Original Article

Imminent Angiotensin-converting Enzyme Inhibitor from Microbial Source for Cancer Therapy

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Abstract

Background: Drugs targeting Angiotensin I-converting enzyme (ACE) have been used broadly in cancer chemotherapy. The recent past coupled with our results demonstrates the effective use of ACE inhibitors (ACEi) as anticancer agents, and they are potentially relevant in deriving new inhibitors. **Methods:** Bacterial strains were isolated from cow milk collected in Coimbatore, Tamil Nadu, India and plated on nutrient agar medium. The identity of the strain was ascertained by 16s rRNA gene sequencing method and was submitted to the NCBI GenBank nucleotide database. Various substrates were screened for ACEi production by the fermentation with the isolated strain. ACEi was purified by sequential steps of ethanol precipitation, ion exchange column chromatography and gel filtration column chromatography. The apparent molecular mass was determined by SDS-PAGE. The anticancer property was analyzed by studying the cytotoxicity effects of ACEi using Breast cancer MCF-7 cell lines **Results:** The isolate coded as BUCTL09 was selected and identified as Micrococcus luteus. Among the seven substrates, only beef extract fermented broth showed an inhibition of 79% and was reported as the best substrate. The peptide was purified and molecular mass was determined. The IC50 value of peptide was found to be 59.5 µg/ ml. The purified peptide has demonstrated to induce apoptosis of cancer cell.**Conclusions:** The results of this study revealed that Peptide has been determined as an active compound that inhibited the activity of ACE. These properties indicate the possibilities of the use of purified protein as a potent anticancer agent.

Keywords: *16S rRNA gene sequence, angiotensin-converting enzyme inhibitor, anticancer activity, antimetastatic, antiproliferative, beef extract, hippuric acid, MCF-7 cell line, Micrococcus luteus*

Introduction

Angiotensin-converting enzyme (ACE) is a dipeptide hydrolase that catalyzes both the formation of the potent vasoconstrictor, angiotensin-II (Ang II), and the deactivation of bradykinin, a vasodilator peptide.[1] Therefore, substances such as synthesized chemical drugs (e.g., captopril) or natural ACE inhibitory (ACEi) peptides can inhibit ACE activity and can cause a drop in blood pressure. These findings have been shown in both hypertensive human participants and spontaneous hypertensive rats. Ang II has a potential role in various aspects of tumor progression and targeting Ang II production, or action could prove useful in anticancer therapy.[2] Given the potential of Ang II in containing the proliferation of the tumor, production of these inhibitors by bacteria could open new doors for anticancer therapy. The protective effect of ACEi cannot be attributed solely to the inhibition of Ang II production.[1]

Reported to suppress vascular endothelial growth factor, which is believed to play a major role in stimulating angiogenesis in human growth.[3]

Researchers hope a better understanding of the angiogenesis process will help them in cancer treatment. There are many reports on ACEi peptides derived from food proteins, their physiological and pharmacological effects, and their prospects for application in preventing hypertension and for therapeutic purposes.[2] Rather, multiple mechanisms that are not yet fully understood could be responsible for the same. ACEi has also been.

Peptides including ACEi or antihypertensive peptides, immunomodulatory, antioxidative, antimutagenic, and anticancer peptides have been released from milk proteins, eggs, meat, and fish as well as in different plant protein sources such as soy and wheat through microbial proteolysis.[4,5]

Among the bioactive peptides, ACEi inhibitory peptides derived from food

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proteins have attracted particular attention and have been studied comprehensively for their ability to prevent hypertension. These peptides could be used as a potent functional food additive and represent a healthier and natural alternative to ACEi drugs. With the exception of *Lactobacillus delbrueckii* and *Lactobacillus lactis* which are used for milk fermentation, the uses of microbes as ACEi source have been less explored. Many research groups have combed for ACEis in microbial sources such as *Doratomyces putredinis*, *Nocardia orientalis*, *Streptomycetes*, *Actinomycetes*, *Spiculospora*, and *Actinomadura*.[6] This study was intended to isolate protease producing organism for the production of ACEi by the fermentation of various proteinaceous substrates. Based on these findings, this research focuses on isolating and identifying ACEi from the fermentation of beef extract using *Micrococcus luteus*.

