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ORIGINAL PAPER

ACE- inhibitory and radical scavenging activities of bioactive peptides obtained from camel milk casein hydrolysis with proteinase K

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Abstract The aim of this study was to evaluate the effects of enzymatic hydrolysis of camel whole casein on their antioxidant and angiotensin-converting enzyme (ACE)-inhibitory properties. Whole camel casein was hydrolyzed by proteinase K (PK), and the hydrolysates were fractionized by ultrafiltration membranes into three fractions. Semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC) was used to differentiate the mixture of peptides in the 3 kDa permeate fractions. A fraction (F4) with potentials of ACE-inhibitory activity (IC50 = 73 $\mu g.mL^{-1}$) and radical scavenging activity (IC50 = 6.8 $\mu g.mL^{-1}$) was selected for further purification and fractionation. The fraction F4C obtained from a second step purification of F4 showed strong ACE-inhibitory activity (IC_{50} = 36 $\mu g.mL^{-1}$) as well as radical scavenging activity (IC_{50} = 3.3 $\mu g.mL^{-1}$). The results of this study suggest that whole camel casein can be considered as a promising source for the production of peptides with potential of ACE-inhibitory and antioxidant activities.

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1 Introduction

Milk proteins and milk protein-derived peptides have important roles as human nutrition and promotion of health (Korhonen and Pihlanto 2007). These proteins are among the main sources of bioactive peptides, which are generally latent within the sequence of food proteins (Clare and Swaisgood 2000). When these peptides are released, either during the food processing (by enzymatic hydrolysis or fermentation) or during the gastrointestinal digestion from parent proteins, they may exhibit important biological functions such as antioxidant, antimicrobial (Salami et al. 2010), antihypertensive (Quan et al. 2008), opioid (Shabo and Yagil 2005), mineral binding (Gorban and Izzeldin 1997), hypocholesterolemic (Ali et al. 2013), immunemodulatory (Takeda et al. 2011), and anti-proliferative activities (Habib et al. 2013).

It has been elucidated that many peptide sequences in milk proteins have angiotensin-converting enzyme (ACE)-inhibitory activity (Jrad et al. 2014; Otte et al. 2007). The angiotensin I-converting enzyme (ACE, EC 3.4.15.1) is a dipeptidyl carboxypeptidase which regulates the blood pressure (Hooper 1991). ACE may inactivate the bradykinin (a potent vasodilator) by removing its carboxyl terminal phenylalanyl-arginine dipeptide. On the other hand, by removing histidylleucine dipeptide from angiotensin I (an inactive decapeptide), ACE catalyzes the formation of angiotensin II (a potent vasoconstriction octapeptide) (Skeggs et al. 1957). Thus, inhibition of this dipeptidyl carboxypeptidase can significantly drop the blood pressure.

Although the bioactivity of the milk-derived peptides from different sources has been extensively studied, only few attempts have been made to investigate the peptides from camel milk proteins (Jrad et al. 2014; Moslehishad et al. 2013; Salami et al. 2011). Jrad et al. (2014) hydrolyzed the camel milk protein by pepsin and pancreatin and evaluated the antioxidant and ACE-inhibitory activities of resulting peptides. Moslehishad et al. (2013), in their study, compared ACE-inhibitory and antioxidant activities of peptide fractions obtained from fermented bovine and camel milk. Salami et al. (2011) studied the effects of digestive enzymes on releasing the ACE-inhibitory and antioxidant peptides from camel milk caseins. Nevertheless, there is no available data concerning the biological functions of camel casein-derived peptides produced by a microbial enzyme, e.g., proteinase K (PK). Due to the presence of many hydrophobic amino acid residues in the camel caseins, which are potential cleavage sites for PK, this microbial enzyme has been considered a potential enzyme for hydrolysis of camel caseins. This study was conducted to investigate the ACE-inhibitory and radical scavenging potential of the peptides from enzymatic hydrolysis of camel milk caseins.

