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Effect of heat treatment on the antioxidant activities of camel milk alpha, beta and total caseins

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ABSTRACT

This study aimed to evaluate the effect of various heating temperatures on the antioxidant activities of camel milk caseins. The samples were processed with three different heat treatments: Pasteurization at low and high temperatures and boiling. Fresh camel milk (unheated) was used as a control. Camel milk caseins were separated by fast ion exchange liquid chromatography (FPLC) and identified by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS page). The antioxidant activities of caseins were measured by three different in vitro methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) radical scavenging activity and ferric reducing power assay (FRAP). The antioxidant activity evaluated by the DPPH assay decreased significantly ($p<0.05$) with the increase in heat treatment of caseins. However, there was no significant difference in ABTS radical scavenging activity and Ferric Reducing Antioxidant Power assay (FRAP) of heat-treated camel caseins compared to unheated onesStill, a decrease was observed in those activities by the increase of temperature in the different casein concentrations. Besides, whatever the concentration tested and the methods applied, the antioxidant activity of beta-casein (β−CN) was more pronounced than the alpha-casein (α-CN). Therefore, camel milk casein could be used as a natural source of antioxidants which may have a potential application in the food and nutraceutical industries. Throughout the different heat treatments applied, pasteurization at low temperature could be the most suitable alternative to preserve the antioxidant properties of camel milk.

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Introduction

Oxidative metabolism is an essential process in all living organisms. During this process, the formation of free radicals and other reactive oxygen species (ROS) is unavoidable. However, uncontrolled generation of ROS or a lack of antioxidants to quench the excess of free radicals can be the reason for different degenerative diseases (1).

Besides, lipid oxidation is one of the main reasons for sensory deterioration and nutrient loss of foods during their food processing and storage (2). Antioxidants can be added to food to avoid food deterioration and foodstuff's shelf life (3, 4).

Several synthetic antioxidants have been used in foods to prevent oxidation (4). However, due to their toxic effect, using these synthetic antioxidants in foodstuffs is strictly regulated (5). Therefore, there is an increased interest from researchers and the food industry to determine the functional value of traditional foods and to develop new and natural antioxidants as an alternative to synthetic ones (6).

Camel milk represents one of the most interesting milks for inhabitants of the arid zone. It contains all essential nutrients found in cow milk. Indeed, several studies demonstrate the potential benefits of camel milk in many health issues and diseases with significant anti-carcinogenic, anti-diabetic, anti-hypertensive, and anti-oxidant properties (7-9).

Caseins are the major protein of camel milk present in the form of macro-molecular aggregates. Various casein fractions (alpha, beta, and kappa) are present in milk due to the difference in phosphate content. Similar to human milk, camel milk contains a low amount of K-CN and a high content of β-CN, which may explain its better digestibility and low allergic incidence in newborns (10).

Different scientific investigations have been carried out to obtain bioactive peptides from camel casein hydrolysates, while these enzymatically hydrolyzed peptides are reported to possess antioxidant activities (10, 11).

Heat treatment is considered one of the essential steps of milk production. The purpose of milk thermal processing is to improve the biological quality of milk and extend its shelf life by either partially destroying microorganisms or completely milk.

However, data concerning the thermal effect on the antioxidant activities of camel milk are still limited in the literature. Thus, the purpose of the current study was to determine the effect of heat treatment at different temperatures on the anti-oxidant activities of camel milk caseins.

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Materials and Methods

Purification of casein

Camel milk samples were obtained from the dromedary (*Camelus dromedarius*) of the experimental herd of the Arid Land Institute, Livestock and Wildlife Laboratory (Medenine, Tunisia). Milk samples were divided into 4 portions, one portion was kept as a control (raw), and the rest were heated to 63°C for 30 min, 90°C, and 100°C for 3 min in a thermostatically controlled water bath.

Milk samples were then defatted by centrifugation at 3000 g for 20 min at 4°C. Casein of raw and heated skim milk was prepared by adjusting their pH to 4.2 with 1M of HCL. Then the mixture was centrifuged (3000 g, 20 min, and 4 °C). The precipitated casein was separated from the whey supernatant, washed three times with distilled water to remove the whey residue, and solubilized at pH 7 with 1 M NaOH. All the samples were freeze-dried and kept at -20°C.

Purification of casein fraction

According to (12), 8 mg of whole camel casein was dissolved in urea buffer (Tris HCL 5mM, pH 8; Dithiothreitol 0.8mM; Urea 4.5M). After homogenization for 15 minutes at room temperature, the homogenate was filtered through a membrane filter (Minisart Sterile filter 0.45 µm, Sartorius Stedim Biotech, Germany) and subjected to fast protein liquid chromatography (FPLC AKTA purifier system) on a GL 5/50 Mono-Q exchange column.

