Chickpea Peptide: A Nutraceutical Molecule Corroborating Neurodegenerative and ACE-I Inhibition

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Abstract: Chickpea seeds are the source of proteins in human nutrition and attribute some nutraceutical properties. Herein, we report the effects of chickpea seed bioactive peptide on albumin, insulin, lactoglobulin and lysozyme amyloid fibril formation. Employing thioflavin T (ThT) assays and circular dichroism (CD), amyloid structural binding transition was experimented to analyze the inhibition of amyloid fibril formation. The purified active peptide with a molecular mass of 934.53 Da was evaluated in vitro for its ACE-I inhibitory, antibacterial, antifungal and antidiabetic activities. Further, in vivo animal studies were carried out in wistar rats for blood pressure lowering action. In hypertensive rats, chickpea peptide decreased 131 ± 3.57 mm of Hg for systolic blood pressure and 86 ± 1.5 mm of Hg for diastolic blood pressure after 8 h intraperitoneal administration. Additionally, the peptide suppressed the fibrillation of amyloid and destabilized the preformed mature fibrils. Data emphasize efficacy of chickpea peptide vis-a-vis ACE-Inhibitory, antibacterial, antifungal, antidiabetic and anti-amyloidogenic activities, allowing us to propose this novel peptide as a suitable candidate for nutraceutical-based drugs and seems the first kind of its nature.

Keywords: ACE-inhibition; anti-amyloidogenic; chickpea; bioactive peptide; neurodegenerative

1. Introduction

Ordered protein aggregates and amyloid-like fibrils are known to induce neurodegenerative pathologies, viz., type II diabetes and Alzheimer’s disease [1,2]. These aggregates represent a cross β-sheet structure [3] and describe injured tissues [4]. In Alzheimer’s diseases, theses aggregates target the brain cells, while in type II diabetes, they remain concentrated in pancreatic tissues leading to β-cell dysfunction [4,5]. Further, proteins such as insulin [6], lysozyme [7] and β-2 microglobulin [8] also can form amyloid-like fibrils and also associated with neurodegenerative diseases. Most of the amyloid and amyloid-like fibrils share a common cross β-sheet structure. An inhibitor of amyloid fibril, therefore, needs to be identified that can target only the cross β-sheet secondary structure without altering the primary structure [1].
Peptides derived from natural sources address potential health benefits owing to low weight ensuing high activity [9–15]. These peptides exhibit antioxidant properties, acting as inhibitors of lipid peroxidation and scavengers of free radicals [16,17]. The enzymatic hydrolysates of legume seed proteins display numerous functional properties [18,19]. Such legume seed protein hydrolysates can be obtained using one [17,20] or multi enzyme hydrolysis employing different proteases to generate bioactive peptides of human health concern [21].

The functional properties studied herein have been reported for the first time in chickpea (Cicer arietinum) that belongs to family leguminoseae. Seeds of chickpea contains protein and essential amino acids needed by the human body. Chickpea seed protein demonstrate various health-promoting functions such as antioxidant, antifungal activity, angiotensin I-converting enzyme (ACE) inhibition and anticancer performance [20,22,23]. Chickpea protein hydrolysate generated peptides and studying their biophysical characterization, ascertaining inhibition of amyloid oligomerization and bioactivity in vivo, is sparsely reported [10,20,21].

The present study, therefore, was designed to examine the effects of chickpea peptide on fibrillation of albumin, insulin, lactoglobulin and lysozyme. The chickpea peptide revealed inhibition of albumin, insulin, lactoglobulin and lysozyme fibril formation under in vitro conditions. This study allows for future molecular interaction employing chickpea peptides.

2. Materials and Methods

2.1. Preparation of Protein Concentrates and Enzymatic Hydrolysis

Defatted chickpea seeds powder was stirred for 8 h in cold and protein extraction was performed in distilled water at pH 8.0. This flour suspension was subjected to centrifugation at 10,000 × g for 15 min. The supernatant adjusted to pH 4.5 was stirred 30 min in cold and recentrifuged at 10,000 × g for 15 min [24,25]. The protein was lyophilized and concentration was estimated following Bradford method [26].

Enzymatic hydrolysis was performed using alcalase with chickpea protein concentrate [24]. Hydrolysis was performed for 120 min by adding 2% (w/v) alcalase (pH 8). Protein hydrolysates were clarified through 0.45 nm filters and maintained at −20 °C until further analysis.

2.2. Ultrafiltration and Purification by FPLC

The alcalase protein hydrolysate was fractionated [27] using ultrafiltration cells of different molecular weight cut-off (MWCO) membranes (30 kDa > 10 kDa > 5 kDa > 3 kDa). The peptide fraction with highest ACE inhibiting activity (3 kDa) was consequently subjected to an FPLC system (AKTA GE Healthcare, Montreal, QC, Canada) equipped with a Hi Trap Q XL column. Employing linear salt gradient, the bound proteins were eluted. The fraction showing prominent ACE inhibitory activity was lyophilized and used further.