2 Materials and methods

2.1 Materials

Fresh camel (*Camelus dromedarius*) milk was collected from 10 local camel farms, and the samples were transported to the laboratory under sterile conditions. PK from

Tritirachium album, bovine serum albumin (BSA), 6-hydroxy-2,5,7-8-tetramethylchromane-2-carboxylic acid (Trolox), butylated hydroxyanisole (BHA), vitamin C (ascorbic acid), 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), lung acetone powder from rabbit as a source of ACE and ophtaldehyde (OPA) were purchased from Sigma Aldrich (GmbH, Munich, Germany).

2.2 Preparation of protein and protein hydrolysates

Camel whole casein was prepared according to the method described earlier (Salami et al. 2011). Briefly, camel milk was skimmed by centrifugation (5000 × g, 15 min at 4 °C). The pH value was then adjusted to 4.6. Keeping the solution at 37 °C for 30 min, the caseins were precipitated. The precipitates were separated from the transparent supernatant containing whey proteins by using centrifugation (5860 × g, 60 min at 4 °C). The latter procedure was repeated three times. Then, the obtained caseins were lyophilized using an Alpha1-2 Lo plus, Christ, Germany, lyophilizer and stored at −20 °C for further analysis.

To produce bioactive peptides, enzymatic hydrolysis was conducted using PK. Camel casein (1250 mg) was dissolved in 50 mL of 20 mM phosphate buffer (25 mg.mL^{-1}). The hydrolysis was initiated by adding PK at pH 6.8, T = 37 °C, and the ratio of enzyme to substrate was 1:250 (*w*/*w*). After each hydrolysis reaction, the mixture was heated (85 °C for 15 min) to inactivate the protease. The precipitates were then removed by centrifugation of protein hydrolysates (10,000 × g, 10 min at 4 °C), and the supernatant were collected. The latter contained the peptides. The peptides were then fractionized by ultrafiltration (UF) membranes (Amicon Ultra-15, Millipore, cutoff of 10, 5, and 3 kDa, Carrigtwahill, Co. Cork, Ireland) into three fractions. The details of fractions are given in the following sections.

2.2.1 Degree of hydrolysis

Degree of hydrolysis (DH) was determined using OPA solution (Church et al. 1983). For preparing the OPA solution, 40 mg OPA was dissolved in 1 mL methanol; this was added to 25 mL of 100 mM tetraborate. After adding 2.5 mL SDS 20% (*w*/*w*) and 100 μL β-mercaptoethanol, the resulting solution was diluted into 50 mL using deionized water. Fifty microliters of the samples were then added to 950 μL of OPA reagent and after 2-min incubation, the absorbance of OPA was measured by UV-Vis spectrophotometer (Shimadzu, Model UV-3100, Kyoto, Japan) (λ = 340 nm).

DH was calculated by the following equation:

$$\mathrm{DH}(\%) = (n/N) \times 100$$

where N is total number of peptide bounds in the substrate, and n is the number of peptide bounds hydrolyzed in a given time.

2.2.2 Purification of ACE-inhibitory and radical scavenging activity peptides

After fractionation of the peptides by UF membranes, semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC) (CECIL 1100 series HPLC, UK) on an ODS C18 column (10 UM, L = 250 mm, ID = 10 mm) equipped with a UV detector was

used to further fractionize the mixture of the peptides with molecular weight (MW) lower than 3 kDa present in a fraction which exhibited significant ACE-inhibitory and radical scavenging properties. Gradient elution was conducted by a mixture of buffer A, 10% acetonitrile containing 0.1% trifluoroacetic acid (TFA), and buffer B, 80% acetonitrile containing 0.1% TFA, at a flow rate of 1.5 mL.min^{-1}. The absorbance was monitored at $\lambda = 215$ nm. Different fractions were collected according to the profile given in Fig. 1 and then lyophilized. Then, they became lyophilized and their activities were measured. For further purification, the most active fraction was subjected again to the column (mobile phase flow rate = 1 mL.min^{-1}). Peptide purity was evaluated by the chromatograms from RP-HPLC analysis of sample on an ODS C18 ($L = 250$ mm, ID = 4.6 mm) column at a flow rate of 1 mL.min^{-1} and under identical elution conditions.