Methods

Isolation of bacteria from milk

Raw unpasteurized milk samples of the cow were collected from the local area of Coimbatore, Tamil Nadu, India. Milk samples were serially diluted and were spread plated on nutrient agar medium.

Screening of protease producing bacteria

The isolates were screened for protease production by gelatin clear zone method using protease specific medium. The diameters of clear zones were measured by flooding the plates with mercuric chloride solution.[7] The largest zone was selected for further study.

Identification of bacteria

Morphological and biochemical characterizations

Identification of bacteria was performed according to their morphological, cultural, physiological, and biochemical characteristics. Bergey's manual of systematic bacteriology was performed to identify the genus of bacteria.[8]

Molecular characterization of bacteria

DNA isolation

Genomic DNA was isolated from the test organism.[9] Amplification of 16s rRNA gene was done with Universal primers (Chromous Biotech, Bengaluru, India). The amplification of the gene was done as per the Table 1. The polymerase chain reaction product was then visualized by running in agarose gel electrophoresis (1%). The amplicon was then sequenced.

Phylogenetic analysis

The construction of phylogenetic tree was based on fast minimum evolution method [Figure 1c]. The phylogenetic tree was constructed using neighbor joining method.[10]

Table 1: Polymerase chain reaction conditions

PCR reaction mixture (31 cycles and final extension at 72°C for 10 min)	
10X Taq buffer	5 µl
dNTP mix (4 mm)	5 µl
Forward primer (5 µm)	5 µl
Reserve primer (5 µm)	5 µl
Genomic DNA template (100 ng/1 µl)	5 µl
Taq DNA polymerase (5 U/µl)	0.4 µl
Nuclease free water	24.6 µl
PCR program is mentioned as follows	
Initial denaturation (96°C)	5 min
Denaturation (94°C)	1 min
Annealing (48°C)	1 min
Extension (72°C)	2 min

PCR=Polymerase chain reaction, dNTP=Deoxynucleotide

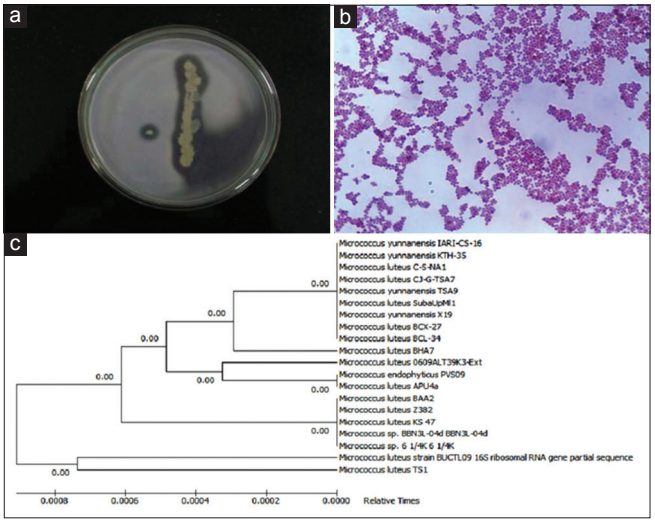

Figure 1: (a) Highest proteolytic activity. (b) Isolation of bacteria. (c) Phylogenetic tree was constructed using neighbor joining method

Screening of substrate for angiotensin-converting enzyme inhibiting peptide production

Various protein substrates such as beef extract, yeast extract, peptone, milk powder, cow milk, goat milk, and buffalo milk were amended in the modified M9 medium. Overnight grown bacterial cultures were harvested by centrifugation at 4°C, 8000 rpm for 20 min. Extracts fermented by different substrates were then screened for ACE inhibition. The protein content of the supernatant was estimated by Lowry's method.[11] Based on the observation, the chosen substrate was utilized for further studies as ACEi source.

