organic phase was evaporated to dryness at 100°C. The residue was dissolved in 3 mL deionized water and absorbance was measured at 228 nm. Captopril (0.01–100 nM) served as the positive control. IC_{50} was calculated from the dose-response curve using non-linear regression (GraphPad Prism 9.0).

2.8 DPP-IV Inhibitory Activity Assay

DPP-IV inhibitory activity was determined using the chromogenic substrate Gly-Pro-p-nitroanilide following the method of Lacroix and Li-Chan (2013) with modifications. In a 96-well microplate, 25 µL of

hydrolysate solution (0.2–5.0 mg/mL in 0.1 M Tris-HCl buffer, pH 8.0) was pre-incubated with 25 µL of 1.6 mM Gly-Pro-p-nitroanilide at 37°C for 10 min. The reaction was initiated by adding 50 µL of DPP-IV (0.01 U/mL) and incubated at 37°C for 60 min. The reaction was stopped with 100 µL of 1 M sodium acetate buffer (pH 4.0), and the absorbance of released p-nitroaniline was measured at 405 nm using a microplate reader (Multiskan SkyHigh, Thermo Fisher Scientific, Waltham, MA, USA). Diprotin A (0.1–100 µM) served as the positive control.

2.9 Antioxidant Activity Assays

DPPH radical scavenging activity was measured according to the method of Brand-Williams et al. (1995). Hydrolysate solutions (0.1–2.0 mg/mL, 100 µL) were mixed with 100 µL of 0.2 mM DPPH in methanol and incubated in the dark at 25°C for 30 min. Absorbance was measured at 517 nm, and scavenging activity was expressed as: [(Acontrol − Asample) / Acontrol] × 100. ABTS radical cation scavenging activity was determined following Re et al. (1999). $ABTS^{\bullet+}$ was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate in the dark for 16 h. The $ABTS^{\bullet+}$ solution was diluted with PBS (pH 7.4) to an absorbance of 0.700 ± 0.020 at 734 nm. Hydrolysate solutions (20 µL) were mixed with 200 µL of $ABTS^{\bullet+}$ solution, incubated at 30°C for 6 min, and absorbance was measured at 734 nm. Ferric reducing antioxidant power (FRAP) was determined by the method of Benzie and Strain (1996). Trolox (0–1 mM) was used as the standard for all antioxidant assays.

2.10 Peptide Identification by LC-MS/MS

The most bioactive fraction (F4, < 1 kDa) was analyzed using a Dionex Ultimate 3000 RSLCnano system coupled to a Q Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with an EASY-Spray ion source. Peptides were loaded onto an Acclaim PepMap 100 C18 trap column (100 µm × 2 cm, 5 µm) and separated on an EASY-Spray C18 analytical column (75 µm × 50 cm, 2 µm) at a flow rate of 300 nL/min using a 90-min linear gradient of 5–40% acetonitrile in 0.1% formic acid. Full MS scans were acquired at a resolution of 120,000 over m/z 200–2000, and the top 20 most intense precursors were selected for HCD fragmentation (NCE = 28%) and MS/MS analysis at 30,000 resolution. Raw data were processed using Proteome Discoverer 2.5 (Thermo Fisher Scientific) with the Sequest HT search engine against a customized Cicer arietinum protein database (UniProt, downloaded November 2025) coupled with a decoy database. Peptide identifications were filtered at a 1% false discovery rate (FDR).

2.11 Molecular Docking

Identified peptide sequences were subjected to molecular docking against human ACE (PDB ID: 1O86) and human DPP-IV (PDB ID: 5J3J) using AutoDock Vina 1.2.3 (Trott & Olson, 2010). Peptide 3D structures were generated using the PEP-FOLD3 server and energy-minimized with the AMBER99SB force field. Receptor preparation involved removal of water molecules and co-crystallized ligands, addition of polar hydrogens, and assignment of Gasteiger charges using AutoDock Tools 1.5.7. The docking grid for ACE was centered on the active site Zn^{2+} ion (x = 40.5, y = 37.8, z = 45.2) with dimensions 30 × 30 × 30 Å. For DPP-IV, the grid was centered on the S1/S2 subsite (x = 10.2, y = 55.8, z =

16.5) with identical dimensions. The exhaustiveness parameter was set to 32, and the top-ranked pose by binding affinity was selected for each peptide-ligand complex. Binding interactions were visualized and analyzed using PyMOL 2.5 and BIOVIA Discovery Studio Visualizer 2021.

2.12 Enzyme Inhibition Kinetics

The mode of ACE inhibition by CPI-ALC was determined by measuring enzyme activity at varying concentrations of HHL substrate (0.5, 1.0, 2.0, 3.0, and 5.0 mM) in the absence and presence of two fixed concentrations of CPI-ALC (0.5 and 1.0 mg/mL). Data were plotted as Lineweaver-Burk double reciprocal plots (1/V vs. 1/[S]). The inhibition constant (K_i) was determined from Dixon plots. The same procedure was applied to determine DPP-IV inhibition kinetics using Gly-Pro-p-nitroanilide substrate (0.1, 0.2, 0.4, 0.8, and 1.6 mM) and CPI-ALC concentrations of 0.4 and 0.8 mg/mL.

2.13 Statistical Analysis

All experiments were performed in triplicate and results are expressed as mean ± standard deviation (SD). Statistical analyses were conducted using SPSS version 28.0 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference (HSD) post-hoc test was used to assess differences among protease treatments and among molecular weight fractions. A p-value < 0.05 was considered statistically significant. For RSM optimization, Design-Expert 13.0 was used for experimental design, regression analysis, and response surface plotting. The desirability function was employed for multi-response optimization.

3. Results and Discussion

3.1 Composition of Chickpea Protein Isolate and Degree of Hydrolysis

The chickpea protein isolate (CPI) prepared by alkaline extraction-isoelectric precipitation exhibited a protein content of 88.7 ± 1.2%, with moisture (6.8 ± 0.3%), ash (3.1 ± 0.2%), and residual lipid (1.4 ± 0.1%). The electrophoretic profile of CPI (SDS-PAGE, data not shown) revealed characteristic bands corresponding to legumin subunits (α-legumin, ~35–40 kDa; β-legumin, ~20–25 kDa), vicilin subunits (~43–53 kDa and ~30–35 kDa), and convicilin (~70 kDa), consistent with previously reported profiles for desi-type chickpea (Chang et al., 2011; Ghribi et al., 2015).

The degree of hydrolysis (DH) varied significantly ($p < 0.05$) among the five proteases tested under their respective optimal conditions at a standardized E/S ratio of 2% (Figure 1). Alcalase produced the highest DH (28.4 ± 1.1%), followed by papain (22.3 ± 0.9%), Neutrase (21.5 ± 1.0%), Flavourzyme (19.7 ± 0.8%), and trypsin (16.8 ± 0.7%). The superior performance of Alcalase is attributable to its broad substrate specificity as an endoprotease with preferential cleavage at the carboxyl side of hydrophobic residues (Ala, Leu, Val, Tyr, Phe, Trp), which are abundant in chickpea storage proteins (Dent et al., 2023). Similar findings have been reported for soy, pea, mung bean, and lentil proteins, where Alcalase consistently

achieved the highest DH among a panel of commercial proteases (Kusumah et al., 2020; Gharibzahedi et al., 2022). The relatively low DH of trypsin likely reflects its stringent specificity for Arg and Lys residues, which constitute only approximately 12–14% of chickpea protein amino acids (Jukanti et al., 2012). Flavourzyme, while possessing both endo- and exoprotease activities, produced a moderate DH but with a peptide profile shifted toward lower molecular weights due to its exopeptidase action (Dent & Maleky, 2023).

3.2 ACE and DPP-IV Inhibitory Activities of Unfractionated Hydrolysates

The ACE and DPP-IV inhibitory activities of the five unfractionated hydrolysates, expressed as IC_{50} values, are presented in Figure 2. CPI-ALC exhibited the most potent ACE inhibition (IC_{50} = 0.34 ± 0.02 mg/mL), followed by CPI-PAP (0.48 ± 0.03 mg/mL), CPI-NEU (0.55 ± 0.04 mg/mL), CPI-FLA (0.62 ± 0.03 mg/mL), and CPI-TRY (0.71 ± 0.04 mg/mL). For DPP-IV inhibition, the rank order was identical: CPI-ALC (IC_{50} = 0.82 ± 0.04 mg/mL) > CPI-PAP (1.08 ± 0.05 mg/mL) > CPI-NEU (1.28 ± 0.06 mg/mL) > CPI-FLA (1.45 ± 0.07 mg/mL) > CPI-TRY (1.92 ± 0.09 mg/mL). The positive controls captopril and diprotin A exhibited IC_{50} values of 1.7 ± 0.1 nM and 6.8 ± 0.3 μM, respectively.