2.3 ACE-inhibitory activity assay

The ACE-inhibitory activity of the samples was measured using the method described previously (Shalaby et al. 2006; Vermeirssen et al. 2002). Three grams rabbit lung acetone extract was dissolved in 3 mL, 50 mM Tris-HCl buffer (pH 8.3) containing 5% (*v*/*v*) glycerol, and the mixture was stirred over night at 4 °C. By ultra-centrifugation of the mixture for 40 min at 40,000*g*, a supernatant with high ACE activity was obtained. For ACE-inhibitory activity measurement, 5 μL of ACE extract and 20 μL of peptide solution or deionized water were added to each of ELISA wells. In addition, 110 μL of FAPGG (1.2 mM) dissolved in 50 mM Tris-HCl buffer containing 400 mM NaCl and adjusted to pH 8.3, was added to each well. The decrease in the absorbance at 340 nm was measured using spectrophotometer equipped with an ELISA reader (for 20 min at 37 °C). The decreases in the absorbance values (ΔA340 nm.min^{-1}) were recorded. The ACE-inhibitory activity of each peptide samples was tested after adjusting the pH values to 8.0. ACE-inhibitory activity was calculated according to Eq. 1:

$$\text{ACE inhibition } (\%) = [1-(\delta A \text{ inhibitor}/\delta A \text{ control})] \times 100 \quad (1)$$

where δA inhibitor and δA control represent the slopes of the decrease in absorbance at 340 nm in the test and control samples, respectively. The ACE-inhibitory

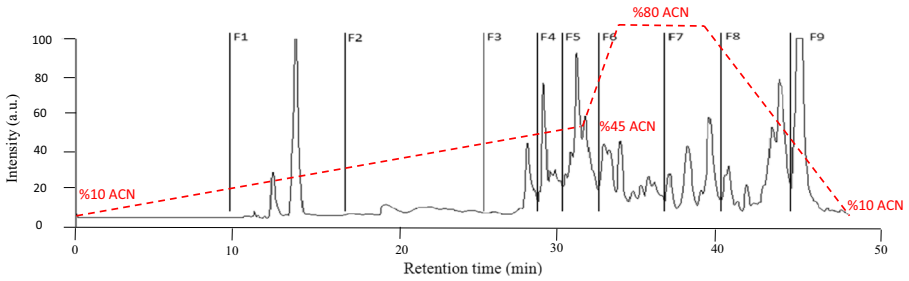

Fig. 1 A representative RP-HPLC chromatogram during fractionation of 3 kDa permeate fraction obtained from cleavage of camel milk casein by proteinase K. For elution of the fractions from ODS C18 column (10 UM, $L = 250$ mm, ID = 10 mm), buffer A (10% ACN containing 0.1% TFA) and buffer B (80% ACN containing 0.1% TFA) were used at a flow rate of 1.5 mL.min^{-1}. The eluted fractions were monitored by UV detector at 215 nm wavelength

potencies of the samples were expressed as the concentration of peptides ($\mu g.mL^{-1}$) required inhibiting the ACE activity by 50%.

2.4 Radical scavenging activity measurement using ABTS assay

Radical scavenging activity of the peptide samples were measured using spectrophotometry as described by Re et al. (1999). ABTS with concentration of 7 mM was prepared. This was then oxidized by adding potassium persulfate (2.45 mM) and kept in a dark room for 12–16 h. The solution was diluted with phosphate-buffered saline (PBS, pH 7.4) to reach the absorbance equal to 0.70 ± 0.02 at wavelength 734 nm. After adding 10 μL of peptide samples to 1 mL of the ABTS radical solution, the absorbance values were monitored continuously at wavelength 734 nm for 6 min. The decreases in the absorbance values were used for calculating the radical scavenging activity. The latter was expressed as the concentration of inhibitory peptides ($\mu g.mL^{-1}$) required quenching the absorbance of the radical cation by 50% (IC_{50}). Trolox, BHA and vitamin C were considered as controls.

2.5 Statistical analysis

Results of the present study were expressed as mean values ± standard deviations. The significance of the differences between the values was determined with *t* test (*P* value <0.05) using SPSS version 15.0.