2.3. Amino Acid Composition Analysis

The lyophilized chickpea peptide fraction was hydrolyzed with 6 mol L⁻¹ HCl at 110 °C for 24 h under nitrogen. The presence of amino acid was stated as g per 100/g protein.

2.4. Assay and Kinetics of ACE-I Inhibition

The determination of ACE-I inhibitory activity of the chickpea and their hydrolysates was measured by the spectrophotometric assay with minor modifications to the method of Cushman and Cheung [28].

Studies on the inhibition kinetics of chickpea and their hydrolysates were performed using substrate Hippuryl-His-Leu (1.0 mM) and analyzed by Lineweaver–Burk’s method [29].
2.5. Biophysical Activity
2.5.1. Far-UV Circular Dichroism Measurements

The circular dichroism measurements were performed using JASCO spectropolarimeter (J-815) using human serum albumin (HSA), human insulin, β-Lactoglobulin (β-L) and human lysozyme (HL) with chickpea peptide. A range of 200–250 nm spectra was scanned. The achieved scanning speed was 100 nm/min and recorded three scans.

2.5.2. ThT Fluorescence Measurements

Protein aggregation was evaluated by ThT fluorescence assay. The assay was executed using human serum albumin (HSA), human insulin, β-Lactoglobulin (β-L), human lysozyme (HL) incubated with or without chickpea peptide as previously described [11]. The ThT was excited at 440 nm and spectra was documented from 450 to 600 nm. Slit widths were set at 3 and 5 nm for excitation and emission [30].

2.5.3. Differential Scanning Calorimetry (DSC)

Peptide fractions prepared in 10 mM phosphate buffer (pH 7.0) were read on DSC thermal analyzer. Peptide fractions (10 µL) were injected into aluminium liquid pans and heated in a calorimeter from 25 to 120 °C at a rate of 10 °C/min. A blank pan was used as a reference. Denaturation temperature (Td) and corresponding enthalpy change of denaturation (DH) were computed using universal analyzer in triplicates.

2.5.4. Infra-Red Spectroscopic Analysis

FTIR spectroscopy was employed to find out the presence of the β-sheet secondary structure. This analysis takes advantage of differentiating β-sheet secondary structure formation. The FTIR spectra of chickpea peptide was noted between 400 and 4000/cm. Further, transmission spectra were documented via a KBr pallet consisting 0.1% of sample.

2.5.5. Identification of Peptide by LC–MS

Chickpea peptide was fractionated by Agilent 1260 Infinity Capillary Pump and coupled to Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Theoretical pI and molecular weight of the identified proteins was estimated using ExPASY pI/Mw tool. The obtained raw data was cross-referenced to the plant species sub-directory within the Swiss-Prot database (UniProt, EBI, Cambridgeshire, UK) and analyzed in Mascot search against NCBI database.

2.6. Biochemical Characterization

2.6.1. Assay of Antibacterial Activity

The chickpea peptide experimented against various bacterial strains, included Escherichia coli, Micrococcus luteus, Staphylococcus aureus, Bacillus licheniformis, Pseudomonas aeruginosa and Bacillus subtilis. Streptomycin (1 mg/mL) was used as reference. The bacterial cultures grown on nutrient agar medium were used to document zone of inhibition [20,31].

2.6.2. Assay of Antifungal Activity

The antifungal activity of chickpea purified peptide showing the highest ACE-I inhibitory activity was measured against unicellular fungal species. This contained Candida albicans, Candida krusei and Saccharomyces cerevisiae by disc diffusion method as per our previous report [20,31].

2.6.3. Assessment of DNA Damage

Efficacy of chickpea peptide for protecting DNA damage was studied qualitatively and visualized under UV-transilluminator [32].
2.6.4. Protein Oxidation Assay

The chickpea peptide was investigated for its protective ability against H$_2$O$_2$/Fe$^{3+}$/ascorbic acid attack as described by Kizil et al. [32,33].

2.7. Antidiabetic Assay

2.7.1. α-Amylase Inhibition Assay

The α-amylase inhibitory activity of the chickpea peptides was estimated following methods by Yu et al. [34] and the results of α-amylase inhibition activity were expressed in terms of inhibition percentage as given below:

\[
\text{Inhibition} \% = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right) \times 100\%
\]

2.7.2. α-Glucosidase Inhibition Assay

The chickpea peptides were assessed for α-glucosidase inhibition following the method of Yu et al. [34] and the results of the activity were expressed as inhibition percentage as given below:

\[
\text{Inhibition} \% = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right) \times 100\%
\]

2.8. In Vivo Studies

2.8.1. Animal Management

Male wistar rats aged 10–14 weeks were randomly selected and acclimatization was performed for one week in the animal facility. Animals were handled carefully as per declaration of Institutional Animal Ethics Committee (IAEC) (824/PO/ERe/S/15/CPCSEA). During the adaptation and investigation phase, rats were housed in a 12 h light–dark cycle and in a quiet room with the relative humidity of 50 ± 10% and temperature of 23 ± 2°C. Standard rat chow and water was fed ad libitum to all the rats. A hypertensive rat model was developed using L-NAME 60 mg/kg/day fed in drinking water for 30 days [35].