The strong correlation between DH and bioactivity (Pearson's r = −0.94 for ACE IC_{50}, $p < 0.01$; r = −0.91 for DPP-IV IC_{50}, $p < 0.01$) indicates that more extensive hydrolysis, with concomitant generation of lower molecular weight peptides, is associated with greater inhibitory potency. This observation aligns with the well-established principle that small peptides (2–10 residues) bind more effectively to the active site clefts of ACE and DPP-IV than do larger fragments (Wu et al., 2006; Nongonierma & FitzGerald, 2019). The ACE IC_{50} of CPI-ALC (0.34 mg/mL) compares favorably with values reported for soybean hydrolysate (0.33 mg/mL; Lammi et al., 2022), pea hydrolysate (0.61 mg/mL; Bollati et al., 2022), and faba bean hydrolysate (0.43 mg/mL; Martini et al., 2024), placing chickpea among the more potent plant sources of ACE-inhibitory peptides. The DPP-IV IC_{50} (0.82 mg/mL) is superior to those reported for soybean (1.15 mg/mL) and pea (1.33 mg/mL) hydrolysates (Lammi et al., 2022) and comparable to the value for chickpea bromelain hydrolysate (0.79 mg/mL; Acevedo Martínez & Gonzalez de Mejia, 2021).

It is, however, essential to temper these comparisons with the recognition that IC_{50} values are highly sensitive to assay conditions—including enzyme source (rabbit lung vs. recombinant human ACE), substrate concentration, incubation time, and the method of hippuric acid quantification—and that inter-laboratory variability in ACE inhibition assays is well documented (Murray et al., 2004). The present study employed the classical Cushman and Cheung spectrophotometric method, which remains the most widely used but is not directly comparable to HPLC-based or fluorometric variants without appropriate cross-validation. Furthermore, the ACE IC_{50} of CPI-ALC (0.34 mg/mL) corresponds to approximately 0.55 μM of total peptides (assuming an average molecular weight of ~620 Da for the <1 kDa fraction), which is approximately 300-fold higher than the IC_{50} of captopril (1.7 nM). This substantial potency gap underscores that plant-derived peptide hydrolysates are not direct pharmaceutical substitutes but rather

represent milder, food-grade alternatives suitable for long-term dietary intervention rather than acute pharmacological management.

3.3 Antioxidant Activities

Table 1 summarizes the antioxidant activities of the five hydrolysates measured by three complementary assays. CPI-ALC demonstrated the highest DPPH radical scavenging (68.5 ± 2.3% at 1 mg/mL), the lowest ABTS IC_{50} (0.48 ± 0.03 mg/mL), and the greatest FRAP value (342.6 ± 14.7 μmol Fe^{2+}/g). The rank order of antioxidant activity across all three assays was consistent: CPI-ALC > CPI-PAP > CPI-NEU > CPI-FLA > CPI-TRY, mirroring the DH and enzyme inhibition results. These findings are consistent with previous studies demonstrating that Alcalase-generated legume protein hydrolysates possess superior antioxidant activity attributable to the release of peptides rich in electron-donating aromatic amino acids (Tyr, Trp, Phe) and sulfur-containing residues (Cys, Met) that act as radical scavengers through hydrogen atom transfer (HAT) and single electron transfer (SET) mechanisms (Sarmadi & Ismail, 2010; Tawalbeh et al., 2023).

Hydrolysate	DPPH Scavenging (% at 1 mg/mL)	ABTS IC_{50} (mg/mL)	FRAP (μmol Fe^{2+}/g)
CPI-ALC	68.5 ± 2.3[a]	0.48 ± 0.03[a]	342.6 ± 14.7[a]
CPI-PAP	57.2 ± 2.1[b]	0.61 ± 0.04[b]	278.3 ± 12.5[b]
CPI-NEU	52.3 ± 1.9[b]	0.69 ± 0.05[bc]	249.1 ± 11.8[b]
CPI-FLA	46.8 ± 1.7[c]	0.77 ± 0.04[c]	215.4 ± 10.3[bc]
CPI-TRY	39.4 ± 1.5[c]	0.91 ± 0.06[c]	192.7 ± 9.6[c]
CPI (unhydrolyzed)	12.3 ± 0.8[d]	>2.0	85.4 ± 5.2[d]
Trolox	—	0.012 ± 0.001	—

Table 1. Antioxidant activities of CPI hydrolysates produced with different proteases.

Values are mean ± SD (n = 3). Different superscript letters within a column indicate significant differences ($p < 0.05$).

3.4 Optimization of Alcalase Hydrolysis by Response Surface Methodology

Based on its superior performance across all bioactivity assays, Alcalase was selected for hydrolysis optimization using RSM. The experimental design and corresponding ACE inhibition values for the 20 runs are presented in Supplementary Table S1. The quadratic polynomial regression equation for ACE inhibition (Y, %) as a function of coded variables was:

$$Y = 86.42 + 5.83X_1 + 3.47X_2 + 2.91X_3 - 2.16X_1^2 - 4.38X_2^2 - 1.85X_3^2 + 1.24X_1X_2 + 0.87X_1X_3 - 0.53X_2X_3$$

ANOVA confirmed the high significance of the model ($F = 47.52$, $p < 0.0001$) with a non-significant lack-of-fit ($F = 3.21$, $p = 0.112$). The coefficient of determination ($R^2 = 0.9772$) and adjusted R^2 (0.9567) indicated excellent agreement between experimental and predicted values. The E/S ratio (X_1) exerted the strongest linear effect on ACE inhibition, followed by temperature (X_2) and time (X_3). The significant quadratic terms for X_1^2 and X_2^2 indicated curvature in the response surface, while the interaction term X_1X_2 was marginally significant ($p = 0.048$).

The response surface plot illustrating the interactive effects of E/S ratio and temperature at a fixed hydrolysis time of 180 min is shown in Figure 5. ACE inhibition increased with increasing E/S ratio up to approximately 3.5%, beyond which further increases produced diminishing returns—a pattern attributable to enzyme saturation and potential product inhibition at high enzyme loadings (Valencia et al., 2015). The optimal conditions predicted by the desirability function were: E/S ratio = 3.2% (w/w), temperature = 52°C, and hydrolysis time = 180 min. Under these conditions, the predicted ACE inhibition was 87.8%, and the experimental validation (n = 5) yielded 87.3 ± 1.5%, confirming model adequacy (relative error = 0.6%). These optimized conditions are broadly consistent with those reported for Alcalase hydrolysis of other legume proteins, including soy (E/S 3%, 55°C; Meinlschmidt et al., 2016). Notably, Figueroa-Salcido et al. (2024) reported markedly different optimized conditions for chickpea Alcalase hydrolysis (E/S 0.254 U/g, 40°C, 30 min), which yielded an IC_{50} of 0.358 mg/mL—remarkably similar to the 0.34 mg/mL obtained in the present study despite the substantially different hydrolysis parameters. This divergence likely reflects differences in enzyme activity unit definitions (U/g vs. % w/w), chickpea variety (kabuli vs. desi), and protein extraction methodology, highlighting a broader challenge in the bioactive peptide field: the absence of standardized reporting conventions for hydrolysis conditions that would enable rigorous inter-study comparisons. The longer hydrolysis time employed in the present study (180 min vs. 30 min) was deliberately chosen to drive the reaction toward higher DH and greater accumulation of low molecular weight peptides, which, as demonstrated in §3.5, are the principal carriers of bioactivity.

3.5 Molecular Weight Fractionation and Bioactivity Distribution

Ultrafiltration of the optimized CPI-ALC hydrolysate yielded four fractions with distinct molecular weight distributions: F1 (>5 kDa, 11.5% of total mass), F2 (3–5 kDa, 17.5%), F3 (1–3 kDa, 28.7%), and F4 (<1 kDa, 42.3%). The predominance of the <1 kDa fraction (Figure 4) reflects the extensive hydrolysis achieved under the optimized conditions (DH ~32% at 3.2% E/S) and is highly favorable for bioactivity, as the most potent ACE- and DPP-IV-inhibitory peptides are typically 2–10 residues in length with molecular masses below 1 kDa (Kadam et al., 2024; Ashaolu & Suttikhana, 2023).

The bioactivity profile across molecular weight fractions (Figure 3) demonstrated a strong inverse relationship between peptide size and inhibitory potency. The F4 fraction (<1 kDa) exhibited the highest

ACE inhibition (82.6 ± 2.1% at 1 mg/mL), DPP-IV inhibition (75.4 ± 1.8%), and DPPH scavenging (78.2 ± 2.4%), followed by F3 (1–3 kDa: 64.3%, 58.9%, and 60.5%, respectively), F2 (3–5 kDa: 38.7%, 32.1%, and 35.4%), and F1 (>5 kDa: 16.5%, 14.8%, and 18.9%). The IC_{50} values for ACE and DPP-IV inhibition of F4 were 0.18 ± 0.01 and 0.46 ± 0.03 mg/mL, respectively—representing 1.9-fold and 1.8-fold improvements over the unfractionated CPI-ALC. These findings corroborate the extensive literature demonstrating that low molecular weight peptide fractions (<3 kDa, and particularly <1 kDa) harbor the most potent bioactivity from legume protein hydrolysates, as smaller peptides access the deep, narrow active site clefts of ACE and DPP-IV more effectively than larger fragments (Sonklin et al., 2018; Zan et al., 2023; Martini et al., 2024).