Microorganism and crude enzyme preparation

The isolated strain *M. luteus* (GenBank accession number Kf303592.1) was inoculated into a protease specific medium broth. The supernatant was filtered through a 0.45 mm cellulose acetate filter paper.[12] The crude enzyme extract was further subjected to the purification process.

Before purifying the protein content, the ACEi activity of the crude extract was estimated.

Measurement of angiotensin-converting enzyme inhibitory activity

The ACEi activity was assayed by the method of Cushman and Cheung[13] with a few modifications. Hip-His-Leu (HHL) was dissolved in 50 mM sodium borate buffer (pH 7.0) containing 1 N NaCl. Following this, 25 µl of 5 mM (HHL) solution was mixed with 10 µl of beef hydrolysate (the pH of which was adjusted to 7.0) and then preincubated for 10 min at 37°C. The reaction was initiated by adding10 µl of ACE and the mixture was incubated for 30 min at 37°C. The reaction was stopped by adding 200 µl of 1 N HCl. The hippuric acid liberated by ACE was extracted with 1 ml ethyl acetate, dissolved by adding 1 ml of the buffer after the removal of ethyl acetate by vacuum evaporation, and the optical density was measured at 228 nm. The extent of inhibition was calculated using the formula

$$\text{Extent of Inhibition} = \frac{(B-A)}{(B-C)} \times 100$$

Result expressed in percentage.

Where, A = the optical density in the presence of ACE and ACEi component; B = the optical density without an ACEi component.

C = the optical density without ACE.

Purification of angiotensin-converting enzyme inhibitory peptide

The crude extract of fermented medium with the selected substrate by test strain was extracted with three volumes of chilled ethanol. The pellet was suspended in Tris-HCl (20 mM; pH 7.0) and further purified by ion-exchange column chromatography (Mono Q) and by size-exclusion chromatography (Sephadex G25). Each fraction was then tested for ACE inhibition activity and protein content. The protein profile of the active fraction with ACE inhibition was studied using 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the molecular weight of the protein was also determined.[14]

Cytotoxicity of angiotensin-converting enzyme inhibitor on breast cancer cell line

Cell line and culture

Breast cancer MCF-7 cell lines used in this study were obtained from King Institute of Preventive Medicine and Research, Chennai, India. The cells were maintained in Minimal Essential Media supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37°C.

Preparation of angiotensin-converting enzyme inhibitor

ACEi was prepared by fermenting the beef extract by *M. luteus*. The purified fraction in Tris-HCl buffer (pH 7.0, 20 mM) with the protein content of 1 mg/ml was used for cytotoxicity analysis. Various concentrations of the ACEi were then analyzed for cytotoxicity activity.

DNA fragmentation assays by agarose gel electrophoresis

The degradation of DNA into multiple fragments of 39–41 base pairs is a distinct biochemical hallmark of apoptosis. DNA fragmentation was analyzed by agarose gel electrophoresis of genomic DNA extracts from the breast cancer MCF-7 cell lines treated with 20 µl of ACEi for 3 h. The DNA was visualized by placing the gel on a ultraviolet transilluminator.[15]

Results

Identification of protease producing bacteria

The proteolytic activities of all 40 strains were assayed using gelatin agar, which was observed as the diameter of the clear zone. Among the forty isolates, the isolate coded as BUCTL09 showed the highest proteolytic activity [Figure 1a]. The potent bacterial strain was identified based on the morphological and biochemical characterization. The bacteria were Gram-positive cocci [Figure 1b]. The morphological and biochemical characteristics of the test organism are presented in Table 2.

Molecular characterization of selected bacteria

The nucleotide amplified the sequence from 16S rRNA gene was found to have around 781 bp. In the present