2.8.2. Antihypertensive Effects

The chickpea bioactive peptide fraction was injected to study antihypertensive activity [36]. Hypertensive rats were randomly divided into four groups: control (distilled water), 5 mg/kg peptide, 10 mg/kg peptide and captopril (10 mg/kg). Measurements of the systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP) were recorded before and after the treatment of test samples at different time intervals, viz., 0, 1, 2, 4, 6 and 8 h. The tail cuff method of non-invasive mode was employed. Captopril was administered as the positive control drug. The average of the measured SBP, DBP and MAP values of each rat was termed the blood pressure (BP) of sample. The difference in value between BP-basal and BP-sample indicated the lowering effects in blood pressure (ΔBP).

3. Result and Discussion

For purification of biologically active peptides, ultrafiltration technology offers the preferred choice and provides convenient access to purification of industrially important food proteins. Chickpeas constitute major food proteins composing into different consumer products of human health [37].

3.1. Chickpea Protein Hydrolysate and Its Ultra-Filtered Fractions

The present study employed ultrafiltration to obtain chickpea peptides of various size, viz., 10, 5–10, 3–5 and 3 kDa. The ACE-I inhibitory activity of these four components and crude chickpea protein ranged from 5–500 µg/mL concentration (Figure 1). The ACE-I inhibitory activity of 3 kDa and 3–5 kDa fractions was significantly higher than that of the crude (without ultrafiltration) as summarized in Table 1. The MW fraction of 3 kDa exhibited comparatively higher ACE-I inhibitory activity (IC$_{50}$ value of 104.9 ± 1.27 µg/mL)
than other fractions. In addition, 5–10 kDa showed the lowest inhibitory potential with IC$_{50}$ value of 125.1 ± 0.22 μg/mL. There was a significant change in the ACE-I inhibitory activity as compared to the positive control (captopril) with an IC$_{50}$ value of 86.79 ± 2.09 μg/mL while crude protein demonstrated 140.9 ± 0.084 μg/mL. This result corroborated with previous reports that ACE-I inhibitory peptides are mainly made up of small peptides. Hence, in our further analyses, 3 kDa peptides were considered for characterization.

![Figure 1. ACE-I inhibitory activity of chickpea protein fractions. * and ** significantly different from each other (p < 0.05) and ns (not significant).](image)

| Ultra Filtered Fraction (kDa) | ACE-I Inhibitory Activity IC$_{50}$ Value (μg/mL) |
|------------------------------|-----------------------------------------------|
| 10                           | 110.3 ± 0.070                                 |
| 5–10                         | 125.1 ± 0.22                                  |
| 3–5                          | 109.7 ± 0.98                                  |
| 3                            | 104.9 ± 1.27                                  |
| Crude protein                | 140.9 ± 0.084                                  |
| Captopril                    | 86.79 ± 2.09                                  |

### 3.2. Separation by FPLC

The fractions showing highest activity were pooled and then loaded on the HiTrap Q chromatography column. The peak of elution under these conditions were referred as P1 and P2 and the ACE-I inhibition was measured (Figure 2). The ACE inhibition rate of P2 fraction was the highest of 94.4 ± 0.57 μg/mL. The yield of P2 in each purification step is shown in Table 2. The final yield of this new peptide was 6.1 mg/g protein.
Table 1. ACE-I inhibitory activity with IC$_{50}$ of chickpea protein fractions.

| Ultrafiltered Fraction (kDa) | ACE-I Inhibitory Activity (µg/ml) |
|-----------------------------|-----------------------------------|
| 10                           | 110.3 ± 0.070                     |
| 5–10                        | 125.1 ± 0.22                      |
| 3–5                         | 109.7 ± 0.98                      |
| 3                           | 104.9 ± 1.27                      |
| Crude protein               | 140.9 ± 0.084                     |
| Captopril                   | 86.79 ± 2.09                      |

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Figure 2. Elution of the HiTrap Q XL column (1 mL) (GE Healthcare).

