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Abstract

The effect of a successive *in vitro* hydrolysis by pepsin and pancreatin on the free radical scavenging activity of camel milk casein was investigated in order to assess the effect of gastro-intestinal digestion. Hydrolysis of camel casein was controlled by reversed-phase high performance liquid chromatography. Anti-oxidant activity was measured by the 2,2'-azino-bis-(3-ethylbensothiazoline-6-sulfonic acid) (ABTS) method. The Trolox equivalent antioxidant capacity (TEAC) values of camel casein and its hydrolysate were 1.6±0.12 μmol TE/mg protein and 0.25 μmol TE/μmol eq. NH_2, respectively. After digestion, the scavenging activity of the casein peptides was more efficient than those reported in the literature regarding digestive hydrolysates of camel milk, colostrum and whey proteins.

Key words: camel casein, protein enzymatic digestion, radical scavenging activity, antioxidant

Introduction

Oxidation is a vital process in all living organisms even though its side effects is the production of free radicals (Ren et al., 2008). The action mechanism of oxidation is related to the inactivation of reactive oxygen species (ROS) by scavenging of free radicals, chelation of pro-oxidative transition metals and reduction of hydroperoxides (Zhou et al., 2012). It is well documented that free radical formation is a major factor involved in the progression of many human diseases, such as heart diseases, stroke, atherosclerosis and cancer, which have been considered as the leading causes of human death (Dávalos et al., 2004).

Butylatedhydroxyanisol (BHA), butylatedhydroxutoluene (BHT) and propyl gallate are synthetic antioxidants used in foods to retard lipid oxidation (Saiga et al., 2003). However, their use is associated with possible health risks (Sakanaka et al., 2004). For this reason, interest in finding natural antioxidants representing an alternative to synthetic ones is high. So, the possible use of many non-enzymatic antioxidants present in fruits and vegetables (polyphenols, flavonoids, iso-flavanones, vitamins A, B, C, E and tocopherols) was well investigated (Kaur and Kapoor, 2001). In the last decade, the production of peptides having antioxidant properties by enzymatic hydrolysis of proteins from animal

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sources has been extensively reported in several studies. Bovine milk is considered as an interesting source of proteins able to generate by enzymatic hydrolysis or during microbial fermentation peptides displaying radical scavenging activity (Pihlanto, 2006).

Camel milk is the most common product in arid regions. The beneficial effects of camel milk, which include the prevention of diseases and conditions such as gastroenteritis, tuberculosis, diabetes and hypertension, have been demonstrated experimentally (Quan et al., 2008 ; Sboui et al., 2010). Therefore, improving the therapeutic value of camel milk has received great attention. Caseins constitutes 75-80 % of proteins of camel milk. Camel casein is very susceptible to proteolysis and able to generate peptides with various biological activities (Salami et al., 2008). Once ingested, one of the conditions for their action on health of the consumers is that the biological activity of these proteins is not impaired by the successive actions of digestive proteolytic enzymes in stomach and in intestine.

The aim of the present work was to assess the effect of an *in vitro* enzymatic digestion procedure mimicking human gastro-intestinal digestion, on the ABTS radical cation scavenging activity of camel caseins.

Materials and methods

Materials

Camel milk was provided from the experimental herd of the Arid Land Institute, Livestock and Wildlife Laboratory (Medenine, Tunisia). Pepsin (from porcine stomach mucosa, EC 3.24.3.1, specific activity of 3260 units/mg) and pancreatin (from bovine pancreas, activity equivalent to 8× U.S.P. specifications according to the supplier Sigma-Aldrich, EC 232-468-9) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals required for the assays including O-phtaldialdehyde (OPA); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid; 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were obtained from Sigma-Aldrich. All solutions prepared with double distilled water were kept at 4 °C until use.

