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Research Article

# Angiotensin-I converting enzyme (ACE-I) inhibitory and antiproliferative potential of chickpea seed protein hydrolysate

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**Abstract**: Chickpea seeds are the preferred source of proteins possessing health care functions in countries across the world. In the present investigation, the chickpea seed protein subjected to enzymatic hydrolysis produced bioactive peptides that were able to inhibit the angiotensin converting enzyme (ACE) and cytotoxic effect. Experimental screening was to test the efficacy of hydrolytic enzymes in chickpea seed for production of bioactive peptides was therefore carried out. The optimum hydrolysis times for each protein hydrolysate prepared by alcalase which inhibited ACE-I with $IC_{50}$ value of 52.22 µg/ml. The protein hydrolysate was further subjected to antiproliferative evaluation for breast cancer cell lines MCF-7 and MDA-MB-231 and the $IC_{50}$ was observed 0.71 mg/ml and 0.78 mg/ml respectively. Study indicated the potential of chickpea proteins as a source of ACE-inhibitory and antiproliferative potential to foresee the application of chickpea proteins into functional foods.

**Key words:** ACE-I inhibitory activity, Chickpea seeds, Hydrolysis.

## Introduction

Food legumes are known to produce functional proteins and ascribed prevention of many health disorders. The fatalities of CVDs presently accounts for 30% human mortality (Erdmann *et al.,* 2008). The bioactive proteins/peptides provides functional principles to various disease lowering concerns that include blood pressure lowering, cardiovascular diseases and anti-inflammatory features (Wu *et al.,* 2006). Therefore, identification and characterization of such bioactive peptides are being explored. Recently, the bioactive peptides like angiotensin I-converting enzyme (ACE) inhibitory peptides derived from plant sources have attracted scientific attention because of their roles in health care (Hua *et al.,* 2011).

ACE, a dipeptidyl carboxpeptidase (EC 3.4.15.1), is involved in peripheral hypertension as well as overall cardiovascular functioning. It catalyses the conversion of the inactive decapeptide angiotensin-I into the potent vaso-constricting octapeptide angiotensin-II and also inactivates the potent vasodilator, bradykinin (Wu *et al.,* 2006). The inhibition of ACE may result in hypotension. Many potent synthetic ACE inhibitors such as captopril, enalapril, lisinopril and ramipril are widely used in the clinical treatment of hypertension related cardiac failures. These synthetic ACE inhibitors however have been reported to lead adverse side effects such as cough, taste disturbances, rashes and angioedema (Erdmann *et al.,* 2008). Therefore, the quest for natural plant products acting as a chief component of the functional foods for the management of hypertension is underway (Wang *et al.,* 2016).

Plant derived ACE inhibitory peptides as generated from enzymatic hydrolysates, demonstrate antihypertensive activity and antiproliferative effect under *in vivo* and *in vitro* systems (Gupta *et al.,* 2018; Wang *et al.,* 2016; Li *et al.,* 2006). Jakubczyk and Baraniak, (2013) have evaluated lentil protein digested peptides for ACE-I inhibitory potential. Wang *et al.,* (2016) have isolated and purified ACE inhibitory peptide of 929 Da molecular weight which had amino acid sequence of Tyr-Val-Pro-His-Trp-Asp-Leu.

Chickpea (*Cicer arietinum*) seed protein contains essential amino acid needed by the human body, showing a nutrition-based health care functions. The major storage proteins of chickpea are globulins (56.0%), glutelins (18.1%), albumins (12.0%) and prolamin (2.8%). The objective of the present work was to evaluate chickpea protein hydrolysates using gastrointestinal enzymes, like alcalase possessing ACE inhibitory activity and antiproliferatve effect *in vitro*.

## Materials and Methods

### Seed Material

The mature and dry seed material was obtained from Indian Institute of Pulses Research (IIPR), Kanpur (U.P.), India under MTA understanding.