Table 2. Yield of chickpea peptide.

| Procedure                              | Yield (mg/g Protein) |
|----------------------------------------|----------------------|
| Crude protein                          | 21.5                 |
| After hydrolysis                       | 17.4                 |
| After ultrafiltration                  | 10.1                 |
| After purification by FPLC system      | 6.1                  |

3.3. Amino Acid Composition of Fractions

Food protein hydrolysate/fractions are now being recognized as potential antihypertensive candidates [21]. The particular combination of amino acid executes antihypertensive effects in vivo due to its structural properties. The composition of chickpea and the potent fractions of hydrolysates obtained after ultrafiltration are summarized in Table 3. Current data revealed aspartic and glutamic acid, arginine, leucine, serine, phenylalanine and lysine in the peptide fraction forms a substantial part of amino acid composition. The presence of amino acid obtained from hydrolysate is in accordance with nutritional recommendations of FAO/WHO/ONU, 1985. Published literature suggest that many naturally occurring ACE inhibitory peptides are mostly hydrophobic [38].

Table 3. Amino acid composition (grams per 100 g of protein) of different chickpea purified peptide fractions.

| Amino Acid | Chickpea Protein | Alcalase Hydrolysate |
|------------|------------------|----------------------|
| Aspartic acid | 14.4 ± 1.24     | 11.2 ± 0.29          |
| Glutamic acid | 18.5 ± 0.21     | 15.6 ± 0.31          |
| Serine     | 7.1 ± 1.20       | 5.8 ± 0.20           |
| Histidine  | 3.0 ± 0.42       | 2.4 ± 0.22           |
| Glycine    | 3.8 ± 0.11       | 3.1 ± 0.11           |
Table 3. Cont.

| Amino Acid | Chickpea Protein | Alcalase Hydrolysate |
|------------|------------------|----------------------|
| Threonine  | 4.0 ± 0.36       | 3.2 ± 0.16           |
| Arginine   | 13.6 ± 0.28      | 10.6 ± 0.14          |
| Alanine    | 3.74 ± 2.60      | 4.1 ± 0.60           |
| Tyrosine   | 3.1 ± 0.31       | 2.9 ± 0.19           |
| Valine     | 3.9 ± 0.29       | 3.0 ± 0.29           |
| Methionine | 1.41 ± 0.27      | 1.01 ± 0.12          |
| Cystine    | 1.3 ± 0.38       | 1.1 ± 0.20           |
| Isoleucine | 4.4 ± 1.08       | 4.2 ± 1.18           |
| Leucine    | 9.7 ± 1.43       | 9.2 ± 0.61           |
| Phenylalanine | 7.3 ± 1.56    | 5.7 ± 0.16           |
| Lysine     | 7.1 ± 0.46       | 6.9 ± 0.12           |

3.4. The Inhibition Pattern of the Purified Peptides against ACE

Kinetic parameters are fundamental to interpret inhibitory potential of peptides against enzymes activities. Lineweaver–Burk plots of ACE reaction with and without peptide inhibitors at two concentrations are presented in Figure 3.

Figure 3. Michaelis_Menten kinetics on ACE-I inhibition by alcalase hydrolysate. Inhibitor concentrations of triangle 0, square 0.1 and diamond 0.25 mg/mL.

The inhibition pattern observed was mixed-type, depicting noncompetitive inhibition. This suggest that chickpea peptides combine with an ACE molecule to produce a dead-end complex binding of substrate. In other words, the peptide binds at a different site from the substrate and hence act as ACE inhibitor by forming enzyme–substrate–inhibitor and enzyme–inhibitor complexes. Ki is a dissociation constant. The Ki values are 4.1 and 4.8 mg/mL for ACE inhibition for 0.1 mg/mL and 0.25 mg/mL of purified peptide fractions, respectively (Table 4). Thus, the peptide 0.25 mg/mL binds more effectively to ACE and depicts the higher inhibitory effects when compared to the 0.1 mg/mL peptides, which have higher values of Ki.
Table 4. Kinetic parameters of hydrolysis of chickpea proteins by alcalase.

| Kinetic Parameters | 0 mg/mL (No Inhibitor) | 0.1 mg/mL | 0.25 mg/mL |
|-------------------|------------------------|-----------|------------|
| Vmax (nm)         | 0.1149                 | 0.1434    | 0.3279     |
| Km (mM)           | 0.08212                | 0.07936   | 0.09130    |
| Ki (mg/mL)        | 00                     | 4.199     | 4.845      |

3.5. ThT Binding Assay

Thioflavin T (TT) fluorescence assay was experimented to estimate amyloid formation of HSA, HI, HL and β-L proteins impact of chickpea peptide fibril formation. ThT fluorescence is widely used to detect cross β-sheet structure present in amyloid fibrils and the growth of amyloid formation of human proteins. A significant rise in fluorescence intensity was observed due to the inhibitory effect of chickpea peptide on fibrillation (Figure 4). In the presence of chickpea peptide (1 mg/mL) and HSA, HI, HL and β-L (20 µM), a decrease in fluorescence intensity was observed. HSA, HI, HL and β-L fibrils show strong ThT fluorescence intensity, indicating the presence of amyloid fibrils.