Casein preparation

Casein were prepared as follows: skimmed camel milk pH was adjusted to 4.2 by the addition of 1 M HCl. The mixture was allowed to stand for 30 min at 30 °C and then centrifuged (5000×g, 30 min, 20 °C). The casein pellet was separated from the whey supernatant, re-suspended and then, washed with distilled water to remove any whey residue. This last operation was repeated twice. Finally, the caseins were obtained by centrifugation at 1500×g for 20 min at 20 °C and then dissolved with 1 M NaOH. In order to remove salts, the caseins were dialyzed (cut-off of dialysis membrane: 100-500 Da, SpectraPor, Spectrum Labs Inc., Rancho Dominguez, CA, USA) against Milli-Q water (Millipore, Bedford, MA, USA) at 4 °C for 48 h and freeze-dried.

Casein hydrolysis

Hydrolysis of camel milk casein was performed according to the *in vitro* enzymatic digestion conditions described by Parrot et al. (2003). First, to mimick gastric emptying, digestion was started by adjusting the pH value of camel milk casein (CN) solution (20 mg/mL) to pH=2.0 with 1 M HCl. Then, a 1 g/L pepsin solution in 0.01 M HCl was added to caseins in order to obtain a final enzyme-substrate ratio (E:S) of 1:200 (w:w). Camel casein was hydrolyzed by pepsin for 30 min at 37 °C in a shaking water bath (160 movements per min). Thereafter, as in human duodenum, peptic digestion products were digested by pancreatin at a slightly basic pH. After the pH of peptic digestion products was adjusted to 7.5, a 2 g/L pancreatin solution in Milli-Q water was added in order to obtain a final 1:400 (w:w) E:S ratio.The pancreatic hydrolysis of the peptic breakdown products was carried out for 4h at 37 °C. The enzymatic hydrolysis was stopped by heating the reaction volume at 85 °C for 5 min.

Ultrafiltration

Samples of casein hydrolysates (CNH) were then subjected to an ultrafiltration step. For this reason, a stirred ultrafiltration cell using membrane (76 mm of diameter) with molecular cut off sizes of 10 kDa (Amicon, Cells Millipore, Saint-Quentin en Yvelines, France) was used. The pressure applied was 2.6 10^5 Pa.The permeate was collected, demineralized by dialysis (100-500 Da c.o., SpectraPor, Spectrum labs inc, U.S.A) against 200 volumes of Milli-Q water during 48 h, lyophilized and kept at -20 °C until analysis.

Reversed-Phase High-performance Liquid Chromatography (RP-HPLC)

A volume of 50 μL containing 5 mg of lyophilized CN or CN-H was loaded onto a Lichrospher C_{18} analytical column (150 × 2 mm, 5-μm particle size, 10-nm porosity; Cluzeau, Sainte-Foy-La-Grande, France) connected to an Alliance HPLC unit 2690 (Waters Corp., Milford, MA, USA) equipped with a photodiode array detector DAD 996 (Waters). Peptides were then eluted for 90 min by a linear gradient from 5 to 50 % of acetonitrile in water and in the presence of 0.1 % trifluoroacetic acid. The flow rate was 0.25 mL min^{-1}, detection was carried out at 215 nm, and the separation was analysed with the Millennium 32 version 3.05.01 software (Waters).

O-phthaldialdehyde (OPA) and Bradford assays

The peptide concentration was measured at 340 nm using a MRX® microplate reader (Thermolabsystems, Chantilly, VA, USA) by the OPA micromethod adapted for small volumes from that of Frister et al.(1988). The calibration curve was established with leucine and the concentration was expressed in μmoL equivalent (eq.) NH_2.

The protein concentration was determined in triplicate by the Bradford method using bovine serum albumin (BSA) as protein standard and expressed as mg/L eq. BSA (Bradford, 1976).

Determination of casein hydrolysis degree

The degree of hydrolysis was calculated as described by Spellman et al. (2003):

$$DH\ (\%) = (n/p) \times 100 \qquad (1)$$

Where n is the average number of peptide bonds hydrolyzed, p the total peptide bonds per protein molecule.

$$n = \Delta A_{340\ nm} Md/\varepsilon c \qquad (2)$$

Where :

$\Delta A_{340\ nm}$ = the absorbance at 340 nm difference between the hydrolysate and the initial sample,

M - the total molecular weight of proteins (Da),

d - the dilution factor,

ε - the molar extinction coefficient at 340 nm (6000 M^{-1} cm^{-1}), and

c - the protein concentration (g/L).