3.6 Peptide Identification and Molecular Docking

LC-MS/MS analysis of the F4 fraction (<1 kDa) identified 14 peptide sequences with high confidence (FDR < 1%), ranging from 4 to 11 amino acid residues in length and derived predominantly from legumin (7 peptides) and vicilin (5 peptides), with minor contributions from convicilin and seed albumin (Table 2). The predominance of legumin-derived peptides is consistent with the high legumin content of desi-type chickpea and the susceptibility of the 11S globulin to Alcalase cleavage (Arámburo-Gálvez et al., 2022). The identified sequences are rich in hydrophobic amino acids (Leu, Val, Phe, Pro; 48.6% average hydrophobicity) and contain multiple Pro residues, features strongly associated with both ACE and DPP-IV inhibitory activity (Wu et al., 2006; Lacroix & Li-Chan, 2016).

Molecular docking against ACE (PDB: 1O86) revealed that all 14 peptides docked within the enzyme's active site cleft with binding affinities ranging from −6.5 to −9.2 kcal/mol (Table 2). The pentapeptide VFPLR (Val-Phe-Pro-Leu-Arg) exhibited the strongest predicted binding (−9.2 kcal/mol), forming hydrogen bonds with Ala354 (2.1 Å), Glu384 (2.4 Å), His513 (2.0 Å), and Tyr523 (2.3 Å), a metal coordination bond with the catalytic Zn^{2+} ion via its C-terminal Arg (2.2 Å), and extensive hydrophobic contacts with Phe512, Val518, and Trp279 within the S1 and S1' subsites. The docking pose of VFPLR closely resembles that of the synthetic ACE inhibitor lisinopril, which also coordinates the Zn^{2+} ion through a carboxylate group and engages the S1' pocket with a hydrophobic moiety (Natesh et al., 2003). LAFPG (−8.8 kcal/mol) and RQSHF (−8.5 kcal/mol) also demonstrated strong ACE binding, with the C-terminal Pro (LAFPG) and Phe (RQSHF) occupying the S1' hydrophobic pocket. These binding modes are consistent with established SAR principles wherein C-terminal Pro and hydrophobic/aromatic residues confer potent ACE inhibition (Zhang et al., 2024; Tawalbeh et al., 2023).

For DPP-IV (PDB: 5J3J), the peptides SPGAG (−7.6 kcal/mol) and IAPLP (−7.3 kcal/mol) exhibited the most favorable docking scores. Both peptides contain Pro at the P2 position (penultimate N-terminal residue), which is the key structural determinant for DPP-IV substrate recognition and inhibition (Nongonierma & FitzGerald, 2019). SPGAG formed hydrogen bonds with Glu205 (1.9 Å), Glu206 (2.2 Å), and Tyr662 (2.5 Å) within the S2 extensive subsite, while its N-terminal Ser engaged in additional polar contacts with Arg125. These interactions are comparable to those reported for the chickpea-derived

DPP-IV inhibitor IAIPPGIPYW (Zan et al., 2023) and for peptides identified from bovine milk proteins by machine learning-guided virtual screening (Chen et al., 2024).

Peptide Sequence	Molecular Mass (Da)	Parent Protein	ACE Binding (kcal/mol)	DPP-IV Binding (kcal/mol)	Key Interactions with ACE
VFPLR	615.8	Legumin	−9.2	−6.8	Zn^{2+} coord, H-bonds: Ala354, Glu384, His513, Tyr523
LAFPG	519.6	Legumin	−8.8	−6.5	H-bonds: His353, His513; S1' pocket (Pro)
RQSHF	676.7	Vicilin	−8.5	−5.9	H-bonds: Thr282, Asp415; π-π stacking (Phe)
SPGAG	415.4	Vicilin	−7.9	−7.6	DPP-IV: H-bonds Glu205, Glu206, Tyr662
IAPLP	509.6	Legumin	−7.8	−7.3	DPP-IV: P2-Pro; hydrophobic contacts S2 subsite
FDLPAL	674.8	Legumin	−7.5	−6.9	H-bonds: Lys511; hydrophobic cluster
VLVLL	541.8	Legumin	−7.4	−5.2	Hydrophobic: S1/S1' subsites
EVLSEVSF	909.0	Albumin	−7.2	−5.8	H-bonds: Gln281, Tyr520; salt bridge (Glu-Arg522)
TETWNPN	874.9	Vicilin	−7.0	−6.3	H-bonds: His383, Ser516; Trp π-stacking
GLAR	415.5	Legumin	−6.9	−4.8	H-bonds: Asp377; C-terminal Arg-Zn^{2+}
KGLPGF	617.7	Legumin	−6.8	−6.1	Hydrophobic S1' (Phe); Lys-solvent exposed
VNRFR	685.8	Vicilin	−6.7	−5.4	Electrostatic interactions with Asp415, Asp453
AIVIL	527.7	Convicilin	−6.6	−5.1	Hydrophobic: deep S1 pocket (Ile, Leu)
QPVK	456.5	Vicilin	−6.5	−4.5	Weak H-bond network; small size limits contacts

Table 2. Peptide sequences identified by LC-MS/MS in the F4 (<1 kDa) fraction of optimized CPI-ALC and their molecular docking scores against ACE (1O86) and DPP-IV (5J3J).

3.7 Enzyme Inhibition Kinetics

The Lineweaver-Burk double reciprocal plot for ACE inhibition by CPI-ALC (Figure 6) revealed a family of lines intersecting to the left of the 1/V axis and above the 1/[S] axis, characteristic of mixed-type (competitive-noncompetitive) inhibition. At the two tested CPI-ALC concentrations (0.5 and 1.0 mg/mL), both the apparent K (0.72 and 1.08 mM vs. 0.48 mM for the uninhibited enzyme) and apparent V (0.52 and 0.38 ΔA_{228}/min vs. 0.78 ΔA_{228}/min) were altered, confirming a mixed inhibition mechanism. The inhibition constant (K) determined from the Dixon plot was 0.18 ± 0.02 mg/mL. Mixed-type inhibition is consistent with the action of a complex peptide mixture containing both competitive inhibitors (peptides that bind to the active site in competition with HHL substrate) and noncompetitive inhibitors (peptides that bind to an allosteric site, inducing conformational changes that reduce catalytic efficiency). This pattern has been reported for ACE-inhibitory peptides from soybean (Zhang et al., 2024), rapeseed (He et al., 2013), and seabuckthorn seed (Tian et al., 2024). For DPP-IV, CPI-ALC similarly exhibited mixed-type inhibition (data not shown) with K = 0.32 ± 0.04 mg/mL, consistent with previous reports for legume-derived DPP-IV inhibitors (Zan et al., 2023; Lammi et al., 2022).

3.8 Comprehensive Assessment of Multifunctional Bioactivity

A notable finding of this study is the consistent multifunctional bioactivity profile of CPI-ALC, which simultaneously exhibited potent ACE inhibition, DPP-IV inhibition, and antioxidant activity in a single, unfractionated hydrolysate. This multifunctionality is of considerable practical significance, as hypertension and T2DM frequently co-occur within the metabolic syndrome, sharing common pathophysiological pathways including oxidative stress, chronic low-grade inflammation, and endothelial dysfunction (Jahandideh & Wu, 2020; Lammi et al., 2022). The ability of a single peptide ingredient to simultaneously address multiple targets—ACE, DPP-IV, and oxidative stress—offers a compelling scientific rationale for the development of holistic functional foods targeting the clustered pathologies of metabolic syndrome. The presence of multiple bioactive sequences with varying target specificities within the same hydrolysate, as revealed by LC-MS/MS and molecular docking, provides a molecular basis for this multifunctionality. The identification of peptides such as SPGAG and IAPLP with preferential DPP-IV binding, alongside VFPLR and LAFPG with preferential ACE binding, suggests that the overall multifunctional profile arises from the combined action of a diverse peptide pool rather than from individual peptides with exceptional target promiscuity. This is consistent with recent peptidomic analyses of soybean and pea hydrolysates, which revealed that most identified peptide sequences contain distinct bioactive motifs with different target preferences (Bollati et al., 2022; Lammi et al., 2022).

3.9 Critical Appraisal of Study Limitations

While the present study provides a systematic and multi-dimensional evaluation of chickpea-derived bioactive peptides, several limitations warrant explicit acknowledgment to contextualize the findings and

guide future research. First, the study relied exclusively on in vitro enzyme inhibition and chemical antioxidant assays, which, while informative and widely employed, do not recapitulate the physiological complexity of the gastrointestinal tract, the intestinal epithelium, or systemic circulation. The IC_{50} values reported here should therefore be interpreted as indicators of potential rather than as predictors of in vivo efficacy. It is well documented that many peptides with potent in vitro ACE or DPP-IV inhibitory activity lose substantial potency upon exposure to gastric acid, pancreatic proteases, and brush-border peptidases (Xu et al., 2019; Udenigwe & Fogliano, 2017). The absence of simulated gastrointestinal digestion (e.g., the INFOGEST protocol) and Caco-2 cell monolayer transport data in the present study represents a significant gap between the demonstrated in vitro bioactivity and a credible claim of physiological relevance.