![Figure 4. ThT fluorescence intensity](image)

ThT results inferred that the chickpea peptide is able to inhibit the amyloid formation and is possibly due to small size peptides that may intercalate between partially misfolded species of the protein and thereby, inhibit their self assembly [2].

3.6. Secondary Structural Modulation

To understand the secondary structure of proteins and peptides, far-UV CD is the conventional method. The spectra of secondary structures provided by CD are unique. The conversion of secondary structures (α-helix/β-sheet or unordered/β-sheet), associated with amyloid fibril formation is monitored by following the advent of negative CD band at 218 nm. This is one of the peculiar features of β-sheet conformation.

Two negative peaks at 208 nm and 222 nm of native HSA, HI and HL indicate the alpha helical nature of protein. Whereas a single peak at 218 nm of native β-L indicates...
that protein is rich in the beta sheet. Under amyloidogenic condition, the peak at 222 nm became diminished, whereas its ellipticity became increased at 208 nm, suggesting the formation of HSA amyloid fibrils. HI, HL and β-L under amyloidogenic condition and a single peak that appeared around 218 nm suggest the formation of beta sheet-rich structure of amyloid fibrils (Figure 5). Although, the intensity is high compared to the native HI. When incubated with the peptide, it resists the formation of the beta sheet structure of amyloid fibrils. Chickpea peptide, therefore, stabilizes the native structure of the protein and/or decreases the beta sheet content.

Figure 5. Far-UV CD spectra.

Alzheimer’s disease is an acute neurodegenerative disease expressed by the aggregation of amyloid formation causing neuroinflammation. Various plant extracts/peptides inhibit aggregation of β-pleated sheets restricting oligomerization. We demonstrate inhibition of aggregation by various means. Chickpea peptides prevent the formation of neurotoxic oligomers and hold potential to reduce neuroinflammation.

3.7. DSC

Being an important marker of heat-induced aggregation and gelation behaviors, the thermal properties of chickpea protein hydrolysates were determined [39]. The higher denaturation temperature (Td) is normally associated with higher thermal stability of a globular protein. The separation of hydrogen bonds responsible for tertiary and quaternary structures of proteins are additionally understood by Td data. Thus, a higher Td for the proteins suggests that the polypeptides have a more compact tertiary structure [40]. Thermal stability of chickpea proteins, using Td as a marker, as evaluated by DSC, is shown in Figure 6. Thermogram depicts a prominent endothermic peak that is attributed to thermal transition of proteins. The highest Td value observed for chickpea purified peptide was 113.9 °C.

Thermal stability is mostly linked to higher disulfide bonding for globular proteins. The thermal stability of a protein may additionally be related to other important parameters, such as its acidic/basic amino acid composition and polypeptide heterogeneity [40].
Besides these differences, the interactions of proteins with residual salts might also be more responsible for high thermal stability than protein structure differences [41].

![DSC profile of chickpea peptide](image)

**Figure 6.** DSC profile of chickpea peptide.

### 3.8. FTIR

The chickpea purified peptide was subjected to the 4000–500 cm$^{-1}$ region for obtaining FT-IR spectra. The spectrum in Figure 7 shows the distinct absorption of hydrolysate. The peak at 1560 and 794.7 cm$^{-1}$ appeared broad and showing nature of purity. The small bands recorded at 1254.0 and 1007.3 cm$^{-1}$ are attributed to C-H anti-symmetrical stretching vibrations. The weak absorption at 3436.01 cm$^{-1}$ indicates the presence of aliphatic C-H bonds [42]. The bands around 1647 cm$^{-1}$ represented the free carboxylate (COO$^{-}$) stretching, indicating the presence of free carboxyl groups and uronic acids in the polysaccharides of chickpea. In addition, FT-IR spectra showed broad absorbance in the region of 1560–763.7 cm$^{-1}$ which was dominated by ring vibrations that overlapped with stretching vibrations of the (C-OH) side groups and the (C-O-C) glycosidic bond vibration [43].

![FTIR spectroscopy of chickpea peptide](image)

**Figure 7.** FTIR spectroscopy of chickpea peptide.

### 3.9. Identification of Bioactive Peptides by LC-MS

Molecular weight of the purified peptide was identified using a LC-MS with a nano LC chip cube source (Agilent Technologies, Santa Clara, CA, USA). Ion peaks with doubly or higher charges were selected for processing. The sequence of amino acids LGEFVLTR derived from the spectra has a high signal-to-noise ratio and showed complete or near-complete backbone fragmentation and indicate a low error (<0.8 Da) in the data (Figures 8 and 9).

