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Structure–activity relationships of α_s-casein peptides with multifunctional biological activities

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Abstract Multifunctional bioactive peptides have a wider role in modulating physiological functions and possess multiple biological activities. Peptides from bovine milk with sequences QKALNEINQF [p10] and TKKTKLTEEEKNRL [p14] from α-$_{S2}$ casein f (79–88) and α-$_{S2}$ casein f (148–161) were identified to be having multifunctional biological activities and were synthesized. These synthesized peptides show various biological activities like angiotensin-converting enzyme inhibition, prolyl endopeptidase inhibition, antioxidant, and antimicrobial activities. The mode of antimicrobial mechanism was studied and p10 shows depolarization of cell membrane, whereas p14 was found to display DNA-binding activity. Structural studies envisaged backbone flexibility, for differences in their mode of action. Peptide structure function studies were correlated to understand their multifunctional biological activity.

Keywords Peptides · Casein · Multifunctional · Circular dichroism · DNA binding · Antiulcer · Antihypertensive · Antimicrobial

Abbreviations

ACE	Angiotensin-converting enzyme inhibition
PEP	Prolyl endopeptidase
DNA	Deoxyribonucleic acid
CD	Circular dichroism
TFA	Trifluoroacetic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
$DiBAC_4(3)$	Bis-(1,3-dibutylbarbituric acid)trimethine oxonol

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Introduction

Milk is a complex mixture of proteins that provides the sole nutrient requirements to newborns. Milk contains proteins and encrypted biologically active peptides, which are released upon hydrolysis [1]. Bioactive peptides can affect numerous physiological functions of an organism. Once they are released in the body, they modulate various regulatory processes in the living cells as in the case of angiotensin-converting enzyme (ACE) inhibitory peptides, opioid peptides, antimicrobial peptides, mineral-binding peptides, immunomodulatory peptides, and cytomodulatory peptides [2, 3]. Since such peptides with biological activity are small in size, screening methods for their biological activity can be easily optimized and their therapeutic potential can be quickly investigated.

Peptides from cow's milk are multifunctional, i.e., they have more than one biological activity. Bioactive peptides are functional foods with health modulating benefits [4, 5]. Biologically active peptides have previously been identified and studied from in vitro enzymatic digests, in vivo gastrointestinal digests, and or by chemical synthesis [6]. Various bioactive peptides from bovine milk α-casein have been structurally characterized and reported in literature [7–9].

Antimicrobial activity has a special role among the various characteristics pertaining to peptides. Antimicrobial peptides are widely distributed in nature and have been characterized both from entire animal and plant kingdoms [10, 11]. Antimicrobial peptides reported in literature function by permeabilizing cell membranes and inhibit DNA, RNA, and/or protein biosynthesis [12]. These peptides are more potent compared to synthetic antibiotics, and are less prone to resistance [13, 14].

The biological activity of peptides can be related to their conformation, hydrophobicity, propensity to form different

structures in different environments, charge, amphipathicity, etc. [15, 16]. Structure–function interrelationships of biologically active peptides will pave the way for understanding mechanisms by which antibiotics work or drug resistance develops [17, 18]. Peptides with more than one activity are preferred over single-activity peptides as they can modulate or inhibit multiple physiological pathways simultaneously. The design and synthesis of new peptides is considered a viable option as compared to natural peptides due to their selectivity, specificity, and affinity for target biomolecules.

In the present paper, work was carried out on the structure and functional characterization of multifunctional bioactive peptides. Peptides from bovine milk α-$_{S2}$ casein with sequences QKALNEINQF [p10] from f(79–88) and TKKTKLTEEEKNRL [p14] from f(148–161) were identified to be having multifunctional biological activities and were identified, characterized, and synthesized [19, 20]. Peptides were then screened for various bioactivities.

Materials and methods

Materials

The following chemicals—trifluoroacetic acid (T6508), pancreatin (P5575) from Hog pancreas, ACE (A6778) from rabbit lung, ACE inhibitor (A0772) peptide containing p-Glu-Trp-Arg-Pro-Gln-Ile-Prp-Pro-Pro (Mol Wt. 1,101 Da), Hippuryl-Histidine-Leucine tetrahydrate (H-1635), 2,2-diphenyl-1-picrylhydrazyl (43180), Kaiser ninhydrin kit (60017), 1,2-ethanedithiol (02390), L-ascorbic acid (A92902), and calf thymus DNA (D1501) were from Sigma Aldrich Chemical Co, St. Louis, MO, USA. *Z*-pro-prolinal, *Z*-Gly-Pro-β-naphthylamide, and prolyl endopeptidase were from Bachem AG, Bubendorf, Switzerland. Acetonitrile, ethyl acetate, and methanol were obtained from E-Merck (India) Limited, Mumbai, India. Dimethyl formamide, dichloromethane, diethyl ether, isopropanol, piperidine, and *N*,*N*-diisopropylethylamine were from Spectrochem India Pvt. Limited, Mumbai, India. Fmoc—aminoacids, rink amide resin, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*,*N*-tetramethyluronium hexa fluoro phosphate (HBTU), and 1-hydroxybenzotriazole (HOBt) were from Chem-impex Limited, Wooddale, IL, USA. Brain heart infusion broth and nutrient agar were from Hi-Media, Labs Pvt. Limited, Mumbai, India. Quartz triple-distilled water was used to prepare reagents and buffers.