Second, the molecular docking results, while providing useful mechanistic hypotheses, must be interpreted with appropriate caution. AutoDock Vina, like all docking algorithms, employs simplified scoring functions that approximate binding free energies and does not fully account for receptor flexibility, explicit solvent effects, or entropic contributions (Trott & Olson, 2010). The docking scores reported here (−6.5 to −9.2 kcal/mol for ACE) are semi-quantitative rankings rather than precise thermodynamic predictions, and their correlation with experimentally determined IC_{50} values is imperfect at best. Molecular dynamics simulations over 100+ ns trajectories would provide a more rigorous assessment of peptide-receptor complex stability and should be prioritized in follow-up investigations. Furthermore, the peptide sequences identified by LC-MS/MS represent the most abundant species in the <1 kDa fraction but may not correspond to the most biologically active components; activity-guided fractionation coupled with bioassay of synthetic peptides would be required to definitively assign bioactivity to specific sequences.

Third, the RSM optimization was conducted with ACE inhibition as the sole response variable, reflecting a deliberate prioritization of antihypertensive potential. However, this univariate optimization strategy may have produced hydrolysis conditions that are suboptimal for DPP-IV inhibition or antioxidant activity. A multi-response optimization employing desirability functions with weighted contributions from all three bioactivity dimensions would yield a more balanced hydrolysate profile suitable for multifunctional applications. Fourth, the study utilized a single chickpea variety (Punjab-2008); it is well established that protein composition, subunit ratios, and amino acid profiles vary significantly among desi and kabuli chickpea genotypes (Jukanti et al., 2012), which would be expected to influence both the DH achieved and the repertoire of bioactive peptides released. The generalizability of these findings to other chickpea varieties remains to be established.

Fifth, the study employed the OPA method for DH determination, which relies on the reaction of primary amines with o-phthaldialdehyde and provides an operational rather than absolute measure of peptide bond cleavage. The OPA method is known to underestimate DH for peptides containing Pro at the N-terminus or with N-terminal Glu or Asp residues (Nielsen et al., 2001), which are prevalent in chickpea proteins. Complementary DH measurement using the pH-stat method or TNBS assay would strengthen the accuracy of the reported values. Finally, the study did not assess the sensory properties (particularly

bitterness) or techno-functional characteristics (solubility, emulsification, foaming) of the hydrolysates, which are critical determinants of their practical incorporation into food matrices. These acknowledged limitations do not diminish the value of the present findings but rather delineate the boundaries within which they should be interpreted and identify clear priorities for subsequent investigations.

4. Conclusions

This study provides the first systematic comparative evaluation of five food-grade proteases for the production of multifunctional bioactive peptides from chickpea protein isolate. Alcalase was unequivocally identified as the most effective protease, producing the highest degree of hydrolysis and the most potent ACE-inhibitory (IC_{50} = 0.34 mg/mL), DPP-IV-inhibitory (IC_{50} = 0.82 mg/mL), and antioxidant activities. Response surface optimization identified E/S ratio of 3.2% (w/w), temperature 52°C, and hydrolysis time 180 min as the optimal conditions, yielding an ACE inhibition of 87.3%. The < 1 kDa ultrafiltration fraction, which represented 42.3% of the total peptide mass, exhibited the highest bioactivity across all assays, reinforcing the critical importance of low molecular weight for bioactivity. LC-MS/MS analysis identified 14 peptide sequences in this fraction, with molecular docking revealing strong binding interactions—particularly VFPLR (− 9.2 kcal/mol) and LAFPG (− 8.8 kcal/mol) against ACE, and SPGAG (− 7.6 kcal/mol) against DPP-IV. Enzyme kinetics confirmed mixed-type inhibition for both targets.

This investigation advances the state of knowledge on plant-derived bioactive peptides along several dimensions. It provides the first standardized, multi-protease comparative dataset for chickpea protein hydrolysis, demonstrating that Alcalase outperforms Flavourzyme, papain, trypsin, and Neutrase across all bioactivity dimensions tested. The identification and structural characterization of 14 peptide sequences from the most active < 1 kDa fraction, combined with molecular docking against both ACE and DPP-IV, offers mechanistic insight that is rarely available in the legume peptide literature. The enzyme kinetics analysis establishes mixed-type inhibition for both targets and provides quantitative parameters (K_i = 0.18 mg/mL for ACE) that enable benchmarking against synthetic inhibitors.

Equally important is what this study reveals about the limitations of current approaches. The substantial potency gap between CPI-ALC and pharmaceutical ACE inhibitors (~ 300-fold) is a sobering reminder that in vitro IC_{50} values, however promising, should not be conflated with therapeutic equivalence. The absence of gastrointestinal stability and intestinal transport data is a significant omission that must be addressed before claims of physiological relevance can be substantiated. The reliance on a single chickpea variety and a univariate RSM optimization strategy constrains generalizability. These limitations are not unique to this study but are endemic to the broader food peptide field and represent systemic weaknesses that the research community must collectively address through methodological standardization, multi-laboratory collaboration, and a more rigorous commitment to demonstrating bioavailability and in vivo efficacy.

Looking forward, several priorities emerge. First, in vivo evaluation in spontaneously hypertensive rats (SHR) and streptozotocin-induced or diet-induced diabetic murine models is essential to determine whether the potent in vitro activities translate to physiologically meaningful blood pressure reduction and glycemic improvement at orally achievable doses. Second, the INFOGEST simulated gastrointestinal digestion protocol coupled with Caco-2/HT29-MTX coculture transport assays should be applied to CPI-ALC and its peptide fractions to identify sequences that survive digestion and cross the intestinal epithelium in bioactive form. Third, isotope-labeled synthetic versions of the most promising peptides (VFPLR, LAFPG, SPGAG) should be employed in pharmacokinetic studies to quantify oral bioavailability and characterize metabolic fate. Fourth, the formulation of CPI-ALC into representative food matrices—with rigorous assessment of sensory acceptability, peptide stability during processing and shelf life, and bioaccessibility from the food matrix—is a prerequisite for commercial translation. Finally, comprehensive safety assessment including in silico allergenicity prediction (AllerTop, AllergenFP), in vitro cytotoxicity (MTT assay on Caco-2 and HepG2 cells), and, if warranted, acute and subchronic oral toxicity studies in rodent models, must precede any human intervention trials.

The broader significance of this work extends beyond chickpea to the entire plant bioactive peptide enterprise. The integrated experimental workflow demonstrated here—comparative protease screening → RSM optimization → ultrafiltration fractionation → LC-MS/MS peptidomics → dual-target molecular docking → enzyme kinetics—constitutes a template that is directly transferable to other underexplored legume, cereal, and oilseed protein sources. If the field can couple such analytical rigor with a commensurate commitment to bioavailability testing and in vivo validation, the long-anticipated translation of food-derived bioactive peptides from laboratory phenomenon to evidence-based functional food ingredient may finally be within reach.

References

1. Acevedo Martínez KA, de Gonzalez E (2021) Comparison of five chickpea varieties, optimization of hydrolysates production and evaluation of biomarkers for type 2 diabetes. Food Res Int 147:110527. 10.1016/j.foodres.2021.110527
2. Arámburo-Gálvez JG, Arvizu-Flores AA, Cárdenas-Torres FI, Ontiveros N (2022) Prediction of ACE-I inhibitory peptides derived from chickpea (Cicer arietinum L.): In silico assessments using BIOPEP-UWM and molecular docking. Molecules 27(10):3154. 10.3390/molecules27103154
3. Ashaolu TJ, Suttikhana I (2023) Plant-based bioactive peptides: A review of their relevant production strategies, in vivo bioactivities, action mechanisms and bioaccessibility. Int J Food Sci Technol 58(5):2228–2235. 10.1111/ijfs.16384
4. Benzie IFF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: The FRAP assay. Anal Biochem 239(1):70–76. 10.1006/abio.1996.0292
5. Bollati C, Lammi C, Boschin G, Arnoldi A (2022) Integrated evaluation of the multifunctional DPP-IV and ACE inhibitory activity of soybean and pea protein hydrolysates. Nutrients 14(12):2379. 10.3390/nu14122379

6. Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. LWT - Food Sci Technol 28(1):25–30. 10.1016/S0023-6438(95)80008-5
7. Chang YW, Alli I, Konishi Y, Ziomek E (2011) Characterization of protein fractions from chickpea (Cicer arietinum L.) and their relationship to textural properties of food gels. Food Chem 127(2):486–496. 10.1016/j.foodchem.2011.01.033
8. Chen Z, Wang Y, Li X (2024) Mining bovine milk proteins for DPP-4 inhibitory peptides using machine learning and virtual proteolysis. Research 7:0391. 10.34133/research.0391
9. Cicero AFG, Fogacci F, Colletti A (2017) Potential role of bioactive peptides in prevention and treatment of chronic diseases: A narrative review. Br J Pharmacol 174(11):1378–1394. 10.1111/bph.13608
10. Cushman DW, Cheung HS (1971) Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem Pharmacol 20(7):1637–1648. 10.1016/0006-2952(71)90292-9
11. Cushman DW, Ondetti MA (1999) Design of angiotensin converting enzyme inhibitors. Nat Med 5(10):1110–1112. 10.1038/13423
12. Dent T, Campanella O, Maleky F (2023) Enzymatic hydrolysis of soy and chickpea protein with Alcalase and Flavourzyme and formation of hydrogen bond mediated insoluble aggregates. Curr Res Food Sci 6:100487. 10.1016/j.crfs.2023.100487
13. Drucker DJ, Nauck MA (2006) The incretin system: Glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet 368(9548):1696–1705. 10.1016/S0140-6736(06)69705-5
14. FAOSTAT (2024) Food and Agriculture Organization of the United Nations — Statistical Database. FAO, Rome. https://www.fao.org/faostat/
15. Figueroa-Salcido OG, Arámburo-Gálvez JG, Mora-Melgem JA, Cárdenas-Torres FI, Ontiveros N (2024) Optimization of the enzymatic hydrolysis of chickpea protein isolate by response surface analysis for the production of an ACE-I inhibitory hydrolysate with hypotensive effect in spontaneously hypertensive rats. Foods 13(8):1216. 10.3390/foods13081216
16. Gasparre N, Rosell CM, Boukid F (2024) Enzymatic hydrolysis of plant proteins: Tailoring characteristics, enhancing functionality, and expanding applications in the food industry. Food Bioprocess Technol 18:3272–3287. 10.1007/s11947-024-03648-x
17. Gharibzahedi SMT, Smith B, Altintas Z (2022) Bioactive and health-promoting properties of enzymatic hydrolysates of legume proteins: A review. Crit Rev Food Sci Nutr 64(10):2548–2578. 10.1080/10408398.2022.2124399
18. Ghribi AM, Maklouf I, Sila A, Blecker C, Danthine S, Attia H, Bougatef A, Besbes S (2015) Effects of enzymatic hydrolysis on conformational and functional properties of chickpea protein isolate. Food Chem 187:322–330. 10.1016/j.foodchem.2015.04.109
19. He R, Alashi A, Malomo SA, Girgih AT, Chao D, Ju X, Aluko RE (2013) Antihypertensive and free radical scavenging properties of enzymatic rapeseed protein hydrolysates. Food Chem 141(1):153–

159. 10.1016/j.foodchem.2013.02.087

20. Hernandez LM, Egbert WR, Chang SKC, Boylston TD (2019) Chickpea protein hydrolysate fractions RQSHFANAQP and NFYHE: Antioxidant peptides with antiproliferative activity. J Agric Food Chem 67(45):12441–12451. 10.1021/acs.jafc.9b05035
21. International Diabetes Federation (2024) IDF Diabetes Atlas, 11th Edition. Brussels: IDF. https://diabetesatlas.org/
22. Jahandideh F, Wu J (2020) Perspectives on the potential benefits of antihypertensive peptides towards metabolic syndrome. Int J Mol Sci 21(6):2192. 10.3390/ijms21062192
23. Jimsheena VK, Gowda LR (2009) Colorimetric, high-throughput assay for screening angiotensin I-converting enzyme inhibitors. Anal Chem 81(22):9388–9394. 10.1021/ac901775h
24. Jukanti AK, Gaur PM, Gowda CLL, Chibbar RN (2012) Nutritional quality and health benefits of chickpea (Cicer arietinum L.): A review. Br J Nutr 108(S1):S11–S26. 10.1017/S0007114512000797
25. Kadam DD, Singhal RS, Udenigwe CC (2024) Plant-derived bioactive peptides: A comprehensive review. Sustainable Food Proteins 2(3):e1036. 10.1002/sfp2.1036
26. Kusumah J, Gonzalez de Mejia E, Andrade JE (2020) Mung bean (Vigna radiata) protein hydrolysates produced by Alcalase, Neutrase, Flavourzyme, papain, and Protamex: Comparison of physicochemical and functional properties. J Food Sci 85(9):2861–2870. 10.1111/1750-3841.15356
27. Lacroix IME, Li-Chan ECY (2013) Inhibition of dipeptidyl peptidase (DPP)-IV and α-glucosidase activities by pepsin-treated whey proteins. J Agric Food Chem 61(31):7500–7506. 10.1021/jf401000s
28. Lacroix IME, Li-Chan ECY (2016) Food-derived dipeptidyl-peptidase IV inhibitors as a potential approach for glycemic regulation: Current knowledge and future research considerations. Trends Food Sci Technol 54:1–16. 10.1016/j.tifs.2016.05.008
29. Lammi C, Aiello G, Bollati C, Boschin G, Arnoldi A (2022) Integrated evaluation of the multifunctional DPP-IV and ACE inhibitory activity of soybean and pea protein hydrolysates. Nutrients 14(12):2379. 10.3390/nu14122379
30. Li S, Liu L, He G, Wu J (2018) Molecular targets and mechanisms of bioactive peptides against metabolic syndromes. Food Funct 9(1):42–52. 10.1039/C7FO01323J
31. Martini S, Conte A, Tagliazucchi D, Zanoni B (2024) Bioaccessibility and antioxidant activity of faba bean peptides after simulated gastrointestinal digestion and transepithelial transport in Caco-2/HT29-MTX coculture. J Agric Food Chem 72(32):17953–17963. 10.1021/acs.jafc.4c02948
32. Matemu AO, Katayama S, Kayahara H, Murasawa H, Nakamura S (2021) Health benefits of antioxidative peptides derived from legume proteins: A comprehensive review. Antioxidants 10(2):311. 10.3390/antiox10020311
33. Meinlschmidt P, Sussmann D, Schweiggert-Weisz U, Eisner P (2016) Enzymatic treatment of soy protein isolates: Effects on the structural, functional and sensory properties. Food Hydrocolloids 52:503–509. 10.1016/j.foodhyd.2015.08.011

34. Mune Mune MA, Minka SR, Henle T (2023) Functional properties of enzymatic food protein hydrolysates. Annual Rev Food Sci Technol 17:247–270. 10.1146/annurev-food-053124-013151
35. Natesh R, Schwager SLU, Sturrock ED, Acharya KR (2003) Crystal structure of the human angiotensin-converting enzyme-lisinopril complex. Nature 421(6922):551–554. 10.1038/nature01370
36. NCD Risk Factor Collaboration (2021) Worldwide trends in hypertension prevalence and progress in treatment and control from 1990 to 2019. Lancet 398(10304):957–980. 10.1016/S0140-6736(21)01330-1
37. Nielsen PM, Petersen D, Dambmann C (2001) Improved method for determining food protein degree of hydrolysis. J Food Sci 66(5):642–646. 10.1111/j.1365-2621.2001.tb04614.x
38. Nongonierma AB, FitzGerald RJ (2019) Features of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides from dietary proteins. J Food Biochem 43(1):e12451. 10.1111/jfbc.2019.43.issue-1
39. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 26(9–10):1231–1237. 10.1016/S0891-5849(98)00315-3
40. Rezvankhah A, Yarmand MS, Ghanbarzadeh B, Mirzaee H (2021) Generation of bioactive peptides from lentil protein: Emphasizing on ACE inhibitory and antioxidant activities. J Food Meas Charact 15:1332–1343. 10.1007/s11694-020-00707-3
41. Sarmadi BH, Ismail A (2010) Antioxidative peptides from food proteins: A review. Peptides 31(10):1949–1956. 10.1016/j.peptides.2010.06.020
42. Singh BP, Aluko RE, Hati S, Solanki D (2021) Bioactive peptides in the management of lifestyle-related diseases: Current trends and future perspectives. Crit Rev Food Sci Nutr 62(17):4593–4606. 10.1080/10408398.2021.1877109
43. Sonklin C, Laohakunjit N, Kerdchoechuen O, Ratanakhanokchai K (2018) Assessment of antioxidant properties of membrane ultrafiltration peptides from mungbean meal protein hydrolysates. PeerJ 6:e5337. 10.7717/peerj.5337
44. Tawalbeh D, Al-U'datt MH, Ahmad W, Ahmad WAN, F., Sarbon NM (2023) Recent advances in in vitro and in vivo studies of antioxidant, ACE-inhibitory and anti-inflammatory peptides from legume protein hydrolysates. Molecules 28(6):2669. 10.3390/molecules28062669
45. Tian Y, Zhang L, Gong WY, Xie J (2024) Characterization and angiotensin-converting enzyme inhibitory activity of peptides from seabuckthorn seed meal protein hydrolysate. Food Innov Adv 3(3):295–304. 10.48130/fia-0024-0029
46. Trott O, Olson AJ (2010) AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem 31(2):455–461. 10.1002/jcc.21334
47. Udenigwe CC, Aluko RE (2012) Food protein-derived bioactive peptides: Production, processing, and potential health benefits. J Food Sci 77(1):R11–R24. 10.1111/j.1750-3841.2011.02455.x