The molecular mass of purified peptide was 934.53 D. Peptide sequencer identified it as an octapeptide, Leu-Gly-Glu-Phe-Val-Leu-Thr-Arg. For binding the ACE active site, peptides containing hydrophobic amino acids at the N-terminal region execute an important role [44,45]. The high amount of hydrophobic amino acids in chickpea peptide, viz., Leu,
Gly, Phe, Val and Leu. The N-terminus hydrophobic amino acids in chickpea peptide, viz., Leu and Gly, might contribute to ACE binding. The presence of hydrophobic amino acids in the peptide improve the solubility of peptides in lipid. This could help to enhance lipid inhibitory activity by facilitating the interaction between peptides and radical species. The hydrophobic amino acid exhibit higher antihypertensive potential [38].

Figure 8. MS-MS spectra of chickpea purified peptide.

| # | b-bH2O | b-bH2O+ | Seq. | y-bH2O | y-bH2O+ | y-2NH2 | y-2NH2+ |
|---|---|---|---|---|---|---|---|
| 1 | G | 303.44101 | E | 402.22435 | 402.71615 |
| 2 | 282.14683 | 141.57605 | E | 746.61955 | 773.71341 | 747.40957 | 774.40542 |
| 3 | 301.25125 | 209.31026 | E | 857.27606 | 303.99232 | 616.30697 | 309.68412 |
| 4 | 328.28208 | 264.64547 | V | 470.30094 | 235.63798 | 471.30269 | 235.14992 |
| 5 | 641.45753 | 321.18650 | Y | 371.24013 | 372.24415 | 186.61571 |
| 6 | 742.41360 | 371.71034 | T | 258.13967 | 259.14008 | 130.57368 |
| 7 | 318.00594 | 158.00290 | N | 76.54984 |

Figure 9. Ion table of spectra of chickpea purified peptide.

Biological activity of peptides is related to the chain length in addition to amino acid composition and sequence [46]. A literature perusal suggests that 2–20 amino acids are the most effective ACE-I inhibitory peptides [46, 47]. Hydrophobic amino acids thus enhance the solubility of peptides in lipid-based conditions of cell membrane, exerting a greater antihypertensive effect [48, 49]. Therefore, the position and type of amino acids present in peptide sequence are key factors determining its activity [50]. Wang et al. [51] studied the rice bran protein hydrolysate and found that a lysine was positioned at C-terminal.

3.10. Antimicrobial Activity

The major concern in medical treatment is antimicrobial resistant. New compounds from plant sources are continuously probed from a safeguard point of view. Plant peptides are projected to plug this void due to their bioactive principles. The antibacterial activities of the chickpea peptide were, therefore, evaluated against five species of bacteria M. luteus, S. aureus, B. licheniformis, P. aeruginosa, and B. subtilis and antifungal against C. albicans, C. krusei and S. cerevisiae.

Antibacterial and Antifungal Activity

The antibacterial activity of chickpea seed peptide using six bacterial strains were assessed. All strains were susceptible to streptomycin and ampicillin [52]. Chickpea peptide
fraction shown inhibition of growth against a broad spectrum of bacteria [53]. Chickpea peptide showed antibacterial activity with minimal concentration in the range of 20 µg/disk. Chickpea peptide performance tested against *E. coli*, *S. aureus*, *B. licheniformis*, *P. aeruginosa* and *B. subtilis* exhibited zone of inhibition at 29.5 ± 1.18 mm, 21.7 ± 0.99 mm, 25.8 ± 0.35 mm, 41.9 ± 0.35 mm, 37.1 ± 0.99 mm and 37.5 ± 1.90 mm, respectively. The standard drug zones of inhibition were 34.5 ± 1.74, 34.3 ± 0.84 mm, 36.4 ± 0.56 mm, 42.7 ± 0.42 mm, 38.8 ± 1.13 mm and 44.8 ± 1.06 mm, respectively (Figure 10).

**Figure 10.** Antimicrobial activity of chickpea peptide (**a**) towards various human pathogen (**b**) zone of inhibition.

The assay for antifungal activity towards *C. albicans*, *C. krusei* and *S. cerevisiae* was equally carried out. Chickpea peptide showed antifungal activity with minimal concentration in the range of 20 µg/disk. Chickpea peptide performance tested against both unicellular fungi showed zones of inhibition of 66.29 ± 3.76 mm, 95.5 ± 4.01 mm and 69.70 ± 4.52 mm, respectively, in comparison to the standard drug of antifungal effect (Figure 11).

The chickpea peptide demonstrates antimicrobial action. Purified peptide of chickpea presented antibacterial activity in several bacterial strains because of its easier diffusion through cellular membrane of bacteria. Our study suggest that short peptide chains penetrate easily through bacterial cellular membrane facilitating the action of such peptides [54,55].

The antimicrobial action is coupled with membrane permeabilization via pore formation initiating cell death [56,57]. In a previous study, an isolated peptide from *P. vulgaris* and *P. lunatus* showed antibacterial and antifungal activities, but studies with lower molecular...
mass peptides have not been carried out [58,59]. Thus, our results expand this knowledge, demonstrating that lower molecular mass peptides execute better antibacterial activity.