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**Protein concentrates preparation**

Ground flour of chickpea seeds was defatted by hexane (1:10 w/v) employing constant stirring for 8 h at 4°C. For protein extraction, isoelectric precipitation method was carried out. Briefly, protein extraction using flour to solvent ratio of 1:10 (w/v) was performed in distilled water at pH 8.0. This flour suspension (25 g flour in 250 mL of water at pH 8) was stirred at 4 °C in magnetic stirrer for 1 h and subsequently centrifuged at 10,000 × *g* for 15 min. The supernatant so obtained was vacuum filtered using 100-160 m pore size filter funnel. Finally filtrates were freeze-dried and stored at -20 °C under vacuum in plastic bags.

**Enzymatic hydrolysis**

Treatments with alcalase protein concentrate hydrolysis was done using the method of Pedroche et al., (2002). Hydrolysis reaction was performed for 120 min by adding 2% (w/v) alcalase (pH 8) in the final volume at 50 °C temperature and pH was kept constant during hydrolysis. The reaction was stopped by heating to 85 °C for 15 min. Protein hydrolysates were clarified filtering through 0.45 nm filters to remove the insoluble matter. The filtrates maintained at 70 °C were lyophilised and named as alcalase-generated chickpea protein hydrolysate.

**Degree of hydrolysis**

The protocol of De Castro and Sato, (2014) was followed to determine DH with slight modification. Briefly, to the different time interval withdrawn hydrolysates (0.5 ml), 12 % trichloroacetic acid (0.5 ml) was added and centrifuged at 10,000 x g for 20 min. Using Lowry *et al.,* (1951) method, the protein content was determined and DH was calculated as the ratio of TCA-soluble protein to total protein in the hydrolysate.

**Extraction of ACE from rabbit lungs:**

Rabbit lungs as donated by the pharmacology laboratory of Defence Research & Development Establishment (DRDE), Gwalior Madhya Pradesh state of India, was used to obtain an angiotensin converting enzyme (ACE) source following the method of Cushman and Cheung, (1971). Briefly, lung sample was ground in liquid nitrogen and homogenized in 10 mM potassium phosphate buffer (pH 8.3), containing 100 µm pepstatin and 0.1mM PMSF. The homogenate was centrifuged at 5000 x g for 10 min and resulting supernatant was dialysed for 2 h against 20 volumes of the same buffer in cold. This dialysed supernatant was used as a source of angiotensin converting enzyme. To verify the absence of undesirable proteases such as carboxypeptidase in ACE extracts, the kinetics of HHL hydrolysis by ACE was followed in the presence or absence of captopril, a potent ACE inhibitor. The hydrolysis is due to ACE only and not to other proteases was confirmed by the following figure 1.

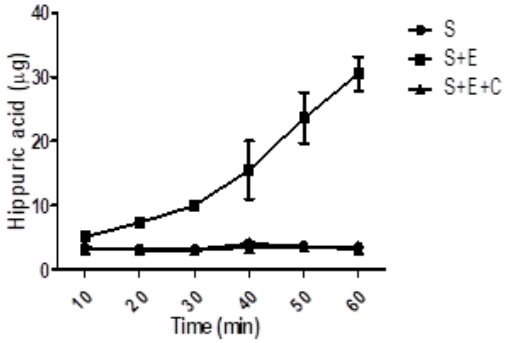


**Figure 1.** Kinetics of hydrolysis of substrate HHL (S) by ACE (E) in presence or absence of captopril (C).

**Spectrophotometric assay for ACE activity:**

Determination of the ACE inhibitory activities of the digests was performed according to the method of Cushman and Cheung, (1971) with minor modifications. One hundred microlitres of CP and ACPH were added to 500 µl mixture containing 100 µl of 100 mM phosphate buffer (pH 8.3), 100 µl of 300 mM NaCl, 200 µl of 5 mM Hippuryl-L-Histidyl-L-Leucine (HHL) and 100 µl of ACE isolated from rabbit lungs. The mixture was incubated at 37°C for 30 min on incubator shaker and the reaction was stopped by adding 500 µl of 1 N HCl. The mixture was added with 3.0 ml of ethyl acetate and mixed through vortex for 15 s. Ethyl acetate layer was obtained and the solvent was allowed to evaporate. The residue was redissolved in 1.0 ml of distilled water and the absorbance of the resulting solution was recorded at 228 nm. Blank was prepared without addition of peptide sample. Pulverized captopril served as positive control. ACE inhibitory activities were expressed as inhibitory activity (U), percent inhibition and $IC_{50}$ values. All values are means of three experimental trials.