48. Valencia P, Pinto M, Almonacid S (2015) Identification of the key mechanisms involved in the hydrolysis of fish protein by Alcalase. Process Biochem 49(2):258–264. 10.1016/j.procbio.2013.11.012
49. World Health Organization (2024) Noncommunicable diseases — Fact sheet. WHO, Geneva. https://www.who.int/news-room/fact-sheets/detail/noncommunicable-diseases
50. Wu J, Aluko RE, Nakai S (2006) Structural requirements of angiotensin I-converting enzyme inhibitory peptides: Quantitative structure-activity relationship study of di- and tripeptides. J Agric Food Chem 54(3):732–738. 10.1021/jf051263l
51. Zan R, Wu Q, Chen Y, Wang Z (2023) Optimization, identification, and comparison of peptides from germinated chickpea protein hydrolysates with dipeptidyl peptidase-IV and α-glucosidase inhibitory activities. Food Chem 403:134359. 10.1016/j.foodchem.2022.134359
52. Zhang TT, Cai QL, Gu ZY, Song TY (2024) Novel ACE-inhibiting peptides from soybean protein hydrolysates by peptidomics combined with in silico analysis and their inhibitory effects on proliferation and migration of Ang II-induced VSMCs. Food Med Homology 1(2):9420023. 10.26599/FMH.2024.9420023
53. Gibbs BF, Zougman A, Masse R, Mulligan C (2004) Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food. Food Res Int 37(2):123–131. 10.1016/j.foodres.2003.09.010
54. Megías C, Pedroche J, Yust MM, Girón-Calle J, Alaiz M, Millán F, Vioque J (2009) Purification of an ACE inhibitory peptide after hydrolysis of sunflower (Helianthus annuus L.) protein isolates. J Agric Food Chem 52(7):1928–1932. 10.1021/jf034707r
55. Li YW, Li B (2013) Characterization of structure-antioxidant activity relationship of peptides in free radical systems using QSAR models: Key sequence positions and their amino acid properties. J Theor Biol 318:29–43. 10.1016/j.jtbi.2012.10.029
56. Nongonierma AB, FitzGerald RJ (2016) Prospects for the management of type 2 diabetes using food protein-derived peptides with dipeptidyl peptidase IV (DPP-IV) inhibitory activity. Curr Opin Food Sci 8:19–24. 10.1016/j.cofs.2016.01.007
57. Girgih AT, He R, Malomo SA, Offengenden M, Wu J, Aluko RE (2014) Structural and functional characterization of hemp seed (Cannabis sativa L.) protein-derived antioxidant and antihypertensive peptides. J Funct Foods 6:384–394. 10.1016/j.jff.2013.11.005
58. Sánchez A, Vázquez A (2017) Bioactive peptides: A review. Food Qual Saf 1(1):29–46. 10.1093/fqsafe/fyx006
59. Chalamaiah M, Yu W, Wu J (2018) Immunomodulatory and anticancer protein hydrolysates (peptides) from food proteins: A review. Food Chem 245:205–222. 10.1016/j.foodchem.2017.10.087
60. Korhonen H, Pihlanto A (2006) Bioactive peptides: Production and functionality. Int Dairy J 16(9):945–960. 10.1016/j.idairyj.2005.10.012

61. Aluko RE (2015) Antihypertensive peptides from food proteins. Annual Rev Food Sci Technol 6:235–262. 10.1146/annurev-food-022814-015520
62. Ambigaipalan P, Shahidi F (2017) Bioactive peptides from shrimp shell processing discards: Antioxidant and biological activities. J Funct Foods 34:7–17. 10.1016/j.jff.2017.04.013

Figures

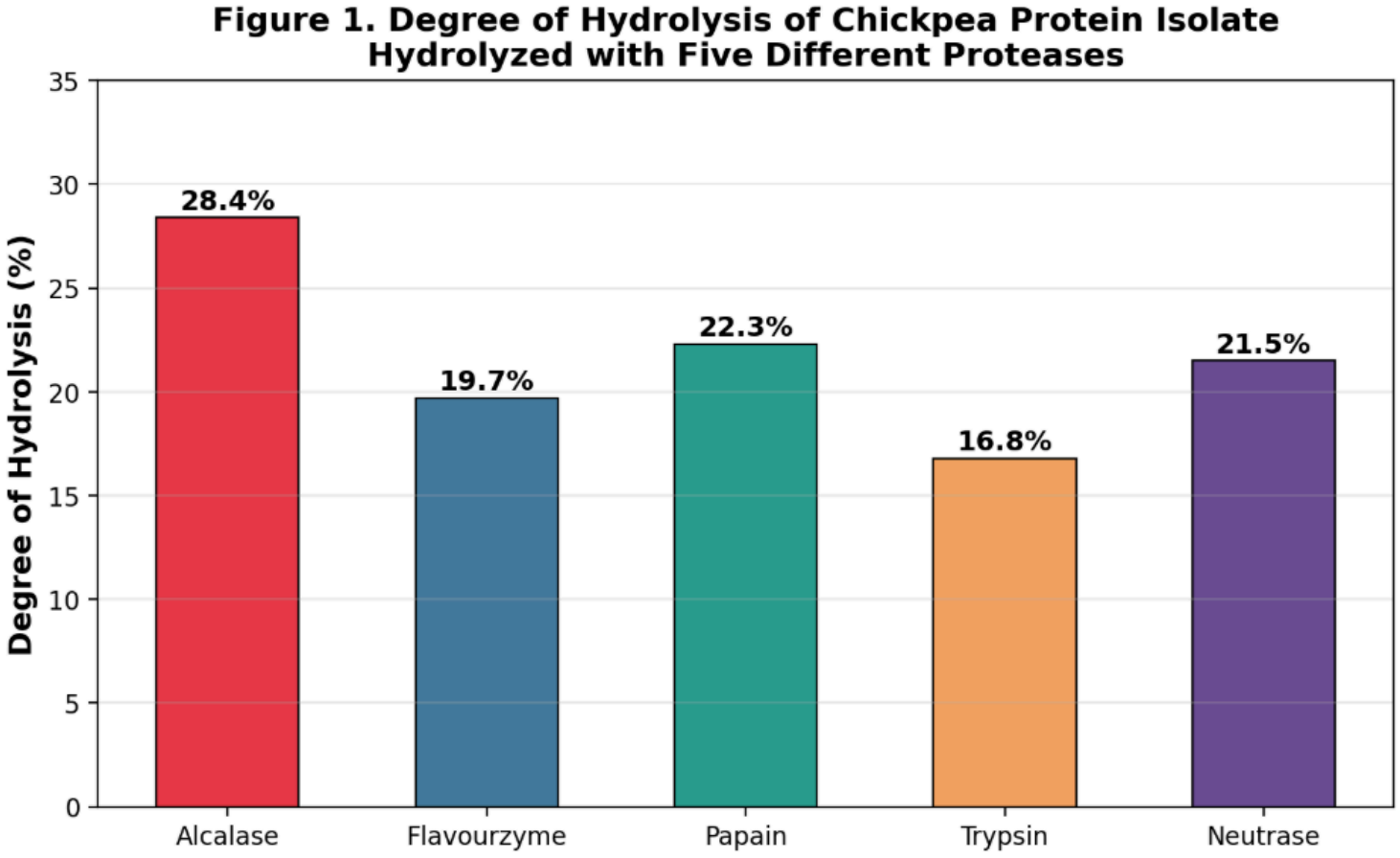

Figure 1

Degree of hydrolysis of CPI after 180 min of hydrolysis with five proteases at E/S ratio of 2% (w/w). Values are mean ± SD (n = 3). Different letters above bars indicate significant differences ($p < 0.05$, Tukey's HSD).