Synthesis of peptides

Peptides were synthesized on the solid-phase amino acid synthesizer (CS136, CS Bio Co., San Carlos, CA, USA). Nitrogen was purged continuously at 10 psi/h. Rink amide MBHA (4-methylbenzhydrylamine) resin was used to synthesize peptides with substitution rate of 0.75 mM. Amino acids were F-moc (9-fluorenyl methoxy carbonyl) protected and secondary side groups were Boc (*tert*-butyloxy carbonyl) protected. F-moc-protected amino acids, HBTU, and HOBT (where required) were added at a 3 times higher concentration to the substitution capacity of the resin. The organic solvents employed during peptide synthesis were dichloromethane and dimethyl formamide. 10 % *N*,*N*-diisopropyl ethylamine were used for decoupling. FMOC-protecting group was removed with 20 % (v/v) piperidine. Both these steps were monitored using Kaiser Ninhydrin reagent. HBTU was used to activate the incoming carboxyl group and to suppress racemization. Peptides resin were washed with two volumes of dimethyl formamide and dichloromethane, and vacuum dried for 24 h. Peptide resin was dissolved in 98 % TFA in water (2 %) and 125 µl ethanedithiol, and stirred at 4 °C for 30 min. Peptide resin was washed with two volumes each of isopropanol, and ice-cold anhydrous distilled diethyl ether [21, 22]. The overall yields of peptides were 89 and 84 % for p10 and p14, respectively. Peptides were analyzed for mass spec and N-terminal sequencing for the exact molecular weight and correct sequence [19].

Angiotensin-converting enzyme inhibition assay

Angiotensin-converting enzyme inhibition activity was determined in vitro as per the protocol discussed elsewhere [19, 20, 23]. Polyglycine was used as the negative control.

Antioxidant activity assay

The antioxidant activity of peptides was analyzed by DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity. L-Ascorbic acid was taken as positive control [19, 24]. Polyglycine was used as the negative control.

Prolyl endopeptidase inhibition assay

Prolyl endopeptidase activity from bovine blood serum was measured using fluorescence [25]. Polyglycine is used as negative control.

Antimicrobial studies

The inhibitory activity of peptides was tested against selected organisms like *Bacillus cereus* (F4801), *L. monocytogenes* (Scot A), and *S. aureus* (Fri722). The organisms were subcultured by growing them in brain heart infusion broth for 12–24 h at 37 °C. The cultured broth was centrifuged in clean sterile centrifuge cups at 6,500 rpm for 30 min. The cell pellet was collected and

dissolved in sterile 9 ml of phosphate buffered saline, pH 7.0 in a screw cap vial. The serial dilution was done from the stock (2×10^9 CFU/ml) to get a viable cell population of 2×10^4, 2×10^5, and 2×10^6 CFU/ml. 1 ml each of viable cultures from the diluted culture were taken and added to sterile 5-ml test tubes containing 0.1 ml of peptide at different concentrations from 1.5 to 4.0 mg/ml (dissolved in PBS, pH 7.4). The tubes containing cell suspension along with peptides were kept in a rotary shaker at 100 rpm for 12 h at 37 °C. The growth of the survived organisms was checked by pour plate method. To the sterile Petri plates, cell suspension containing peptides was poured along with nutrient agar (15 ml) and incubated for 24 or 72 h at 37 °C. The number of colony forming units that survived was counted manually and the percentage survived was calculated based on the survived organisms. Using the percentage survival measure the minimum inhibitory concentration (MIC) was calculated. Appropriate controls along with buffer, saline, and DMSO were also checked in the similar way to know their role in inhibition [26, 27]. The effect of bacitracin was also studied simultaneously and compared with the natural peptide as the standard. Polyglycine was used as the negative control.

Antimicrobial activity against *H. pylori*

Helicobacter pylori cultures were obtained from the clinical isolate of a patient with peptic ulcer using endoscopy from Karnataka Cardio Diagnostic Center, Mysore, India and cultured on F-12 nutrient agar (Gibco BRL, USA) with 5 % fetal bovine serum at 37 °C for 24 h (Medium composition: 10 g/L F12 nutrient mixture with 5 % fetal bovine serum). *H. pylori* strains were confirmed by Gram staining, colony morphology studies, motility test, and urease positivity test. A 100 µl aliquot of *H. pylori* culture was added into 10 ml broth media and incubated at 37 °C for 2 or 3 days. Peptides, positive control (amoxicillin), and negative control (polyglycine) were coated on sterile filter paper disks and were applied on F12 nutrient agar plates (seeded with test *H. pylori* from 2 to 3 days old culture) and incubated at 37 °C for 3 days. The assay for each culture was determined in duplicate. After incubation, the plates were observed for the zone of inhibition which were measured and then expressed as the antimicrobial activity of peptides [28].