3 Results and discussion

3.1 ACE-inhibitory activity

By addition of PK to whole camel casein, the DH increased significantly till 60 min and then reached a constant value (data not shown). At the end of enzymatic reaction, the DH value was about 12%.

To investigate the bioactivity of produced peptides from enzymatic hydrolysis of camel milk caseins, the casein hydrolysates was subjected to the UF membranes with the cutoff of 10, 5, and 3 kDa, and fractionated into three portions (3 kDa permeate, 3 kDa retentate, and 5 kDa retentate). The obtained fractions showed high ACE-inhibitory activity ($IC_{50} = 91–157$ $\mu g.mL^{-1}$) in comparison with parental unhydrolyzed casein ($IC_{50} > 600$ $\mu g.mL^{-1}$ protein) (Table 1). The permeate with MW 3 kDa and the retentate with MW 3 kDa exhibited higher ACE-inhibitory activity ($IC_{50} = 91$ and 99 $\mu g.mL^{-1}$, respectively) than the retentate with MW 5 kDa ($IC_{50} = 157$ $\mu g.mL^{-1}$). The permeate fraction with MW 3 kDa was further subjected to semi-preparative RP-HPLC (Fig. 1). The higher concentration of acetonitrile (ACN) for elution shows the higher hydrophobicity. Thus, it means that F4 was more hydrophobic than F1–F3, but less hydrophobic than F5–F9. Nine fractions were collected, freeze-dried, and stored for biological activity analysis. Six of the collected fractions (i.e., F3–F8), exhibited ACE-inhibitory activity ($IC_{50} = 71–186$ $\mu g.mL^{-1}$) (Fig. 2). The concentration of primary protein

Table 1 ACE-inhibitory and radical scavenging properties of enzymatic hydrolysates from camel milk casein

Samples	ACE I inhibitory activity IC_{50} ($\mu g.mL^{-1}$)	Radical scavenging activity IC_{50} ($\mu g.mL^{-1}$)
Whole camel casein	No[c]	35.0 ± 0.3^{B}
5 kDa retentate	157 ± 11^{b}	14.0 ± 0.5^{A}
3 kDa retentate	99 ± 9^{a}	13.5 ± 0.4^{A}
3 kDa permeate	91 ± 20^{a}	12.6 ± 0.9^{A}

The data marked with different small letters have significantly different IC_{50} values for ACE-inhibitory activity from each other ($P < 0.05$). The data marked with capital letters have significantly different IC_{50} values for ABTS radical scavenging activity from each other ($P < 0.05$). Values represent average of three independent measurements ± standard deviation

No IC_{50} value was not observed up to 600 $\mu g.mL^{-1}$

that was subjected to hydrolysis was 25 $mg.mL^{-1}$ and the concentration of these nine fractions were F1 = 3.6 $mg.mL^{-1}$, F2 = 2.8 $mg.mL^{-1}$, F3 = 3.2 $mg.mL^{-1}$, F4 = 4.8 $mg.mL^{-1}$, F5 = 5.7 $mg.mL^{-1}$, F6 = 2.8 $mg.mL^{-1}$, F7 = 2.51 $mg.mL^{-1}$, F8 = 4.3 $mg.mL^{-1}$, and F9 = 1.4 $mg.mL^{-1}$. At least the final quantity of fraction F4C was equal to 2 $mg.mL^{-1}$. The results demonstrated that ACE-inhibitory activities of F3, F4, and F7 were the highest (IC_{50} = 79, 73, and 71 $\mu g.mL^{-1}$, respectively). F4 fraction was selected for further fractionation and subjected to the same RP-HPLC column. The fraction was then split into three portions (F4A, F4B, and F4C) (Fig. 3), and the bioactivity of the latter were measured (Table 2). Among those, F4C showed the highest ACE-inhibitory activity (IC_{50} = 36 $\mu g.mL^{-1}$). The purity of this fraction was verified by analytical RP-HPLC (Fig. 3).