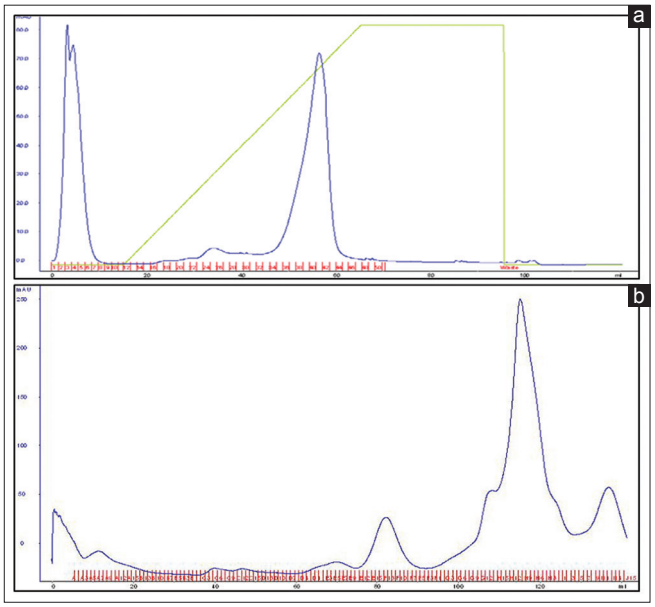

Figure 2: (a) Ion-exchange column chromatogram of angiotensin-converting enzyme inhibitory peptide, (b) Size-exclusion column chromatogram of angiotensin-converting enzyme inhibitory peptide

Table 2: Morphological and biochemical properties of strain BUCTL09

Reactions	Observation
Morphology	Cocci
Gram's reaction	Positive
Catalase test	Positive
Indole	Negative
Methyl red test	Negative
Voges–Proskauer	Negative
Citrate	Negative
Gas production from glucose	Positive
Growth at	37°C
Growth under aerobic condition	Positive
Carbohydrate fermentation	
Glucose	Positive
Lactose	Weakly positive
Sucrose	Positive
Mannitol	Negative
TSI agar	Acids-no H_2S
Skim milk	Negative
Starch hydrolysis	Negative
Urease test	Positive
Oxidase	Negative

TSI=Triple sugar iron

Table 3: Screening of substrate for angiotensin-converting enzyme inhibitor production

Substrates	ACE enzyme activity inhibition (%)*
Beef extract	79.67±4.73
Yeast extract	72.00±3.61
Peptone	53.33±1.53
Commercial milk powder	56.67±9.87
Cow's milk	62.67±4.04
Goat's milk	51.33±2.52
Buffalo's milk	61.33±2.08

*Results expressed are means and SD of triplicate measurements. SD=Standard deviation, ACE=Angiotensin-converting enzyme

Table 4: Purification table of angiotensin-converting enzyme inhibitory peptide

Purification step	Volume (ml)	Protein content (mg/ml)	ACE inhibition (%)
Crude extract	2000	8.66	78.55±0.42
Ethanol precipitation	50	4.00	74.96±0.85
Ion-exchange column (fraction 39-41)	6	0.92	84.22±0.79
Size-exclusion column (fraction 111-113)	6	0.40	85.43±1.08

ACE=Angiotensin-converting enzyme

study, 16S rRNA gene sequence of BUCTL09 showed 100% similarity with *M. luteus* strain in Figure 1c.

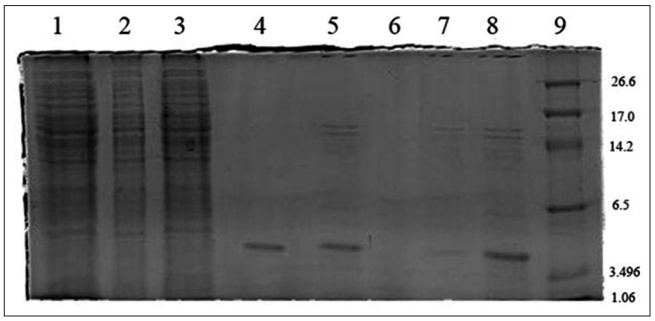

Figure 3: Sulfate-polyacrylamide gel electrophoresis profile of angiotensin-converting enzyme inhibitory peptide purification process

Screening of substrate for angiotensin-converting enzyme inhibitor production

The ACE inhibition by the bacterial extracts ranged from ~51 to ~79% [Table 3]. The purification scheme is shown in Table 4.

Purification of angiotensin-converting enzyme inhibitory peptides

In the present study, the peptides were concentrated using ethanol precipitation. On precipitation, the ACE inhibition and purification scheme are shown in Table 4.