Fluorometric assay for membrane potential

Bacillus cereus was grown in nutrient broth to an absorbance (600 nm) of 0.5 and incubated with 1 µM of bis-(1,3-dibutyl barbituric acid) trimethine oxonol [$DiBAC_4(3)$], (Molecular Probes, USA) a membrane potential-sensitive probe for 15 min. Peptides (10 mg/ml) were added to bacterial cells and the change in the relative fluorescence intensity of oxonol dye was followed at 492 and 515 nm excitation and emission wavelengths, respectively [18]. Amphotericin B was used as positive control.

Gel shift mobility assay

The bacterial genomic DNA of *B. cereus* F4810 strain (Public Health Laboratories, London, UK) was isolated by the method of Schraft and Griffiths [29]. The studies on binding of peptides with DNA were carried out using the gel mobility shift assay. Peptides (10 mg/ml) were incubated with *B. cereus* DNA (100 µg/ml) in 10 mM Tris and 1.0 mM EDTA buffer, pH 8.0 for 2 h and peptide DNA complex was loaded onto a 0.8 % agarose gel. The electrophoresis was run in TBE buffer at a constant voltage of 100 V for 45 min. The DNA in the gel was stained with ethidium bromide and visualized by using *Gene snap* software (Syngene, USA).

Time kinetics of peptide binding

The binding of peptide p14 to *B. cereus* DNA was studied by time kinetics mode on a UV spectrophotometer. The change in absorbance of DNA was monitored in time kinetics mode at 260 nm before and after addition of peptide. The time kinetics was also monitored with calf thymus DNA [30].

Measurement of apparent T_m

The thermal denaturation studies of peptides in the presence of *B. cereus* DNA was carried out in Cary100 Bio UV–Visible spectrophotometer (Varian, Australia). DNA was dialyzed against peptide p14 at 4 °C for 12 h prior to recording the spectra. The concentration of DNA solutions used was 20 µg/ml. The spectra were recorded at 260 nm over a temperature range 25–95 °C with 1 °C increment using corresponding blanks. Thermal denaturation temperature (apparent T_m) was calculated using van't Hoff equations [31]. The precision in values estimated in triplicate was within ±0.05 °C.

Far-UV circular dichroism spectroscopy

Circular dichroism measurements were performed from 190 to 260 nm at 25 ± 1 °C using a Jasco J-810 automatic recording circular dichroism spectropolarimeter fitted with a xenon lamp, calibrated with d-10-camphor sulfonic acid. The instrument slits were programmed to give 1-nm bandwidth. Peptides were also studied in the range of 0.3–0.5 mg/ml dissolved in 10 mM PBS pH 7.4 for far-UV CD spectra. Spectra were collected with a scan speed of 10 nm/min with at least three accumulations for each

sample. The mean residue ellipticities were calculated using a mean residue weight of 115. Secondary structure analysis of the data was done according to Yang et al. [32]. The molar ellipticity values [θ] are calculated according to equation:

$$[\theta] = [\theta]_{obs} \times MRW/10 \times d \times c \quad (1)$$

where $[\theta]_{obs}$ is the observed ellipticity (°), d is the path length of the cell (cm), c is the protein concentration (g/ml), and MRW is the mean residue weight of the protein.

Infrared spectroscopy

Fourier transform Infrared (FTIR) measurements of peptides were recorded at 8.2 μM for p10 and 5.3 μM for p14. Peptides and KBr were mixed and grinded well and the pellets were made. The pellets were kept between two Teflon spacers and were inserted into KBr windows and the scans were recorded. The spectra were obtained on a Nicolet 950 FT-IR spectrometer (Thermo-Nicolet, Madison, WI, USA) operated with Thermo-Nicolet OMNIC software (Version 5.2). Resolution was 1 cm^{-1} with 256 double-sided interferograms collected for each spectrum. Two levels of zero fill were employed. The double-sided interferograms were co-added, phase corrected (Mertz), apodized (Happ-Genzel), and fast-Fourier transformed. The spectra were deconvoluted and the nonlinear curve iterative curve fitting procedure was used to fit the Gaussian bands using Origin 7.0 software [33].

Results and discussion

The peptides with 10 and 14 amino acids containing sequences QKALNEINQF [p10] from the α_{S2}-casein f(78–89) and TKKTKLTEEEKNRL [p14] from α_{S2}-casein f(147–157) were synthesized. HPLC profiles and N-terminal sequencing of peptides show 98 % purity [19]. Peptides were freeze-dried and stored at −80 °C until further use.