As CN contained a heterogeneous mixture of many different proteins, the average number of peptide bonds per mole of CN and theaverage molecular mass of the proteins in CN were estimated as follows. Values were calculated taking the approximate protein content of CN in camel milk to be 31.5% of α_s–CN ; 65 % of β–CN and 3.5 % of κ–CN according to Kappeler (1998). Taking these values into account, the average molecular weight of the proteins in CN was calculated to be 30916 Da (M), with an average of 204 peptide bonds per protein molecule (p) according to Spellman et al. (2003).

Determination of radical scavenging activity

The free radical scavenging activity of camel whole casein (CN) and their hydrolysates (CNH) were analyzed according to the method described by Re et al. (1999) and adapted for small volumes by Sadat et al. (2011).

The $ABTS^{+\bullet}$ radical cation was produced by dissolving 7 mM of cation $ABTS^+$ in 2.45 mM potassium persulfate allowing the mixture to stand in the darkness for 15 h at room temperature before use. The $ABTS^{+\bullet}$ radical cation reagent was then diluted with 5 mM sodium phosphate buffer, pH 7.4 to reach an absorbance of ca. 0.7 at 740 nm. The radical was stable for at least 1 h at 22 °C. A volume of 150 μL of each sample (CN or CNH in 20 % acetonitrile in water corresponding to different concentrations) was added to 150 μL of the $ABTS^{+\bullet}$ radical reagent, and incubated for 10 min at 30 °C. All the analyses were performed in triplicate. The absorbance was measured at 740 nm with an MRX® microplate reader. The radical scavenging activity of the tested samples, expressed as an inhibition percentage (%), was calculated with the equation:

$$\text{Activity}\ (\%) = [1 - (A_r - A_b) / (A_i - A_b)] \times 100 \qquad (3)$$

Where A_i is the absorbance of the initial $ABTS^{+\bullet}$ radical cation, A_r is the absorbance of the remaining radical, and A_b is the absorbance of the blank (in the case of phosphate buffer, A_b = 0.09).

The IC_{50} value is defined as the concentration of sample which is able to transform 50 % of $ABTS^{+\bullet}$ to $ABTS^+$, i.e. when the absorbance of the remaining radical (A_r - A_b) was equal to that of the scavenged radical (A_i - A_r). Thus, logIC_{50} corresponded to the x-intercept of the curve log [(A_r - A_b) /(A_i - A_r)] *vs.* log (Concentration of sample).

The Trolox equivalent antioxidant capacity (TEAC) was calculated as described by Re et al. (1999).

Statistical analysis

Statistical analysis was performed using MS Excel software and results were presented as mean value ± standard error of mean (SEM). The t-test (two-samples, assuming unequal variances) and P value <0.05 were used for statistical evaluation.

Results and discussion

Assessment of in vitro camel casein hydrolysis

Camel milk casein (CN) and its hydrolysate generated by the successive actions of pepsin and pancreatin (CNH) were analyzed by RP-HPLC (Figure 1).

In the chromatogram of intact camel casein, no peaks with important areas were eluted before 60 min. This confirms that the soluble nitrogen fraction of camel casein before digestion contained few small peptides. After hydrolysis, a number of large peaks were eluted between 5 and 70 min, whereas the casein's peak at 70 min disappeared in totality showing that the caseins were completely hydrolysed into peptides. The number of peptide bonds cleaved through the hydrolysis is often estimated by measuring the degree of hydrolysis (DH). The DH value reflects the content of amino groups liberated, the higher the DH, the higher the content of released amino groups. The DH value of camel milk casein reached 19 % after peptic and pancreatic digestion, which is lower than DH of bovine CN (25 %) obtained after 6 h of digestion by pancreatin, but higher than the DH of yak's (4 %) and ovine (4.5 %) milk casein hydrolyzed by microbial proteases for 4 h (Su et al., 2012; Jiang et al., 2007; Correa et al., 2011). The high DH of camel CN might be attributed to pepsin-specific target sites on camel milk CN (41, 36, 45 and 23 for α_{s1}-CN; α_{s2}-CN; β-CN and κ-CN, respectively; Swiss-Prot. accession numbers: O97943,O97944,Q9TVD0,and P79139, respectively). Indeed, camel caseins were more readily digested with chymotrypsin than trypsin (Salami et al., 2008). Recently, Si Ahmed et al. (2013) reported that β-CN of camel milk is rapidly hydrolyzed by pepsin but it is more resistant to trypsin, chymotrypsin