Electrophoretic analysis of angiotensin-converting enzyme inhibitory peptide

On SDS-PAGE analysis using 15% gel, several bands were found to appear in the crude extract [lane 1 and 2 of Figure 3], confirming the presence of unwanted impurities and thus warranting further purification. The fractions (fractions 39–41 in Figure 2a) of ion-exchange column [lane 5, 7, and 8 of Figure 3] showed three prominent bands. The purified fractions of gel filtration column [lane 4 of Figure 3 showed a single band]. The apparent molecular weight was found to be around 4.5 kDa.

Lane 1, 2, 3: Crude extract; Lane 4: Protein fraction of Sephadex G25 column (size exclusion chromatography); Lane 5, 7, 8: Protein fraction of ion-exchange column; Lane 9: Molecular weight markers (26.6, 17, 14.2, 6.5, 3.496, and 1.06 kDa).

In vitro anticancer analysis

The peptide isolated in the present study was observed to have a cytotoxic effect on the MCF-7 cell line. The IC50 value of the peptide was determined to be 59.5 µg/ml. The cytotoxic effect was found to have a linear relation with the concentration of the peptide, which explains the sequential reduction in viability percentage of cancer cells (MCF-7 cell line) in the graph [Figure 4a]. The control cells which were not treated with ACEi were observed to be elongated and proper confluence growth was observed [Figure 4b]. The cell after treatment with ACEi was seen as round shaped which confirmed the detachment of the cells

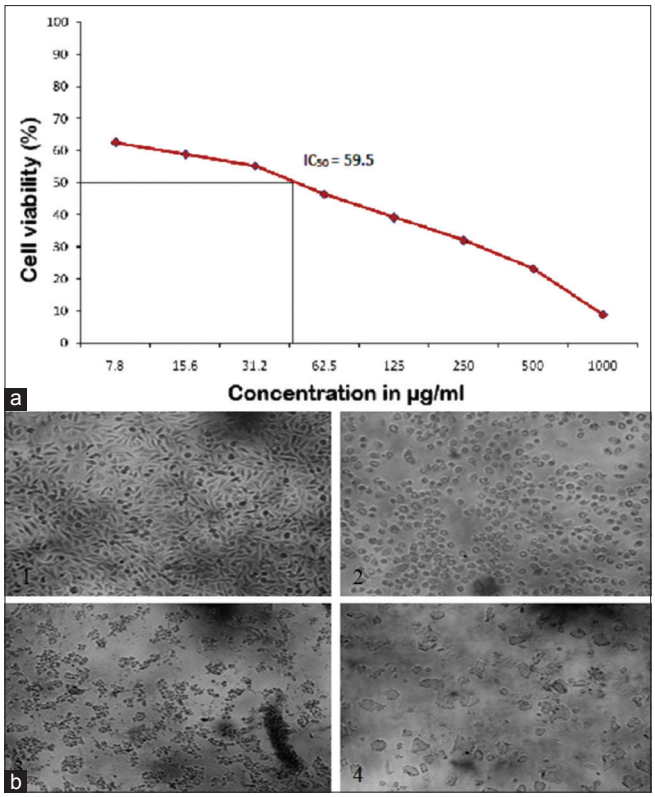

Figure 4: (a) Cytotoxicity of angiotensin-converting enzyme inhibitor showed the viability percentage of cancer cells (MCF cell line). (b) Cytotoxicity effect of angiotensin-converting enzyme inhibitor on breast cancer cell line MCF-7

from the surface. These rounded cells were indicators of apoptotic cells. The cells treated with 62.5 µg/ml were found to be mostly rounded, and as the concentration of ACEi was increased further the total cell count itself was observed to be reduced [Figure 4a].