The synthesized peptides were screened for biological activity. These synthesized peptides were found to be multifunctional with the various biological activities as shown in the Table 1. The table shows IC_{50} values for various biological activities of the synthesized peptides. IC_{50} value of ACEI activity of p10 was found to be 20 nM compared to that of 60 nM for the standard ACE inhibitor. p10 is three times more potent compared to the standard ACE inhibitor. The antioxidant activity of p10 is 50 nM compared to ascorbic acid with 150 nM. p10 is 3 times more potent compared to ascorbic acid, a well-known antioxidant. Similar experiments when performed with the p14 shows the IC_{50} values for ACE inhibition and antioxidant activity as 37 and 6 nM, respectively. The IC_{50} values are comparatively lower than the standards like ACE inhibitor and ascorbic acid of 60 and 150 nM, respectively. Peptides are 1.6 times and 25 times more active compared to the standards. Peptides were studied for the inhibition of prolyl endopeptidase enzyme activity. The p10 and p14 show considerable inhibition with values being 6 and 58 nM, respectively. The standard *Z*-prolyl-prolinal shows 500nM.

Peptide shows ACE inhibition, antioxidant, and PEP inhibition activities. Multifunctional behavior for peptides from milk and other proteins/peptides was reported by Hou et al. [4, 24]. The presence of phenylalanine at the C-terminal end for p10 may be the reason for ACE inhibitor activity and PEP inhibition activity [34]. The hydrophobicity and molecular bulkiness in p10 was also one of the reasons for the ACE inhibition and PEP inhibition activity of peptides [35]. Peptide 14 ACE inhibition is perhaps due to the charged amino acids present in the C-terminal end of the sequence which might bind to zinc metal present in the active site of the ACE enzyme and reduce the activity. Antioxidant activity depends mainly on basic amino acids present in peptides [36–38]. Peptide p14 has charged and basic amino acids that might contribute to the enhanced antioxidant activity compared to peptide p10.

Peptides p10 and p14 show no biological activity for the following assays like, ribonuclease inhibition, red blood cell agglutination assay, inhibition of mammalian lipoxygenase, effect of aflatoxin release by *Aspergillus* sp., and antifungal activities.

The other important biological activity identified in these peptides is antimicrobial activity. Peptides were analyzed for the growth-inhibiting nature of the microorganisms. Peptides were studied for antimicrobial activity against *B. cereus, S. aureus, L. monocytogenes*, and *H. pylori.* Peptide p10 inhibits *B.* cereus at 2×10^4 and 2×10^5 CFU/ml. The percent survival was <0.5 % at peptide concentration of 1.5 mg/ml and indicates efficient bactericidal effect. At higher cell numbers of 2×10^6 CFU/ml, the peptide is still effective in preventing the bacteria from growing. MIC value of peptide has been calculated based on the lowest concentration required to efficiently inhibit the growth of bacteria (Table 2).

Similar studies using p14 on different bacteria were carried out. The p14 shows inhibition on *B. cereus, Listeria*, and *Staphylococcus*, respectively. Peptide MIC values are shown in the Table 2. Bacitracin is effective on all bacteria studied. MIC values of peptides along with bacitracin were calculated and values are shown in Table 2. Similar multiple antimicrobial activities of the synthesized peptides were reported earlier in casein by McCann et al. [3, 9, 39].

Table 1 Biological activity and IC_{50} values of the synthesized peptides from α-casein

Assay	Peptide 10 (nM)	Peptide 14 (nM)	Standard	IC_{50} (nM)
Angiotensin-converting enzyme inhibition assay	20 ± 2	37 ± 2	ACE inhibitor from Sigma	60 ± 5
Antioxidant activity (DPPH)	50 ± 5	6 ± 0.2	L-Ascorbic acid	150 ± 7
Prolyl endopeptidase activity (PEP)	5.8 ± 1	36 ± 2	Z- prolyl prolinal	500 ± 20

Values represent mean ± SD from three measurements

Table 2 The antimicrobial activity of synthetic peptides from α-casein

Organism name	Peptide 10 (MIC) μM	Peptide 14 (MIC) μM	Standard name	Standard (MIC) μM
B. cereus	0.87 ± 0.02	2.99 ± 0.3	Bacitracin	0.12 ± 0.01
S. aureas	1.75 ± 0.5	2.3 ± 0.7	Bacitracin	0.12 ± 0.01
L. monocytogenes	1.75 − 2.38	2.99 ± 0.9	Bacitracin	0.12 ± 0.01
H. pylori	0.083 − 0.25	NAD[a]	Amoxicillin	0.13 ± 0.01

Values represent mean ± SD from *n* experiments or measurements

[a] No activity detected

Table 2 shows the antimicrobial activity of peptides on *H. pylori*. The activity was assessed by observing the zone of incubation and calculating MIC values. The table shows inhibition of *H. pylori* by p10 with an IC_{50} value of 0.25 μM. Peptide p14 has no effect on *H. pylori*. The standard amoxicillin was also measured and shows an IC_{50} value of 0.13 μM. Figure 1 shows zone of inhibition of bacteria in the presence of p10. Peptide p10 and the amoxycillin show inhibition as can be observed from the figure. There are reports available in the literature on the inhibiting potential of bovine milk peptides and proteins on *H. pylori* [40, 41]. The mechanism of action by these peptides is under study.

Peptides were also checked for biological activities against methicillin-resistant *Staphylococcus aureus* (MRSA) strains. There was no biological activity observed in this case by any peptides.