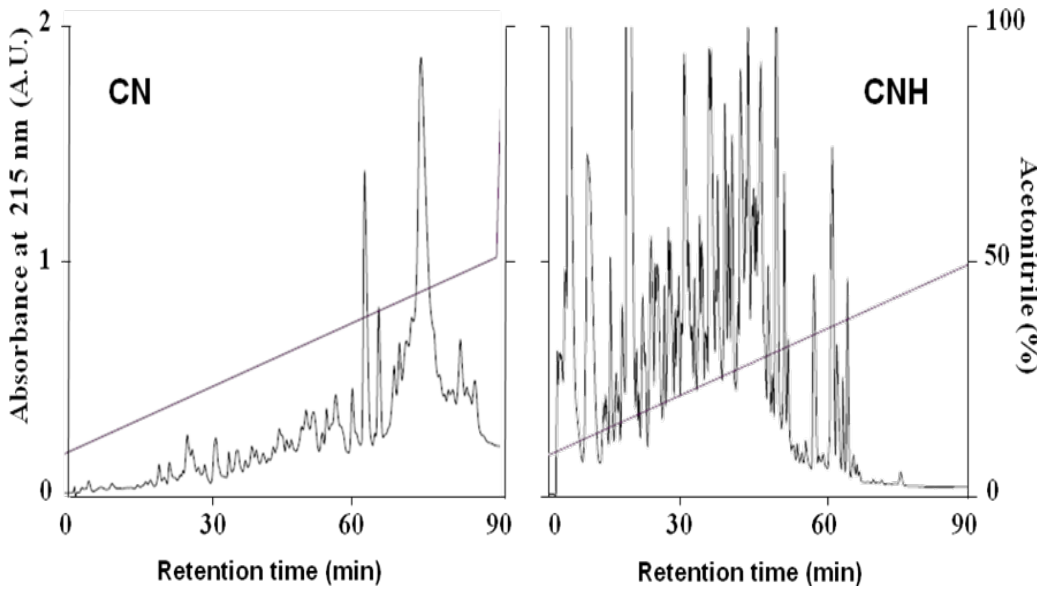

Figure 1. RP-HPLC chromatograms of camel milk casein (CN) and its hydrolysate (CNH) generated by successive actions of pepsin and pancreatin. See the Materials and Methods section for HPLC conditions. A.U.: Absorbance Unit

and papain digestion than its counterpart which is purified from bovine milk.

Free radical scavenging activity

Antioxidant mechanisms of peptides include radical-scavenging (both hydrogen-donating capability and free radical quenching) activity, inhibition of lipid peroxidation, metal ion chelation, or a combination of these properties are well investigated. In this study, scavenging activity was determined using the radical cation $ABTS^{+\bullet}$ which is reduced by concomitant conversion to a colorless product in the presence of antioxidants by hydrogen-donating or chain-breaking properties. Prior to the investigation of the radical scavenging-activity of camel casein and its hydrolysate, two strongly antioxidant molecules, gallic acid and Trolox, were used as positive controls. Figure 2 showed a linear concentration response curve in the range of 0-5 and 0-10 μM of gallic acid and Trolox, respectively. Gallic acid has a higher radical scavenging activity than that of Trolox with a Trolox Equivalent Antioxidant Capacity (TEAC) value of 3 μM and an IC_{50} of 2.0 μM, the latter was similar to that reported by Sadat et al. (2011).