**Sulphorhodamine B (SRB) assay for cytotoxicity assessment**

The SRB assay was performed as reported earlier by Skehan *et al.,* (1990). Briefly, the cells were fixed with 10% chilled trichloroacetic acid (TCA) for 1 hr at 4°C. The supernatant was aspirated and plates were washed thrice with chilled deionized water and subsequently air-dried. 100µL of 0.4% (w/v) SRB in 1% acetic acid was added to each well and incubated for 30 minutes at room temperature. Unbound SRB was removed by 3 washes with 1% acetic acid till the plates get air-dried. 200 µL of unbuffered 10mM tris base, pH (10.5) was added for extracting the bound stain. The absorbance was read at 560nm in microplate reader (Bio-Rad, USA). The % cell inhibition was calculated using the following formula:

Cell proliferation inhibition (%) =

$$[1- (A_s/A_c)] \times 100$$

Where: As – absorbance of sample; Ac – absorbance of control

## Results and Discussion

Being a promising source of protein chickpea seed demonstrated ACE-I inhibitory property at different levels. The defatted chickpea seeds flour was used as a source for the preparation of chickpea protein (CP) and tested the total protein contents in the seeds of all chickpea accessions before hydrolysis. It was found that total protein content was ranged 20-24.2% in chickpea. In order to obtain extensive hydrolysates with DH greater than 50%, it was necessary to use more than one protease. In general, one enzyme can't achieve high DH in reasonable period of time. The inhibitory activity of hydrolysates varied from 56.2 ± 2.7 to 44 ± 5.0%. The 2 hr incubation was a benchmark for chickpea protein hydrolysate, generating active peptides. The activities of protein hydrolysates depend on the protein substrate, proteolytic enzyme, time and temperature of hydrolysis as well as amino acid composition and sequence as per literature evidances (Daskaya *et al.,* 2017).

### Enzymatic hydrolysis

Depending on the use of enzyme system at subsequent reaction time, the degree of hydrolysis differed. For example, alcalase at 2 hr reaction time catalyzed highest degree of hydrolysis in desi, kabuli and wild type chickpea seeds. Alcalase is an alkaline protease that produced chickpea protein hydrolysates with better functional characteristics than the original protein. Alcalase is known to generate bioactive peptides with ACE-I inhibitory activity consequently been used as a purified fraction as described in earlier reports. The bacterial origin *Bacillus licheniformis*, alcalase exhibits the subtilisin Carlsberg serine group in its active site, which has endopeptidase activity. To the extreme C-terminal end, this enzyme hydrolyzes peptides with a wide specificity and releases hydrophobic amino acids such as Phe, Tyr, Trp, Leu, Ile, Val and Met (Markland *et al.,* 1971). It is obvious that potent ACE-inhibitory peptides have a hydrophobic or aromatic amino acid residues in each of the three C-terminal positions are therefore undergo catalysis (Hong *et al.,* 2005). For the above reasons, it is quite probable that the hydrophobic amino acid content of the studied legume seeds is similar to *Phaseolus radiatus* L. (39.75%), meaning chickpea seeds studied here are appropriate substrates for producing peptides with ACE-I inhibitory action using alcalase enzyme (Hong *et al.,* 2005).

### ACE-I Inhibitory activity

The crude protein hydrolysate of chickpea seeds prepared by alcalase enzyme digestion were measured for ACE-I inhibitory activity. The $IC_{50}$ values of alcalase hydrolysate was found significant ($P < 0.05$). The hydrolysate prepared by alcalase at 2 h revealed highest ACE-I inhibitory activity (lowest $IC_{50}$) with $IC_{50}$ value 52.22 µg/ml, when 1 mg protein was used. The most potent ACE-I inhibitory activity of protein hydrolysate was compared to the inhibitory activity of the standard antihypertension drug captopril.