The antimicrobial peptides were studied for their mode of action [42]. Peptides were studied for their cell-penetrating behavior. Peptides were studied in the presence of fluorescence membrane potential-sensing probe DI-$BAC_4(3)$ to monitor the effect of peptides on the bacterial membrane potential. Hyperpolarization of bacterial cell membrane decreases fluorescence [43]. The significance of this dye is that it senses the changes in the membrane potential, which is an indication of the movement of peptide into the cell membrane. This dye enters cells and binds to intracellular proteins or membranes and exhibits enhanced fluorescence. Increased depolarization results in further influx of the dye and an increase in fluorescence. The study using this dye is shown as Fig. 2. Gram-positive organisms *B. cereus* and *S. aureas* were selected based on the potency of the peptides in this study. Figure 2a shows influence of peptide on *Bacillus* as studied by the fluorescent probe. The fluorescence intensity changed from 162 to 190 AU in 15 min. Peptide p10 shows a 20 % change in fluorescence intensity whereas p14 does not show any change in RFI. The increase in the fluorescence is possible due to penetration of the probe into membrane and further due to changes in the electrostatic potential across the membrane. The figure shows that peptide p10 changes membrane potential i.e., depolarization, as seen in the increase of fluorescence whereas peptide p14 does not cause any change in the fluorescence intensity and suggesting that it does not change membrane potential. This indicates that the peptide p10 moves across the cell membrane as opposed to peptide p14.

Similarly, changes in the membrane potential of *S. aureas* have been studied (Fig. 2b). The fluorescence intensity increases from 197 to 206 AU in 15 min. The change in the fluorescence is 9 %, which is an indication of the changes in the location of the dye. Peptide p14 does not show any change in RFI in 15-min time. Figure 2b suggests that in the presence of p10 the membrane is getting depolarized whereas the p14 shows no effect on the membrane. This shows that the p10 is damaging the membrane (depolarized) of cells under study as studied with $DiBAC_4(3)$ [43]. p10 shows similar mechanism on both the bacteria. p14 does not have any effect on the bacterial membranes.

In order to study the binding of p10 and p14 to DNA, *Bacillus* genomic DNA was isolated and incubated with the peptide p14 for 2 h. After incubation with the peptide, DNA was run on an agarose gel and the change in the mobility of DNA was observed. The gel shift mobility assay shows changes in the mobility of the DNA when

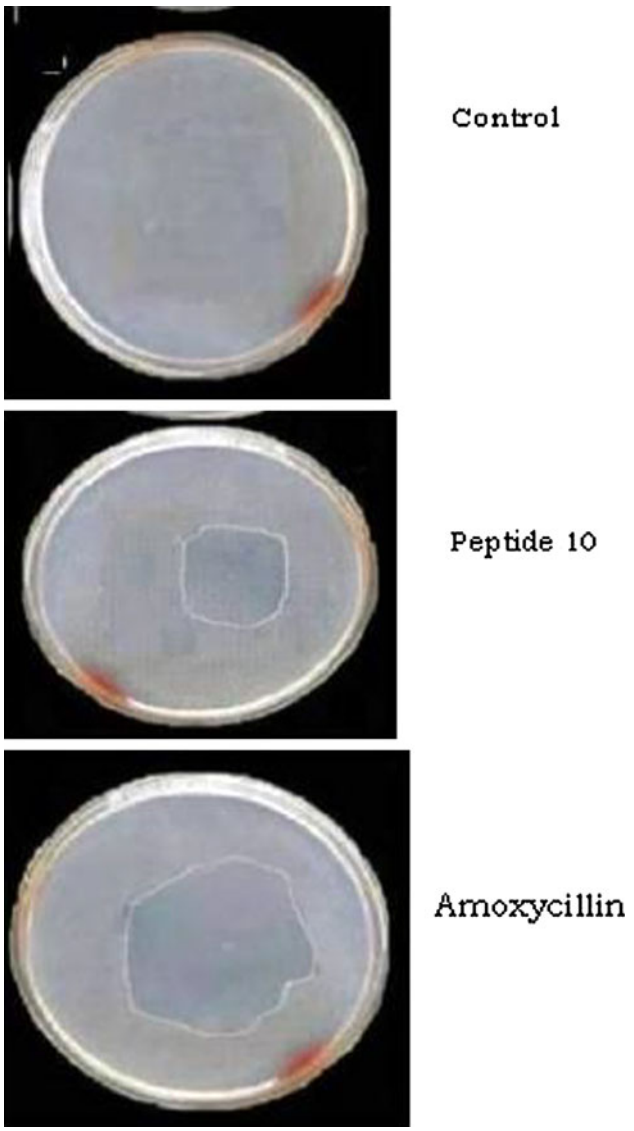

Fig. 1 The growth inhibition of *H. pylori* in the presence of peptide p10. Sterile filter paper disks containing peptide 10 were applied to the surface of F12 nutrient agar plates (which were seeded with test *H. pylori* from 2–3 days old culture) and incubated at 37 °C for 3 days. To each filter paper disks, 75 µl of peptide and amoxicillin as standard were added. After incubation, the plates were observed for diameter of the clear zone around the disk in the bacterial lawn and expressed as the antimicrobial activity of the peptides