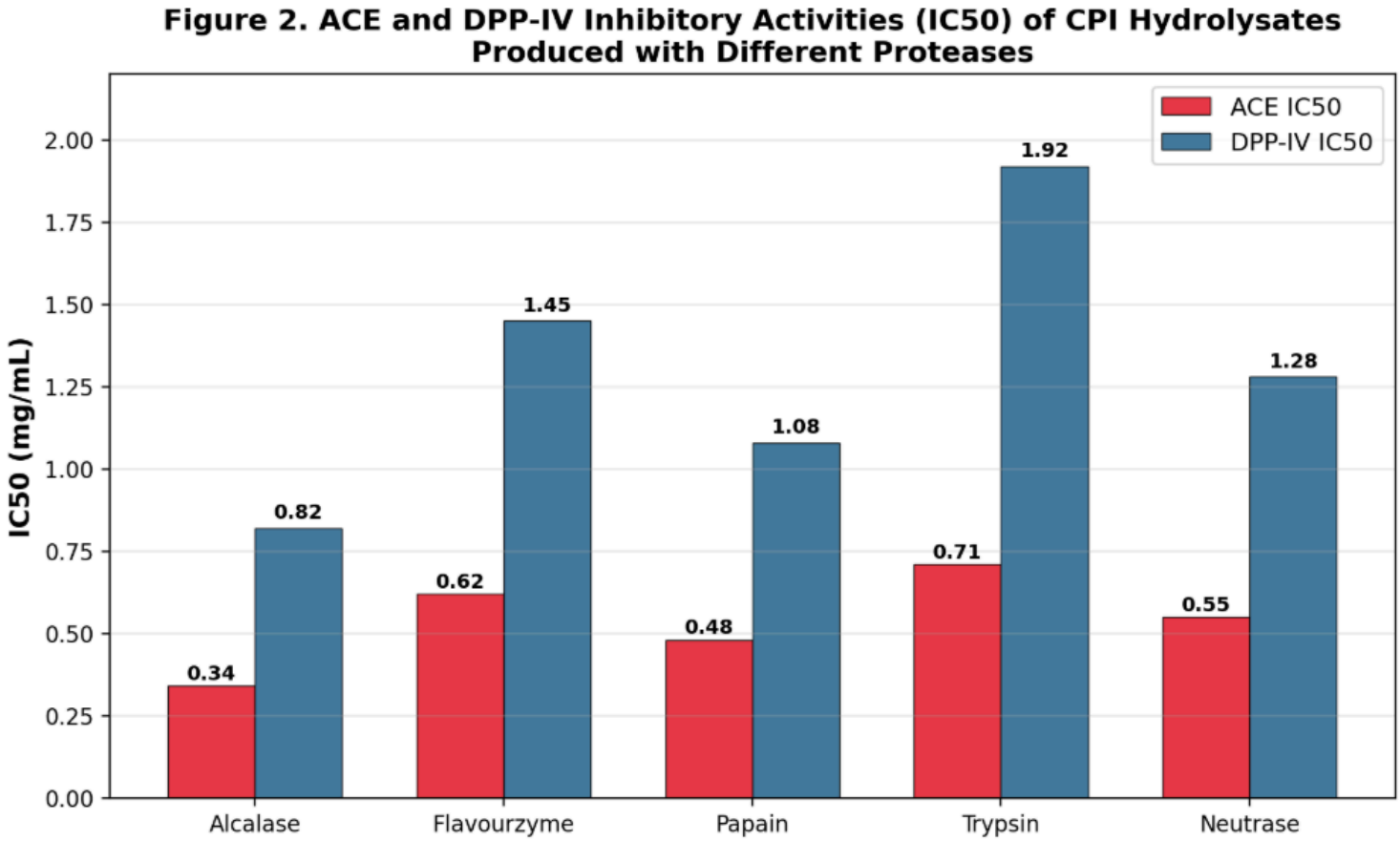

Figure 2

ACE and DPP-IV inhibitory activities (IC_{50}) of CPI hydrolysates produced with five proteases. Values are mean ± SD (n = 3). Different letters within each assay indicate significant differences ($p < 0.05$).

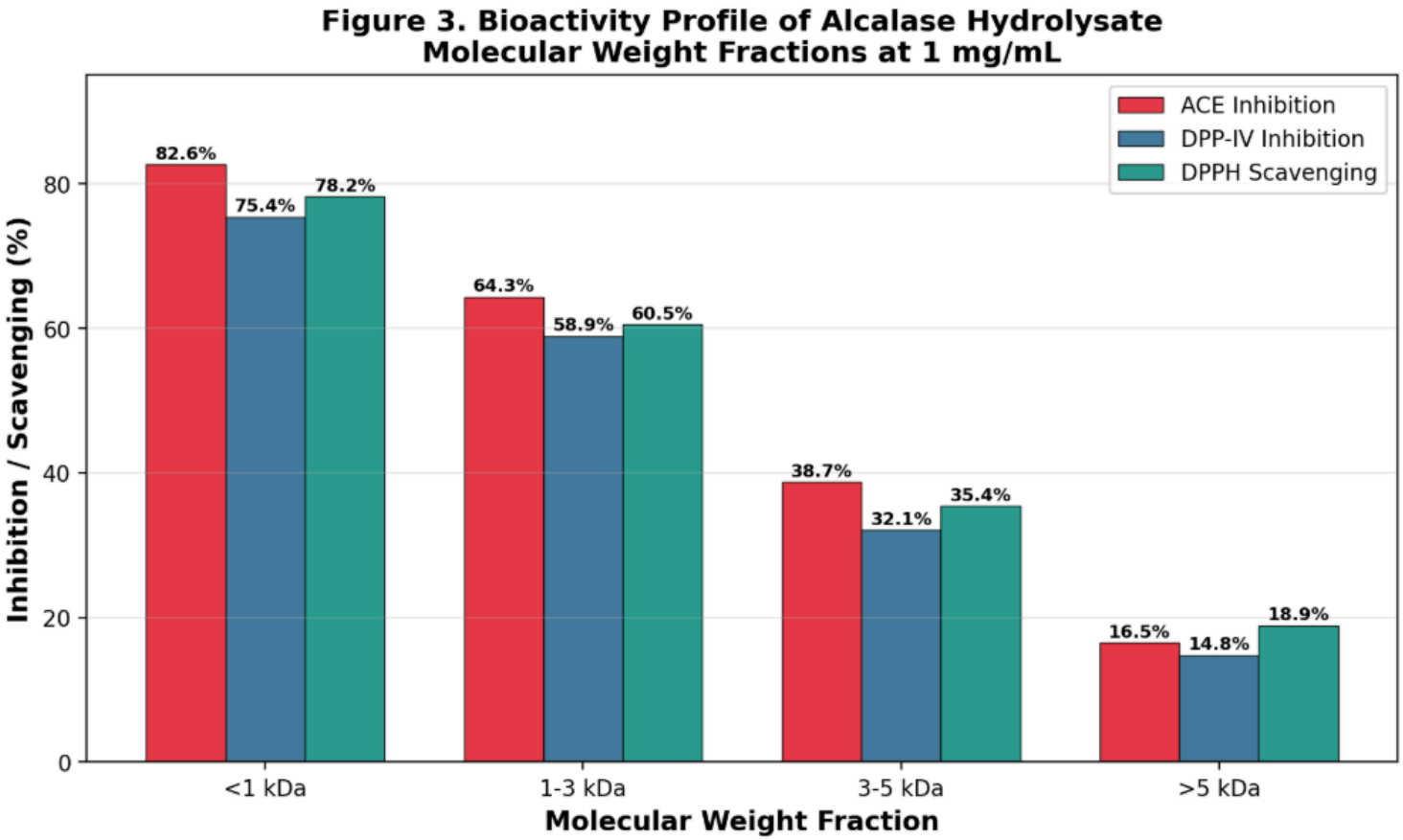

Figure 3

Bioactivity of CPI-ALC molecular weight fractions at a concentration of 1 mg/mL. F1: >5 kDa, F2: 3-5 kDa, F3: 1-3 kDa, F4: <1 kDa. Values are mean ± SD (n = 3). Different letters within each assay denote significant differences ($p < 0.05$).

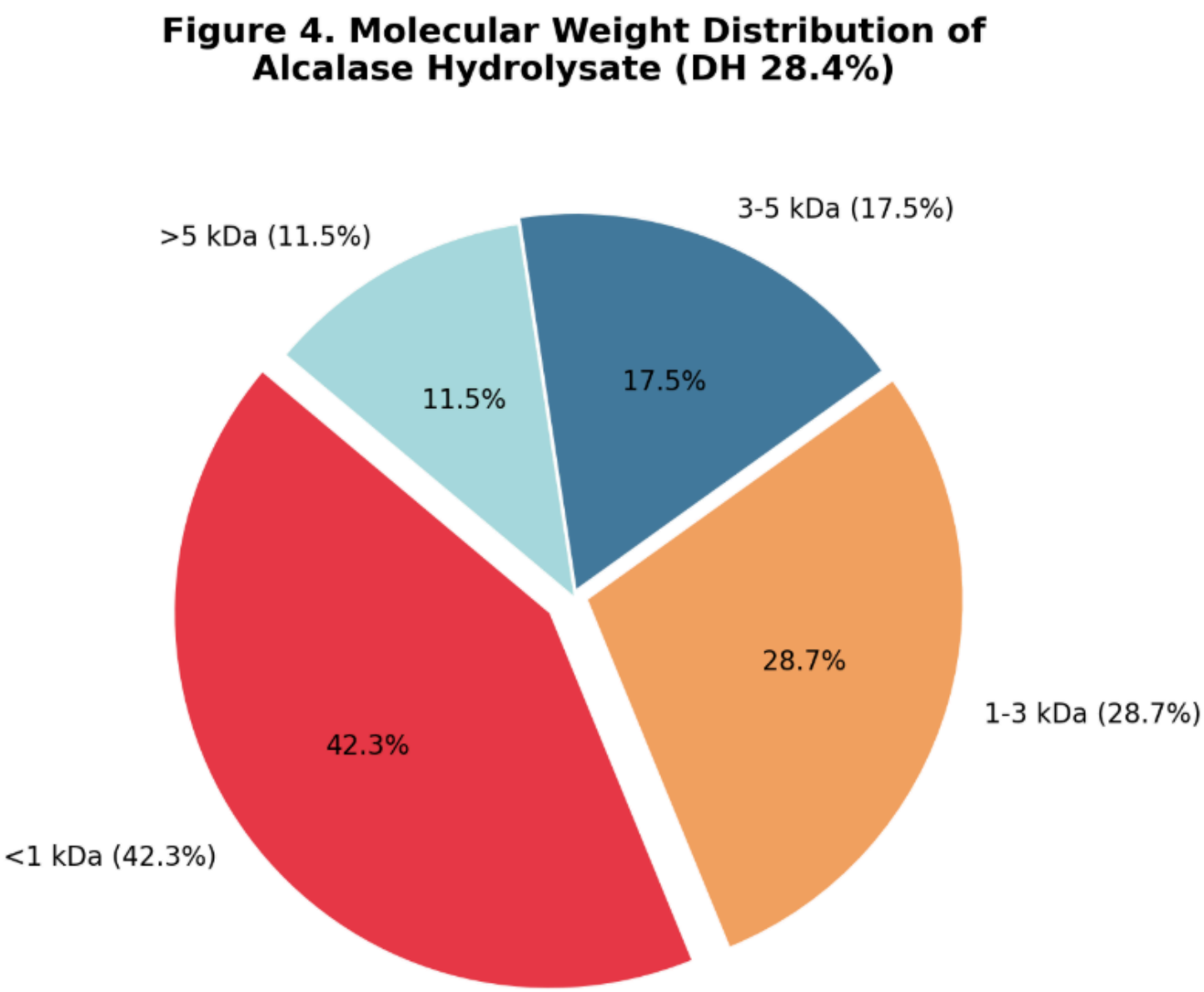

Figure 4

Molecular weight distribution of optimized CPI-ALC hydrolysate determined by ultrafiltration fractionation.