As previously indicated, differences in ACE inhibitory activity reported from earlier studies may attribute to sample composition as well as differences in the peptide composition of the hydrolysates. In addition, proteolytic enzymes used in the digestion of parent proteins and the experimental conditions used in processing play a significant role. Previous studies corroborate the influence of enzymatic digestion on generation of ACE inhibitory activity obtained from food proteins (Yust et al., 2003; Vermeirssen *et al.,* 2003). The parameters of the hydrolytic process such as pH, temperature, enzyme-to-substrate ratio and hydrolysis time, also influence the release of peptides from food proteins that show ACE-I inhibitory reactions (Vermeirssen *et al.,* 2003). As already mentioned in the introduction, limited data is available on ACE-inhibiitory activity in the seeds of chickpea. It is noticed that alcalase is more efficient endopeptidase than papain and pancreatin cleaving bonds of hydrophobic amino acids pool of chickpea seeds (Medina-Godoy *et al.,* 2012). Boschin *et al.,* (2014) worked on ACE-I inhibitory activity of enzymatic protein hydrolysates from other legumes that included pea, lentils, bean and chickpea also. Apart from this, *in-vitro* ACE-I inhibition, *in-vivo* antihypertensive activities of bioactive peptides derived from various plant sources are available in literature domain (Yust et al., 2003; Vermeirssen *et al.,* 2003). Most of these studies revealed that ACE-inhibitory peptides are of non-competitive nature and exert antihypertensive ability through protecting vascular endothelial cells from reactive oxygen species mediated damage.

### Antiproliferative effects of chickpea protein in human breast cancer cells

The morphological changes of different concentrations of extracts (0.2mg-1.0mg) showed dose-dependent antiproliferative activities against these cell lines. After 48 h treatment the $IC_{50}$ of alcalase extracts was 0.71 mg/ml in MCF-7 cells similarly the $IC_{50}$ of 0.78 mg/ml in MDA-MB-231 compared to chickpea protein with $IC_{50}$ of 0.89 mg/ml (Figure 2). Interestingly chickpea hydrolysate showed significant dose dependent antiproliferative effect against both cancer cells, compared to chickpea protein. The results on IC50 values of chickpea hydrolysate for the cancer cells compared to the chickpea protein, suggests that chickpea hydrolysate may possibly have potential as an anti-cancer agent. However further studies are required to prove the safety and efficacy of chickpea hydrolysate as a potential chemopreventive agent in clinical practice.

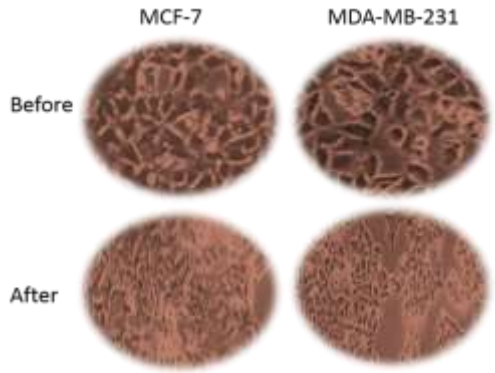


**Figure 2.** Morphological changes of breast cancer cell line MCF-7 and MDA-MB-231 treated with chickpea protein hydrolysate

## Conclusion

This study shows that defatted chickpea seeds can be regarded as a value-added by-product of the food industry. Hydrolysis by proteolytic enzyme like, alcalase produced high ACE-inhibitory activity and antiproliferative effect. According to our results, chickpea seed protein is a promising protein source for the production of ACE-inhibitory peptides which might be utilized to develop foods for hypertension prevention and chemopreventive agent. Further research is still necessary to elucidate the possible *in-vivo* effects of chickpea seed peptide fractions.

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