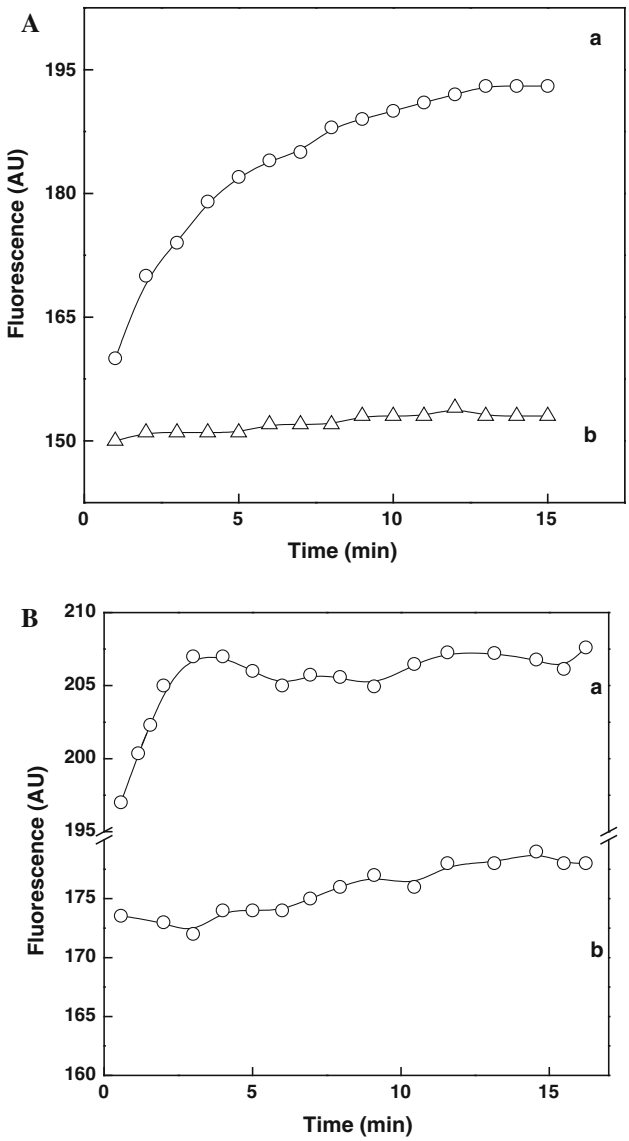

Fig. 2 Studies on membrane polarization: **a** *B. cereus* in the presence of *a* p10 and *b* p14. **b** *S. aureas* in the presence of *a* p10 and *b* p14 *B. cereus* was grown in half-concentrated nutrient broth to an absorbance 600 nm of 0.5 OD and incubated with 1 µM of the membrane potential-sensitive fluorescent probe bis-(1,3-dibutyl barbituric acid) trimethine oxonol [$DiBAC_4$ (3)], for 15 min. Peptide (10 mg/ml) was added to the cell suspension and the change in the relative fluorescence intensity of the oxonol dye was followed at 492 and 515 nm excitation and emission wavelengths, respectively

peptide is bound. The gel shown in Fig. 3 inset suggests that the control DNA and peptide p10 show no change in mobility, whereas DNA incubated with p14 shows retardation in movement and subsequently stays back in the well. The retardation of Bacillus DNA by p14 shows that peptide binds to DNA, which is an indication of intrinsic DNA-binding ability of peptide [17].

Binding of peptide to DNA was further studied by time kinetics on UV spectrophotometer. Figure 3 shows the binding of p14 to DNA as studied by time kinetics mode on spectrophotometer. The kinetics is monitored at 260 nm for the changes in the DNA upon binding with peptide after addition. The study was carried out for 15 min and the change in the absorbance was observed as a function of time. As shown in the figure, absorbance of DNA reduces as a function of time. There is a decrease in the absorbance of *Bacillus* DNA and calf thymus DNA, respectively, over 15-min time. The change in absorbance was fit with the first order and the resultant rates for microbial DNA and

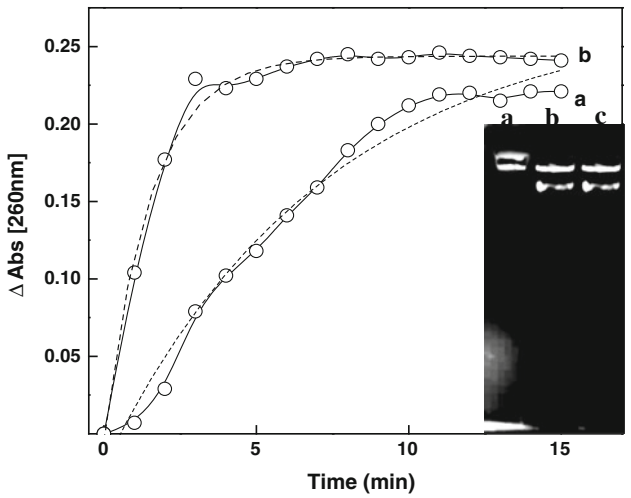

Fig. 3 The binding of p14 to DNA as studied by time kinetics mode using UV spectrophotometer. **a** Binding of p14 to *B.cereus* genomic DNA, **b** Binding of p14 to calf thymus DNA. The dashed line indicates the first-order exponential fit of the data as generated using Origin 7.0. The concentration of DNA used was 20 μg/ml. *Inset* Gel shift mobility assay of peptide interaction with *B. cereus* DNA as studied on 0.8 % agarose gel electrophoresis (*a*) DNA with p14 (*b*) control DNA. (*c*) p10