Figure 5. Response Surface Plot: Effect of E/S Ratio and Temperature on ACE Inhibitory Activity of Alcalase Hydrolysate

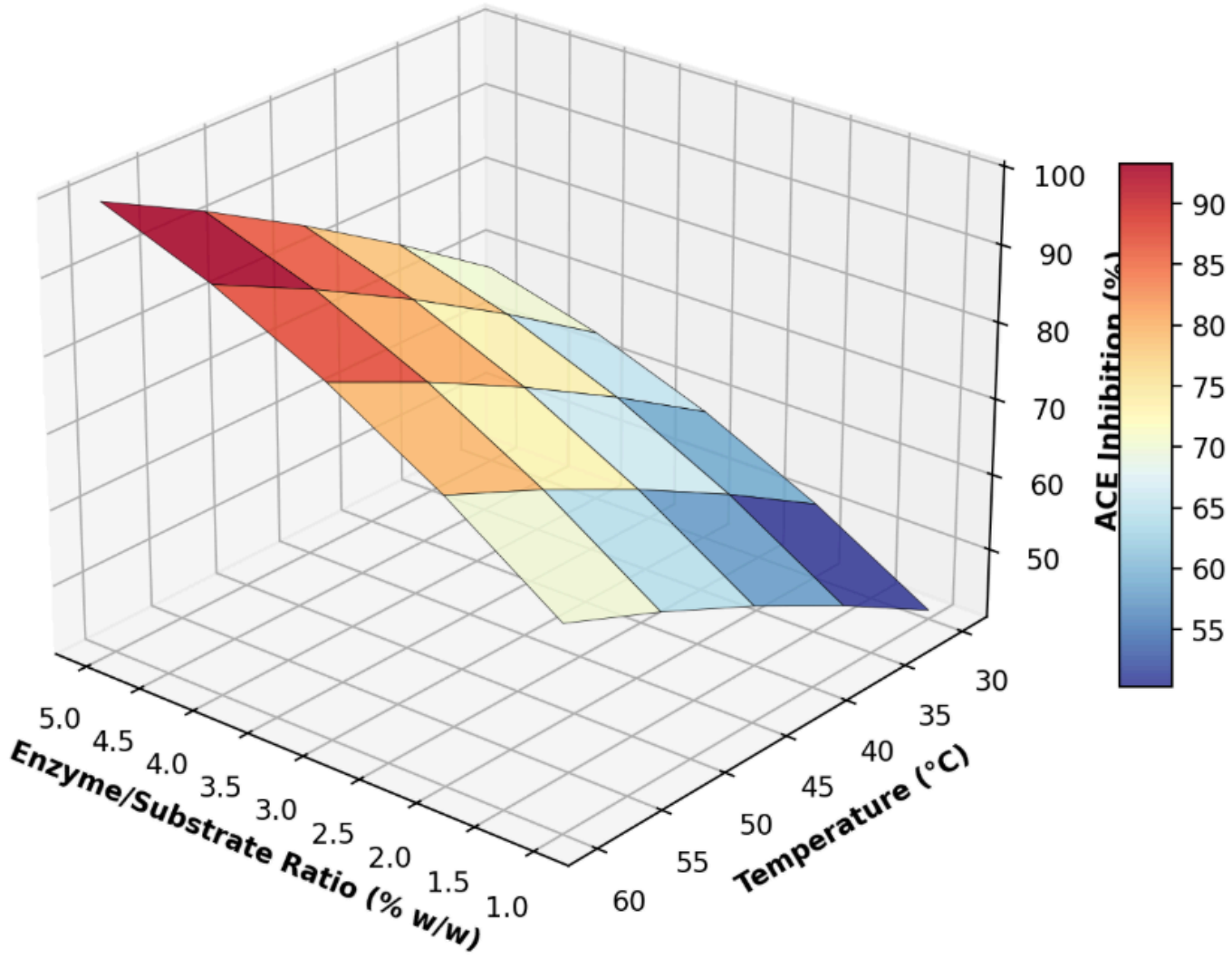

Figure 5

Response surface plot showing the effect of E/S ratio and temperature on ACE inhibitory activity at a constant hydrolysis time of 180 min.

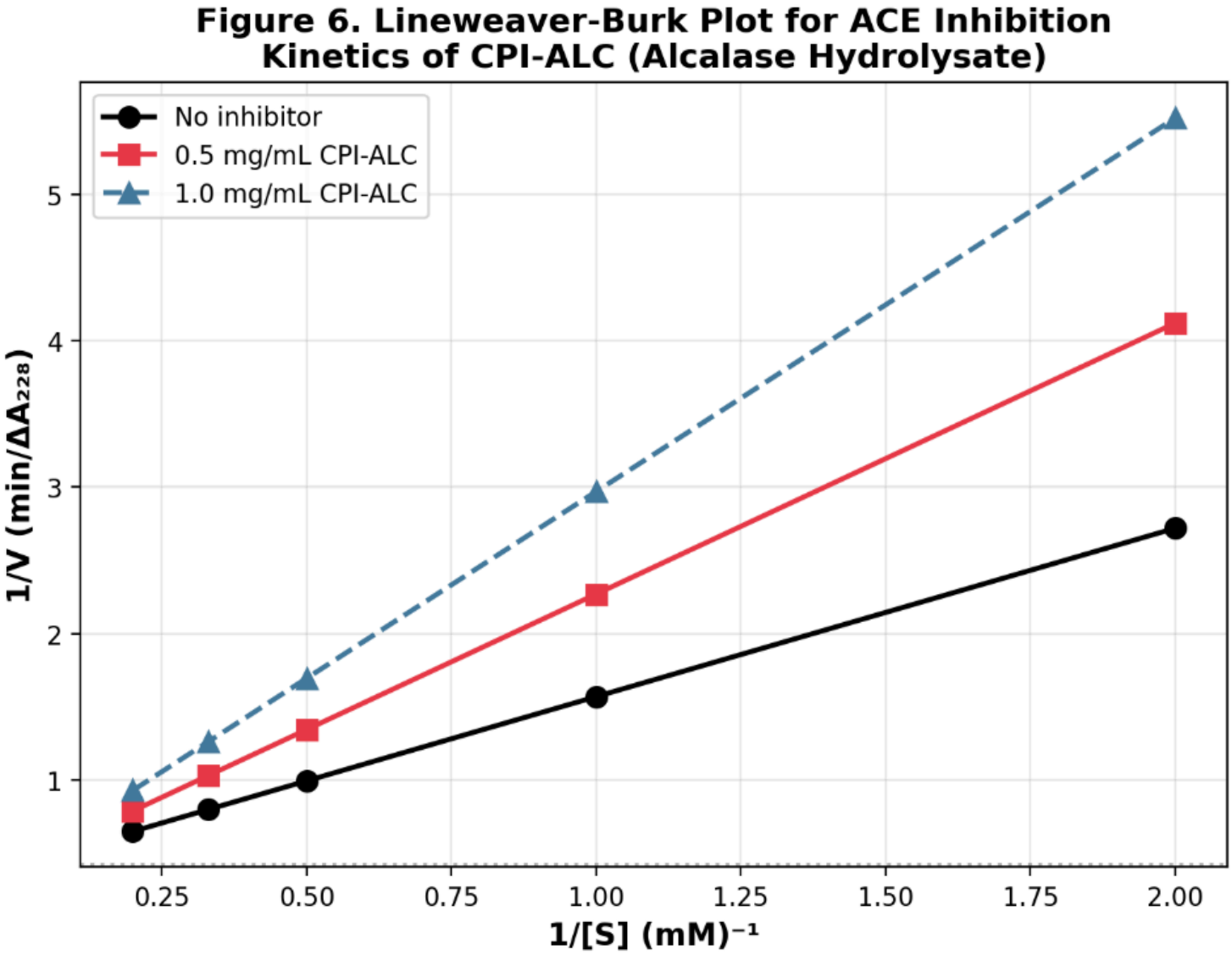

Figure 6

Lineweaver-Burk plot of ACE inhibition by CPI-ALC. HHL substrate concentrations: 0.5–5.0 mM. CPI-ALC concentrations: 0 (circles), 0.5 (squares), and 1.0 mg/mL (triangles). The intersection of lines indicates mixed-type inhibition.