![Figure 11](image1.png)

**Figure 11.** Antifungal activity of chickpea peptide towards (a) various human pathogen (b) zone of inhibition.

To the best of our knowledge, this is the first report showing the antibacterial activity of *C. arietinum* peptides. However, the antibacterial mechanism is not clear in peptides derived from plant sources. Published evidence indicate that the plant peptides act on intracellular targets [60,61]. The antimicrobial effect is, therefore, associated with membrane permeabilization through pore formation causing cell death.

### 3.11. Inhibition of Oxidative DNA Damage

The antioxidant action of the purified peptide was assessed in vitro using calf thymus DNA. The Fenton’s reagent is known to cause oxidative breaks in DNA strands yielding DNA fragments [62]. The peptides of chickpea were able to protect DNA damage as shown in Figure 12. Compared to control DNA (Line 1), DNA was fragmented form due to the generation of hydroxyl radical from the Fenton’s reaction (Line 2). DNA treated with the chickpea peptide ranging from 5 to 500 µg/mL and protected hydroxyl-radical-induced DNA damage dose-dependently (Lines 3–7), indicating its antioxidant effect. DNA is a sensitive biotarget of ROS-mediated oxidative damage, and ascertained herein is the protective effect of the chickpea peptide against such oxidative damage [63–65].

![Figure 12](image2.png)

**Figure 12.** DNA gel electrophoresis depicting effect of chickpea peptide on protection of DNA damage.

### 3.12. Protein Oxidation Assay

Protein oxidation assay was performed using SDS-PAGE to monitor qualitative protein damage. Electrophoretic pattern of BSA is presence and absence of different concentrations of peptide fraction after 3 h incubation with Fe\(^{3+}/\)H\(_2\)O\(_2\)/ascorbic acid system as shown in
Figure 13. SDS-PAGE; Effect of chickpea peptide protection against -OH generated photolysis of H$_2$O$_2$.

3.13. $\alpha$-Amylase Inhibitory Activity of Chickpea Peptides

$\alpha$-amylase, also known as diastase, is the key enzyme responsible for dietary starch digestion. It releases oligosaccharides that further digests to glucose, which is swiftly absorbed by the body [66]. Therefore, inhibition of $\alpha$-amylase is considered as one of the better means for diabetes management. The $\alpha$-amylase inhibitory activities of purified chickpea peptide ranged from 5 to 500 µg/mL (Figure 14). Acarbose is used as one of the standard drugs for the control of diabetes [66]. Chickpea purified peptides at 500 µg/mL revealed significant $\alpha$-amylase inhibitory activities with IC$_{50}$ 122.95 ± 1.20 µg/mL in comparison to acarbose with an IC$_{50}$ value of 90.81 ± 0.55. An earlier report observed that barley protein hydrolysates were able to inhibit $\alpha$-amylase [67–69].

Figure 14. Inhibitory effects of chickpea peptide against $\alpha$ amylase. ** significantly different from each other ($p < 0.05$).

3.14. $\alpha$-Glucosidase Inhibitory Activity of Chickpea Peptides

$\alpha$-glucosidase is another key enzyme involved in starch digestion that breaks terminal $\alpha$-1 to 4-linked glucose releasing a single glucose molecule that can be absorbed by the body. The assay for $\alpha$-glucosidase inhibitory activity of chickpea peptides was measurable with IC$_{50}$ 101.25 ± 0.63 µg/mL in comparison to acarbose (IC$_{50}$ value 85.06 ± 1.75 µg/mL),
as shown in Figure 15. This suggests that chickpea protein hydrolysates have the potential to be developed into functional ingredients for diabetic care [69,70].

![Figure 15](image)

Figure 15. Inhibitory effects of chickpea peptide against α-glucosidase. ** significantly different from each other (p < 0.05).

Erkan and EI [71] reported that chickpeas are potent inhibitors of α-amylase and α-glucosidase and Uraipong and Zhao [72] informed that rice bran protein hydrolysates exhibit strongly in vitro. In humans, dietary carbohydrates are hydrolyzed by pancreatic α-amylase. Further, intestinal α-glucosidase enzymes exert breakdown of oligosaccharides and disaccharides into monosaccharides that are suitable for absorption. The inhibition of these enzymes is specifically useful for the treatment of noninsulin-dependent diabetes, because it will limit the speed of the release of glucose into the stream of blood [71–73]. It is known that α-amylase breaks down starch into disaccharides acted upon by isomaltase especially α-glucosidase to release glucose. The presence of potent α-glucosidase inhibitory activity hence requisite in monitoring the release of glucose from disaccharides in the gut than α-amylase inhibition. However, moderate α-amylase inhibition with potent α-glucosidase inhibitory activity provide a better therapeutic approach to slowdown the availability of dietary carbohydrate substrate for glucose production in the gut [74]. Literature perusal suggests that the major drawbacks associated with synthetic α-glucosidase inhibitors are their strong α-amylase and α-glucosidase inhibitory properties. These synthetic molecules further result in excessive inhibition of pancreatic α-amylase that leads to abnormal bacterial fermentation of undigested saccharides in the colon [75]. Therefore, peptides of natural origin/food ingredients may provide better options to available drugs. However, this needs to be further evaluated at molecular levels in vivo.