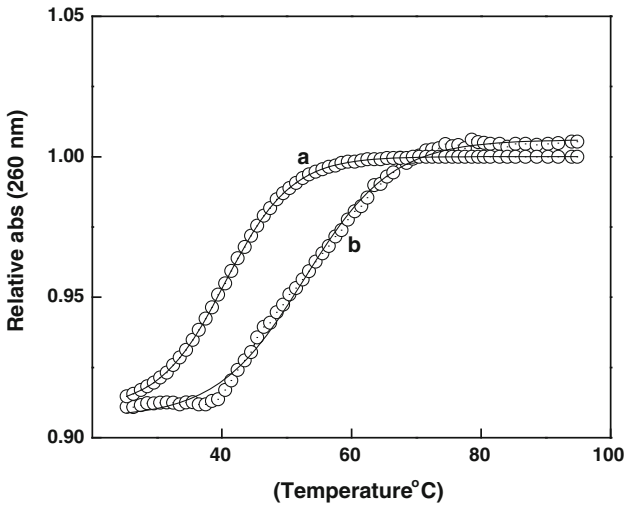

Fig. 4 UV melting curves of *B. cereus* DNA in the presence of peptide. *a* Control DNA and *b* DNA in the presence of p14. The concentration of DNA used was 20 μg/ml

Table 3 Thermodynamic parameters of peptide binding to DNA

Thermodynamic parameters	DNA control (kcal/mol)	DNA in the presence of peptide 14 (kcal/mol)
ΔG	−1.5	−1.3
ΔH	−23.3	+100
$-T\Delta S$	+22	−62

The parameters were calculated from thermal-melting UV profiles

calf thymus DNA are 7 and 1.5 s^{-1}, respectively. The binding to calf thymus DNA by p14 is much faster compared to binding to microbial DNA. The decrease in the absorbance may be due to the binding or aggregation of bacterial DNA upon interaction with p14. Yu et al. [30] carried out similar experiments with amyloid β-peptide and DNA. According to their studies, the binding of amyloid β-peptide with DNA reduces the DNA absorbance. This according to them is due to the condensation of DNA. DNA condensation can influence gene expression and transcription in cells.

For example, lysine and arginine can make two H-bond contacts to guanine, and asparagine and glutamic acid can contact Adenine through two H-bonds. Arginine and lysine residues facilitate the binding of peptides by electrostatic interactions [44]. The presence of lysine and arginine in the p14 can lead to binding of p14 to DNA as observed by the studies using the mobility shift and the time kinetics on the spectrophotometric studies. In the case of peptide p10 there is no binding which could be due to the presence of one lysine residue. The change in the rate upon binding to two different DNA could be due to the differences in the GC content of bacterial and calf thymus DNA (33 and 42 % GC content, respectively) or could be due to the complexity in the coiling of the mammalian and bacterial DNA [45].

Thermal profile of *B. cereus* DNA in the presence and absence of p14 was studied to understand the binding energetics (Fig. 4). The T_m of the *B. cereus* DNA was found to be 40 °C whereas in the presence of p14, the T_m changed to 53 °C. We observe a shift in T_m to 13 °C, which indicates the denaturation of DNA in the presence of p14. The thermodynamic parameters ΔG, ΔH, and $-T\Delta S$ were calculated for the control DNA and DNA–p14 complex and are depicted as Table 3. The thermodynamic values indicate changes to DNA and also indicate that peptide might be a groove binder. Similarly, Chaires [46] had reported the comparison of the binding enthalpy (ΔH) against binding entropy ($-T\Delta S$) for 26 drug–DNA interaction model compounds and indicated that groove-binding interactions have favorable entropy contributions to the free energy. Groove binding is predominantly entropically driven.