Discussion

Breast cancer is one of the most common malignant tumors in women.[16] During the past decade, there has been increasing scientific interest in understanding the complex relationship between breast cancer and renin-angiotensin system (RAS).[17,18] The RAS plays an important role in blood pressure and cardiovascular homeostasis.[19,20] However, recently published data indicated that Ang II, the main biologically active peptide of RAS, contributed to breast cancer development and progression.[21,22] The production of Ang II is regulated by ACE which the serum levels is governed by genetic variation at the ACE locus.[23,24] The biological effects of Ang II are mediated by two subtypes of receptors (AT1R and AT2R) present at the cell membrane. Therefore, ACEis act by reducing AngII production and upregulating BK levels. In addition, ACEis containing a thiol group can directly inhibit the activity of zinc metalloproteases MMP2 and MMP9.[2] A study on the mechanism of action revealed that the modulation of hydrophobicity of peptides plays a crucial role against

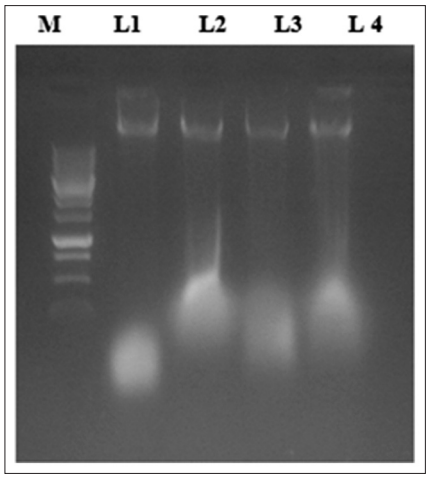

Figure 5: DNA fragmentation of MCF-7 cells treated with an angiotensin-converting enzyme inhibitor M-Marker1000 bp (base pairs) DNA ladder; L1-l; L2–250 µg/ml; L3–125 µg/ml; L4–62.5 µg/ml

cancer cells. As stated earlier, the mode of action of the majority of ACEi peptides proves to be competitive substrates for ACE. Most ACEi peptides found in meat can be classified as true inhibitor type peptides.[25-27] These peptides may act in one of the two ways: first, the peptide may bind to the active site of the ACE enzyme, or it may bind to an inhibitor site located on the ACE, thus modifying the protein conformation and preventing the substrate (Ang I) from binding to the enzyme active site.[28] A similar mechanism has been observed by cyclic depsipeptide coibamide A which was isolated from *Leptolyngbya* sp. Human lung cancer cell line[29] and Lyngbyabellin B isolated from *Lyngbya majuscula* on human Burkitt's lymphoma cells.[30] Linear pentapeptide dolastatin 10 and symplostatin 1 were isolated from *Symploca* sp. which exhibited a cytotoxic effect on human lung cancer cell line and human breast carcinoma cell line by both Bcl-2 phosphorylation and Caspase-3 protein activation.[31,32] Besides, there were also different types of anticancer peptides isolated from *Lyngbya* sp. and *Nostoc* sp., which exhibited inhibiting activity against cancer on different cell lines through microfilament disruption, secretory pathway inhibition, etc.[33,34] Apratoxin A is cyclic depsipeptide extracted from *L. majuscula* which exhibited cytotoxic effects on human HeLa cervical carcinoma between inhibiting protein synthesis in cell lysates and in human adenocarcinoma MCF-7 cells was observed.[35] In view of the earlier reports, the present study was attempted to find the anticancer efficacy of ACEi produced by fermentation of beef extract by *M. luteus*.

The colonies in raw milk sample are expected to be little higher than real microflora. This is due to contamination from the animal, especially the exterior of the udder and the adjacent areas; bacteria found in manure, soil, and water may be present in milk.[36] The morphological and biochemical characterization showed that the selected strain was of genus *Micrococcus* sp. classified under the