The IC_{50} values of the peptide fractions obtained in the present study were in the range of 36–600 $\mu g.mL^{-1}$, compared with 0.03–0.23 $mg.mL^{-1}$, 21–684 $\mu g.mL^{-1}$, and 0.32–2.65 $mg.mL^{-1}$ for lysozyme hydrolysate, bovine casein hydrolysate, and peptides obtained from amaranth grain, respectively (Asoodeh et al. 2012; Jiang et al. 2010; Tovar-Pérez et al. 2009).

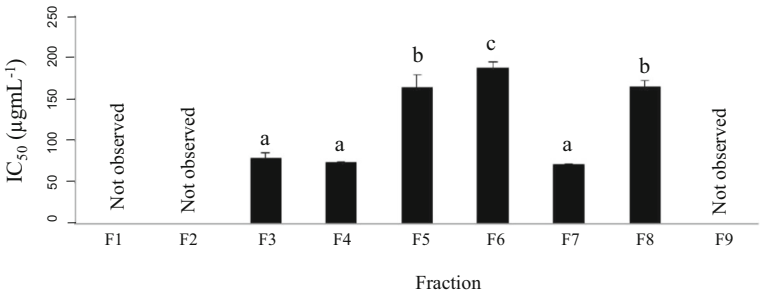

Fig. 2 ACE-inhibitory activity of the collected fractions expressed as IC_{50} (the concentration of inhibitory peptides needed to inhibit the ACE activity by 50%). IC_{50} values for F1, F2, and F9 fractions were not observed up to 300 $\mu g.mL^{-1}$. IC_{50} values are the average of three independent measurements and *error bars* are standard deviations of the experiments. The data marked with *different letters* have significantly different IC_{50} values from each other ($P < 0.05$)

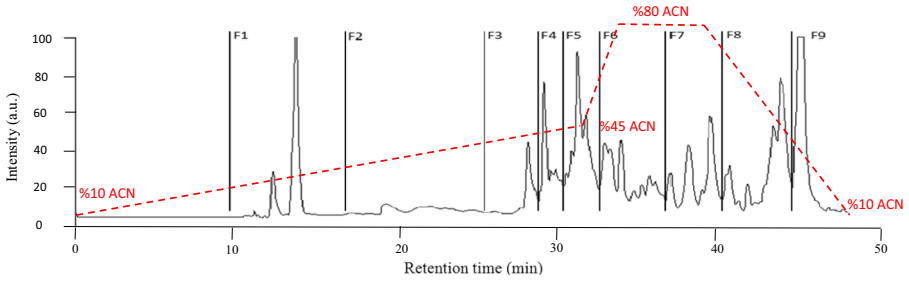

Fig. 3 A representative RP-HPLC chromatogram during fractionation of F4 by use of an ODS C18 column ($L = 250$ mm, ID = 4.6 mm). For elution of the fractions, buffer A (10% ACN containing 0.1% TFA) and buffer B (80% ACN containing 0.1% TFA) were used at a flow rate of 1.5 mL.min^{-1}. The eluted fractions were monitored by UV detector at 215 nm wavelength. Small insertion represents the chromatogram obtained by injection of F4C into the column

The high ACE- inhibitory activity of the camel casein hydrolysates corresponds to the high content of proline in camel milk (Moslehishad et al. 2013; Salami et al. 2011). Small peptides with a proline residue at carboxyl terminal have been found to possess strong inhibitory activity against ACE (Korhonen and Pihlanto 2007). It has also been reported that the presence of aliphatic and aromatic amino acid residues in the three carboxyl terminal positions of ACE-inhibitory peptides is important in enhancing their binding to the active site of ACE (Cheung et al. 1980). The results of the present study revealed that PK is very useful to liberate the bioactive peptides from camel milk casein. PK (EC 3.4.21.64) is a highly proteolytic but low specific serine protease. This microbial enzyme with strong affinity to the hydrophobic amino acid residues hydrolyzes proteins at peptide bounds adjacent to the carboxylic group of aliphatic and aromatic amino acids (Ebeling et al. 1974; Otte et al. 2007). Several anti-hypertensive peptides produced by PK have a proline residue at their carboxyl terminal (Abubakar et al. 1998). Therefore, the high ACE-inhibitory activity of the peptide fractions derived from camel milk casein seems to be due to their small size and the proline located at carboxyl termini.