The microbial activity of peptides is due to their cationic nature and rigidity in structure and hydrophobicity [47, 48]. The presence of arginine and the charged amino acids contribute to antimicrobial activity. The factors that contribute to the antimicrobial activity like charge, hydrophobicity, hydrophobic moment, conformation, and the nature of the peptides are shown for the two peptides in Table 4. This shows that peptide p10 is more hydrophobic than p14 whereas p14 is cationic in nature. The calculation of hydrophobic moment for peptides can help us predict

Table 4 Structural parameters for peptides

Sequences	No. of residues	Molecular weight	Hydrophobicity	Hydrophobic moment (μ)	Charge
QKALNEINQF	10	1,204.6	−0.850	0.636	0
TKKTKLTEEEKNRL	14	1,717.9	−2.043	0.349	+2

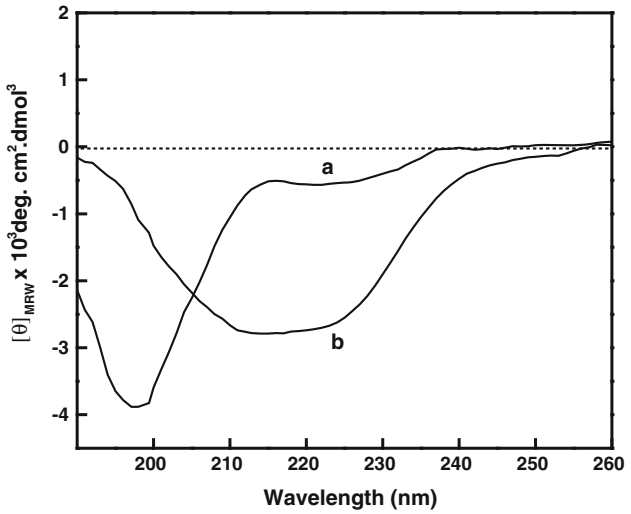

Fig. 5 Far-UV CD spectra of two peptides. The peptides were scanned from 190 to 260 nm with a concentration of 0.4 mg/ml. *a* p10, *b* p14

whether the peptides can form an amphipathic helix. Hydrophobic moment was calculated by using a software available on the web, *hmoment* [48]. Based on this analysis peptide p10 appears to be membrane-liking or surface-liking peptide with a hydrophobic moment of 0.636, whereas peptide p14 has a much lower hydrophobic moment of 0.349, which suggests that peptide p14 may not have any activity on cytoplasmic membranes.

The structure–function relationship of peptides was studied by characterizing the peptides using far-UV circular dichroism spectroscopy. Figure 5 shows the CD spectra of peptides. The spectrum shows that peptide p10 has negative minima at 200 and 235 nm. The presence of such minima suggests the formation of beta turns. Peptide p14 is having negative minima at 215 and 245 nm. This peptide can be a beta sheet-rich peptide. The secondary structure estimation of peptides is shown in Table 5. The secondary structural characteristics of peptide p10 are 7 % alpha helix, 50 % beta structures, and 43 % aperiodic; and that of peptide p14 are 0 % alpha helix, 73 % beta structures, and 27 % aperiodic.

Peptides were also analyzed for their secondary structural parameters by IR. The secondary structural contents of peptides were done by FTIR spectra. The iterative curve fitting of the Gaussian bands for peptide p10 shows bands at 1,553; 1,630; and 1,700 cm^{-1}; respectively. The bands

Table 5 Secondary structural data of peptides as analyzed by CD and IR

	Secondary structure (%)		
	α-helix	β-structures	Aperiodic
Peptide 10	7 ± 1 %	50 ± 2 %	43 ± 2 %
Peptide 14	0 %	73 ± 3 %	27 ± 2 %

	Secondary structure (%)			
	α-helix	β-sheets	β-turns	Aperiodic
Peptide 10	1,553 cm^{-1}	1,630 cm^{-1}	1,700 cm^{-1}	Nil
Peptide 14	Nil	1,663 cm^{-1}	1,517 cm^{-1}	Nil

corresponding to 1,553 cm^{-1} can be due to the alpha helix. The bands corresponding to 1,630 and 1,700 cm^{-1} are due to the presence of beta sheets or beta turns in peptides [33]. A similar study on p14 shows bands at 1,663; 1,517; and 1,674 cm^{-1}. The bands corresponding to 1,663 cm^{-1} can be due to the beta sheets. The bands at 1,674 cm^{-1} can be due to the TFA presence in the peptide. The bands corresponding to 1,517 cm^{-1} can be due to the type II beta turns.

Multifunctional peptides are peptides with more than one biological activity and this is due to the banding sequences present in the parent protein. Peptides QKALNEINQF and TKKTKLTEEEKNRL show more than one biological activity. Multifunctional nature of peptides has been previously reported in the literature [4, 49]. This behavior is due to overlapping peptide sequences that exert different biological activities. The regions are called as "strategic zones," which are partially protected from proteolytic breakdown [50, 51]. Second, adopting a very flexible conformation and dynamic structural conformations in different environments could be another reason for the multifunctional behavior in these peptides.

Conclusions

From this we can conclude that peptides from α-casein are found to possess multifunctional biological activity. Peptides show ACE inhibition, PEP inhibition, antioxidant activity, and antimicrobial activity. Peptides activities were compared with their respective standards and peptides were found to be more potent compared to the standards. The

p10 antimicrobial activities could be due to cell depolarization whereas antimicrobial activity of p14 could be due to binding to DNA. The structural studies show very less ordered secondary structure in p10 whereas p14 is a beta-rich peptide. These biological activities of peptides may be due to the flexible conformations of the peptides.

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