The radical scavenging activities of CN and CNH were investigated also by the determination of the IC_{50} and TEAC values and compared with those of camel milk protein (MP) determined in our previous study (Table 1). Thus, CN exhibited a higher free radical scavenging activity than the whole camel milk proteins (MP), with a 6 folds higher TEAC value and with IC_{50}values c.a. 2 fold lower than that of MP. After subsequent hydrolysis by pepsin and pancreatin, the IC_{50} value determined for the CNH hydrolysate was in the same order of magnitude than that of the protein fraction (the IC_{50} value of CNH was about 2 fold lower than that of the camel milk protein hydrolysate MPH). The TEAC value of CNH was 2 fold higher than that of MPH, indicating that the CNH displayed stronger radical scavenging activities than MPH. The increase of free radical scavenging activity of camel milk proteins and of

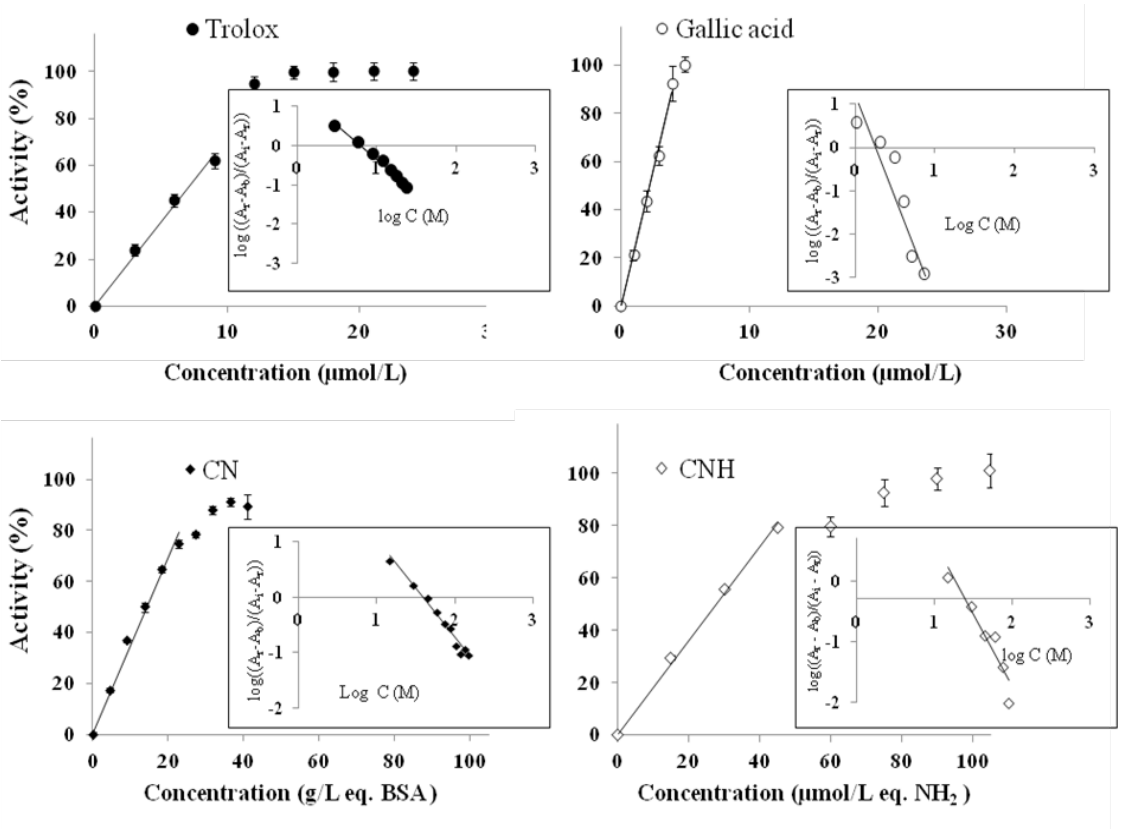

Figure 2. ABTS radical scavenging activity of Trolox, gallic acid, camel milk casein (CN), and its hydrolysate(CNH)

Table 1.TEAC and IC_{50} of gallic acid, camel casein (CN), camel milk protein (MP), camel casein hydrolysate (CNH) and camel milk proteins hydrolysate (MPH)

Sample	TEAC ± SEM	IC_{50} ± SEM
	μmol TE/mg protein[a]	mg/L eq. BSA
Gallic acid	3 μmol/L	2 μmol/L
CN	1.60±0.12	12.03 ± 2.17
MP	0.26±0.03[b]	24.7±0.27[b]
	μmol TE/μmol eq. NH_2[a]	μmol/L eq. NH_2
CNH	0.25±0.01	24.70±2.98
MPH	0.15±0.03[b]	43.4±0.18[b]