Table 2 ACE-inhibitory and radical scavenging properties of the fractions obtained from F4

Samples	ACE-I inhibitory activity IC_{50} (μg.mL^{-1})	Radical scavenging activity IC_{50} (μg.mL^{-1})
F4A	317 ± 13^{b}	8.5 ± 0.2^{B}
F4B	No^{c}	$>100^{C}$
F4C	36 ± 2^{a}	3.3 ± 0.1^{A}

The data marked with different small letters have significantly different IC_{50} values for ACE-inhibitory activity from each other ($P < 0.05$). The data marked with capital letters have significantly different IC_{50} values for ABTS radical scavenging activity from each other ($P < 0.05$). Values represent average of three independent measurements ± standard deviation

No IC_{50} value was not observed up to 300 μg.mL^{-1}

3.2 ABTS radical scavenging activity

In this study, radical scavenging potencies of the samples were measured using the ABTS decolorization assay. The UF fractions exhibited high radical scavenging activities (IC_{50} = 12.6–14 μg.mL^{-1}) in comparison with the parental unhydrolyzed protein (IC_{50} = 35 μg.mL^{-1}) as shown in Table 1. There were no significant differences between the radical scavenging activities of the obtained fractions. Evaluation of the ABTS radical scavenging potency of the peptide fractions obtained from RP-HPLC showed that fractions with moderate hydrophobicity had the most potent inhibitory activity (Fig. 4). The result of this study is in agreement with study of Tang et al. (2009). The fractions F2–F7 showed higher radical scavenging activity than the parental 3 kDa permeate fraction (IC_{50} = 6.7–12 μg.mL^{-1}). On the other hand, F1, F8, and F9 exhibited lower activities (Fig. 4). Among the collected fractions from RP-HPLC, F4 and F5 (IC_{50} = 6.8 and 7 μg.mL^{-1}, respectively) exhibited the most potent scavenging activity against the radical. Because of its relatively high ACE-inhibitory and radical scavenging activity, F4 was selected for further fractionation and was subjected to the same column. As mentioned earlier, F4 was then divided into three parts, and the bioactivity of the obtained fractions were measured (Table 2). Among the fractions obtained from further fractionation of F4, F4C possessed the most potent radical scavenging capacity (IC_{50} = 3.3 μg.mL^{-1}). IC_{50} values of Trolox, BHA, and vitamin C were also calculated (Fig. 5). These IC_{50} values, as well as the IC_{50} value reported previously for vitamin E (α-tocopherol) (IC_{50} ≈ 15 μM or 6.4 μg.mL^{-1}) (Re et al. 1999), were used for comparison with IC_{50} values of our fractions. The results revealed remarkable ABTS radical scavenging activity of the obtained peptide fractions F3–F8 in this study. As shown in Fig. 5, F4C (IC_{50} = 3.3 μg.mL^{-1}) is comparable in ABTS radical scavenging activity with BHA (IC_{50} = 2.1 μg.mL^{-1}), vitamin C (IC_{50} = 2.1 μg.mL^{-1}), and Trolox (IC_{50} = 2.9 μg.mL^{-1}). Also, in comparison with α-tocopherol (IC_{50} ≈ 6.4 μg.mL^{-1}), F4C has a potent ABTS radical scavenging activity. Better understanding of the biological functions of these peptides requires in vivo tests. Some clinical and experimental studies provided convincing evidence that reactive oxygen species (ROS) play an important role in development of hypertension (Touyz 2004). Therefore, in vivo anti-hypertensive activity of the compounds such as F4C (showing both ACE-inhibitory and antioxidant properties) might be much higher than those expected for in vitro ACE-inhibitory activities.