3.15. Antihypertensive Effects of Chickpea Bioactive Peptide in Hypertensive Rats

The wistar rat strains have extensively been employed with a rationale of hypertension development to mimic human system. Post intraperitoneal administration of 5 mg/kg and 10 mg/kg of body weight provided noticeable steady variation and there was noteworthy change in the mean arterial pressure (MAP) at 2, 4, 6 and 8 h (Figure 16). The changes in the SBP, DBP and mean arterial pressure of hypertensive rats during the 8 h observation period was observed.

In vivo experiments were carried out with hypertensive rats to visualize the hypertension modulation induced by the protein hydrolysates of chickpea seeds as a result of alcalase activity. Employing a slightly modified procedure of Chalé et al. [27], such proteins were evaluated for their hypotension modulation vis-à-vis variable doses. Post intraperitoneal administration of 5 mg/kg and 10 mg/kg of body weight over 8 h observation
period provided noticeable steady variation in the mean arterial pressure (MAP) at 2 to 8 h.

Figure 16. Decrease in mean arterial pressure (MAP) in hypertensive rats.

The chickpea bioactive peptide at 10 mg/kg dose at 8 h (−53 mmHg) after administration resulted in the most potent decrease in the SBP. The mean SBP of 5 mg/kg, 10 mg/kg and captopril occurred at 8 h for 148 ± 2.08 mmHg, 131 ± 3.57 mmHg and 126 ± 3.07 mmHg, respectively. The positive control captopril exerted the lowest point of the SBP that appeared at 8 h (−62 mmHg) after administration (Figure 17a,b). Maximum DBP reduction of −60 mmHg was observed 8 h after administration. Further, decline in DBP caused by 5 mg/kg and captopril were −43 and −67 mmHg at 8 h post-administration, respectively (Figure 18a,b). L-NAME is a potent blood pressure inducer and was employed creating a hypertensive model in the present investigation.

The alcalase hydrolysate of chickpea showed a significant suppressive effect on SBP, DBP and also the MAP of hypertensive rats. Among the two doses of hydrolysate, 10 mg/kg demonstrated a higher antihypertensive activity than 5 mg/kg (p < 0.05). Being a by-product of chickpea, alcalase hydrolysate has a potential role in the prevention of hypertension.

Earlier studies related that such kind of pulse peptides are sparsely studied. However, the peptides derived from milk and some animal sources are being intensively studied for ACE inhibition. The peptides of pulse source proteins obtained after alcalase treatment such as soybean, mung bean and pea showed measurable antihypertensive properties. The peptides of these pulses showed a decrease in SBP of 31.8 mmHg at 600 mg/kg, 25.6 mm Hg at 600 mg/kg, 7 mmHg at 100 mg/kg and 44.4 mmHg at 50 mg/kg, respectively [22,23,76]. The inhibition of the 3 kDa fraction of chickpea was similar to the intervals of reduction of SBP and DBP in hypertensive rats such as that of vegetable protein hydrolysates. Studies relevant to this kind must be performed to further demonstrate its pharmacology and toxicology effects by oral and intraperitoneal administration. To the best of our knowledge, this is the first evidence showing the potential antihypertensive effect in vivo of chickpea-derived bioactive peptides.
Figure 16. Decrease in mean arterial pressure (MAP) in hypertensive rats.

Figure 17. (a,b): Decrease in systolic blood pressure (SBP) in hypertensive rats.

Figure 18. (a,b): Decrease in diastolic blood pressure (DBP) in hypertensive rats.

4. Conclusions

Proteins under stress conditions can lead to misfolding and aggregation referred to as amyloid fibrils. Amyloid aggregation forms the nexus of neurodegenerative pathologies vis-à-vis Type II diabetes, Alzheimer’s, Parkinson’s and Huntington’s, etc. One of the attractive approaches to treatment of the aforesaid disorders is to search for natural molecules that inhibit amyloid aggregation. In addition, the peptide also efficiently suppressed the fibrillation of amyloid and destabilized the preformed mature fibrils. The present study emphasizes the efficacy of chickpea peptide in terms of ACE-I Inhibitory, antibacterial, antifungal, antidiabetic and anti-amyloidogenic activities, allowing us to propose these peptides as a suitable candidate for the development of nutraceutical-based drugs and reports the first of its nature observed in chickpea.
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