family *Micrococcaceae*. The phylogenetic tree showed the location of isolated strain BUCTL09 close to *M. luteus* as depicted in Figure 1c. Beef Extract is derived from the infusion of beef and provides an undefined source of nutrients, which is a mixture of peptides and amino acids, nucleotide fractions, organic acids, minerals, and some vitamins.[37] Beef extract being produced from meat might have some active peptides which might not be present in other protein substrates used which justify the present finding. These facts agree with the present finding that beef extract was the best source of ACEi peptides and beef extract may be chosen for further ACEi production. In earlier reports, intense proteolysis by endogenous muscle enzymes resulted in an accumulation of peptides and free amino acids at the end of the dry-curing process of meat. Similarly, ACEi peptides were identified in muscle tissues after hydrolysis of pork meat or derived *in vitro* from the digestion of pork meat. Based on these findings and observations in the present study, beef extract was used as a substrate for hydrolyzing using *M. luteus*. Among the fixed stationary phase, diethylaminoethyl (DEAE) is being extensively used in the conventional anion-exchange chromatography during ACEIP purification. In the present study, the strong anion-exchange Mono Q column was used. The soluble crude protein extract was dissolved in a minimal volume of 20 mM Tris-HCl buffer pH 7 and fractionated on Mono Q column. After washing the unbound protein with 20 mM Tris-HCl buffer, the column-bound protein was eluted with 100 ml linear salt gradient (0-100 mM NaCl in 20 mM Tris-HCl, the flow rate was 3 ml/min). The active fraction (Fraction 39-41, Figure 2a) which displayed ACEi activity were pooled, dialyzed against 20 mM Tris-HCl and was taken for Sephadex G25 chromatography. The protein content of the pool fractions was observed to be 0.92 mg/ml [Table 4]. The ACE inhibition of the active fraction was observed to be 84.22% ± 0.79%. It is a known fact that the strong anion-exchange column tends to bind tightly to the positively charged molecules and release only at a higher concentration of NaCl. Hence, in the present study, the active fractions which were eluted at higher NaCl gradient (~70%) revealed that the peptides may be highly positively charged. The pooled fractions were further purified on a Sephadex G25 column and eluted with 20 mM Tris-HCl buffer containing 100 mM NaCl. The chromatogram showed three peaks [Figure 2b]. The fractions (111–113) under the highest peak were found to have an ACEi activity which was pooled and checked for purity. The protein content of the purified fraction was observed to be 0.4 mg/ml with ACE inhibition of 85.43% ±1.08%. Similar results from earlier work after peptide fractionation through size-exclusion chromatography[38] revealed that the strongest ACEi activity (85% of ACE inhibition) was obtained from eluted fractions. It may be assumed that the peptides with a lower molecular weight exhibit a higher molecular mobility and diffusivity when compared to these peptides with a higher molecular weight, which appears to improve interactions with cancer cell components and enhances anticancer activity.

In the purification process during ion-exchange chromatography using Mono Q column, a strong anion-exchange column, the peptides were eluted only at higher NaCl concentration could be attributed to its higher positive charges. This positive charge and hydrophobicity might not only impart the ACEi activity but also facilitate higher interaction with cancer cells as reported earlier. The size of the generated peptides is crucial to the ACEi effect as previously reported. The peptides with lower MW were easily absorbed compared to peptides with higher MW.[39]

In agreement to the earlier report, the peptides of smaller molecular weight seem to exhibit ACEi activity.

In the present study, the DNA laddering assay revealed that the peptides-induced DNA fragmentation was not clearly seen in the agarose gel pattern of DNA from treated cells [Figure 5]. Even though an earlier report stated that the ACEi produce DNA fragmentation, there are several reports which suggest cell death by an alternate mechanism as well as Didemnin depsipeptides were reported to have a cytotoxic effect on cancer cell lines by inhibiting protein synthesis *in vitro*.[40]

Conclusions

ACEis are used in the treatment of heart diseases, hypertension, kidney diseases, and recent interest has focused on the possible role of these compounds in anticancer therapy.[41] The provocative hypothesis that ACE is might have a protective role in cancer was first suggested by Lever *et al*.[42] needs to be explored. The search for natural ACEis as alternatives to synthetic drugs is of great interest to prevent several side effects, and bioactive peptides are one among the best alternatives which can be developed as potential pharmaceutical ACEis. Currently, many studies are being done to search for more suitable anticancer agents, including ACEis, from natural products. This property enthuses to explore further the possibilities of the purified ACEi as a potent anticancer agent.

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Conflicts of interest

There are no conflicts of interest.

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