[a]TE - a calculated Trolox equivalent expressed in mg^{-1} protein for undigested samples and μmol^{-1} eq. NH_2 for the hydrolysates. The TEAC is the ratio of the slope of the plot of scavenging activity *vs* concentration of the sample over the slope of the plot of Trolox; [b]Results from Jrad et al. (2014)

casein after their hydrolysis suggested that antioxidative peptides were released and these peptides were mainly generated from the caseins rather than from the whey proteins.

It is now well-known that in bovine milk, β–CN produced radical scavenging peptides during gastro-intestinal digestion or fermentation (Kansci et al., 2004). As camel milk casein fraction is rich in β–CN (65 % of total caseins), this component might be the best source of antioxidant peptides (Jrad et al., 2014). Camel β-CN showed high antioxidant activity after hydrolysis with chymotrypsin (Salami et al., 2011). The camel β–CN (Swiss-Prot accession number Q9TVD0) includes 8 residues of Phe, a highly antiradicalar aromatic amino acid, whereas bovine β–CN contains 5 Phe residues (Swiss-Prot accession number P02666) and might be one explanation of the potential antioxidant activity of the camel protein. Tsopmo et al. (2011) have reported that the presence of aromatic residues confers high free radical scavenging activity to synthetic peptides, the most efficient residue being Trp.

The antioxidant activity of peptides has been attributed to certain amino acid sequences (Suetsuna et al., 2000) as well as to the presence of peptide bonds present or structural conformation arrangement (Hernandez-Ledesma et al., 2007). The hydrophobicity of the peptide also appears to be an important factor for its antioxidant activity due to increased accessibility to hydrophobic targets (Chen et al., 1998). In fact, camel β-CN hydropathy is -0.339, which is the highest hydrophobicity among other camel caseins (www.expasy.org).

Thus, another factor must be taken into account to explain the radical scavenging activity of CN which is their polar domains that contain phosphorylated serine residues. Those phosphorylated CN and their caseinophosphopeptides (the so-called CPPs) exhibited antioxidant property towards direct free radical quenching activity (Kitts, 2005).

Conclusion

Camel milk has developed a high reputation as a healthy nutrition with most of its therapeutic value ascribed to its biological properties such as antioxidant activity. In this study, improvement of the free-radical scavenging activity of camel casein was unveiled after *in vitro* hydrolysis by pepsin and pancreatin to simulate the gastro-intestinal digestion. These preliminary results showed the generation of antioxidative peptides from camel caseins. Therefore, further studies are necessary to identify the antioxidant peptides and to investigate the possibility of the gastro-intestinal system to generate camel milk antioxidant peptides *in vivo*.

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Antioksidativnost kazeina devinog mlijeka prije i nakon in vitro simulirane enzimske razgradnje

U radu je istražen utjecaj *in vitro* hidrolize kazeina devinog mlijeka sa pepsinom i pankreatinom na koncentraciju slobodnih radikala i antioksidativnu aktivnost s ciljem procjenjivanja utjecaja gastrointestinalne razgradnje. Hidroliza kazeina praćena je sa RP-HPLC (tekućinska kromatografija visoke djelotvornosti na obrnutim fazama). Antioksidativna aktivnost je praćena sa 2,2'-azino-bis-(3-ethylbensothiazoline-6-sulfonic acid) (ABTS) metodom. Ekvivalent troloks antioksidativnog kapaciteta (TEAC) devinog kazeina iznosio je 1.6±0.12 μmol TE/mg proteina, dok je TEAC hidrolizata iznosio 0.25 μmol TE/μmol eq. NH_2. Nakon digestije, utvrđena antioksidativna aktivnost kazeinskih peptida bila je učinkovitija od literaturnih navoda za hidrolizate devinog mlijeka, kolostruma i sirutkinih proteina.

Ključne riječi: devin kazein, protein enzimska probava, radikalna aktivnost čišćenja, antioksidans

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