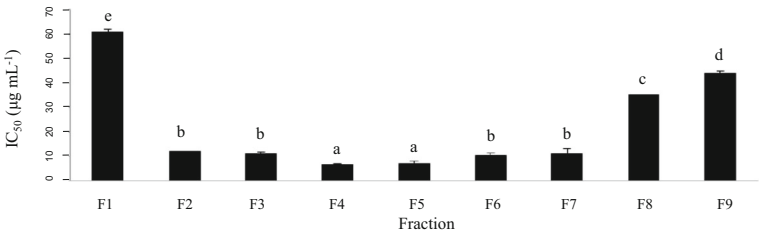

Fig. 4 ABTS radical scavenging activity of the collected fractions expressed as IC_{50} (the concentration of inhibitory peptides needed to scavenge the ABTS radical by 50%). IC_{50} values are the average of three independent measurements and *error bars* are standard deviations of the experiments. The data marked with *different letters* have significantly different IC_{50} values from each other ($P < 0.05$)

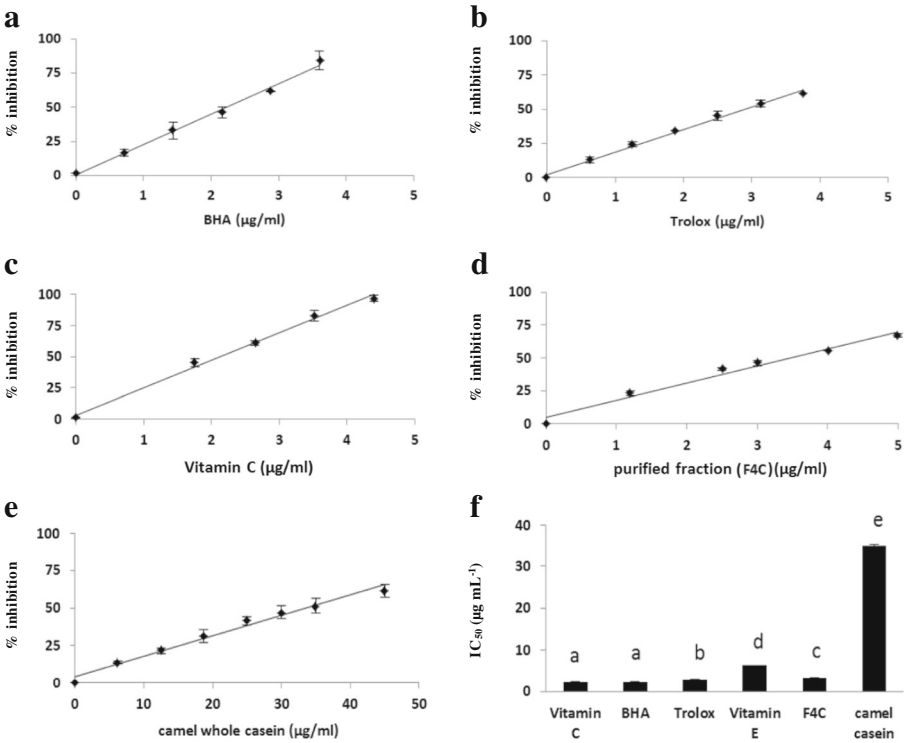

Fig. 5 Comparison of the radical scavenging activities of vitamin C (**a**), BHA (**b**), purified fraction F4C (**c**), Trolox (**d**), and camel casein (**e**). **f** Comparison between radical scavenging activities of vitamin C, BHA, Trolox, vitamin E, camel milk casein, and purified peptide from camel casein (F4C). The data marked with *different letters* have significantly different IC_{50} values from each other ($P < 0.05$). IC_{50} values are the average of three independent measurements and *error bars* are standard deviations of the experiments

4 Conclusion

The results of the present showed that PK is a proper enzyme for liberating peptides with ACE-inhibitory and radical scavenging properties from whole camel casein. Peptides produced by PK are heterogeneous in their biological activities; some of those like F4C show high ACE-inhibitory and antioxidant activity. ACE-inhibitory activity of F4C was almost the same as ACE-inhibitory peptides reported in the literature. In addition to its action on ACE, F4C has also high ABTS radical scavenging activity comparable to that of the most important antioxidants such as vitamin C, BHA, Trolox, and vitamin E. Present work demonstrates that camel casein can be used as a valuable source to produce bioactive peptides with high ACE-inhibitory and antioxidant activities. Comparison to other sources, the IC_{50} values of the peptide from the whole casein of camel milk was remarkable.

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