The separation was carried out by applying a linear salt gradient from buffer A (Tris HCl 5mM, pH 8.00, Dithiothreitol 0.064 mM, Urea 4.5 M) to buffer B (which was buffer A with 0.35 M NaCl) at room temperature and a flow rate of 1 ml/min.

The protein peaks established at 280nm were collected, desalted on the Hitrap desalting column (Cytiva, France), lyophilized, and stored at -20°C for later analysis.

The identity and purity of the eluted fractions were established by SDS polyacrylamide gradient gel electrophoresis (PAGE).

Electrophoresis (SDS–PAGE)

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to (13) using 15% (w/v) separating gel and 5% (w/v) stacking gel. All purified fractions were dissolved in Tris-HCl buffer (62.5 mM, pH=6.8), glycerol (10 % (*V/V*)), SDS (2 % (*m/V*)), β-mercaptoethanol (5 % (*V/V*)) and 0.0025 % (*m/V*) bromophenol blue), at 2:1 (v/v) ratio and boiled at 100°C for 4min.

A volume of 10µl of each sample was loaded in the gel. After 2h of electrophoresis running, the gel was stained in a mixture of 0,1% (w/v) Coomassie blue R-250, 40% (v/v) methanol, 10% (v/v) acetic acid and 50% (v/v) water for 3 hours and de-stained in 10% (v/v) acid acetic and 50% (v/v) ethanol solution.

Antioxidant activity

Determination of DPPH radical-scavenging activity

The determination of radical scavenging activity by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was measured according to (14) with some modifications. In a 96-well microplate, a volume of 100 µL of different samples was added to 100 µL of 0.2mM methanolic DPPH reagent and incubated for 30 minutes in a dark room. Then, the absorbance was measured at 517 nm using a microplate reader. For the control, 100 µl of distilled water was used instead of the sample. The percentage of radical scavenging activity was expressed as:

DPPH radical scavenging activity (%) = (Ac - As/Ac) X 100

Where Ac is the Absorbance of control and As is the Absorbance of samples.

Determination of ABTS radical-scavenging activity

The ABTS radical scavenging activity of samples was determined according to a method adapted from (15), with an MRX microplate reader instead of a spectrophotometer. ABTS radical caption was obtained by dissolving a 7 mM stock solution of ABTS in 2.45 mM potassium persulphate solution and kept it in the dark for 12-16h at room temperature. This solution was diluted with 5 mM sodium phosphate buffer (PBS) to reach an absorbance of 0.7 units at 734 nm. A volume of 100µL of each sample was added to 100 µL of the ABTS radical reagent and incubated for 10 min after mixing.

The absorbance was measured at 734 nm using a 96-well microplate reader. Each assay for each sample was carried out in triplicate. Percentage inhibition of all samples was calculated according to the following equation:

Inhibition of ABTS (%) = (Ac - As/Ac) X 100

Where Ac is the Absorbance of control and As is the Absorbance of samples.

Ferric reducing power assay

Ferric Reducing Antioxidant Power assay (FRAP) is a calorimetric method based on reducing a colorless Fe3+-TPTZ complex into intense blue Fe2+-TPTZ once it interacts with a potent antioxidant.

The reducing power was assessed according to the method of (16). A volume of 1.25 ml of each sample at different concentrations was added to 1.25 ml of phosphate buffer (0.2 mol/L, pH 6.6) and 1.25 ml of 1% (w/v) potassium ferricyanide. After incubation at 50°C for 20 minutes, 1,25ml of TCA (10% w/v) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 1.25 ml of the supernatant was mixed with 1.25 ml of distilled water and 0.25 ml of $FeCl_3$ (0.1% w/v). The absorbance was read at 700nm.

Higher absorbance of the reaction mixture indicates higher reducing power.

Statistical analysis

All experiments were performed in triplicate. The results were statistically assessed using XLSTAT (version 2014.5.03, Addinsoft, Pearson edition, Waltham, MA, USA). One-way analysis of variance (ANOVA) was done with Tukey's test. A P-value of 0.05 was used to indicate a significant difference.

Results and Discussion

Separation of caseins by FPLC

The different chromatographic profiles of the casein fraction are shown in Fig. 1. The complete separation of caseins was achieved after 40min. Camel milk caseins are

elected on two principal peaks (a and b).

The different chromatograms showed that heating impacted the intensity of the peaks. A reduction in the intensity of the peaks was observed especially at a temperature of 100°C.

Electrophoretic profile of camel milk casein

The SDS-PAGE profile of alfa lane (1-4) and beta lane (5-8) caseins fractions is shown in Fig.2. The migration of each fraction showed the presence of a single-well defined band with a molecular weight of 38kDa for the peak b and 35kDa for the peak a. Thus, FPLC reveals proteins that are specific to the camel milk caseins, as they are known by their lower electrophoretic mobility compared with bovine caseins. (17). The absence of a protein band corresponding to the kappa casein due to its low content in camel milk as previously indicated (18,19).

It is observed that heating time and temperature impacted the intensity of the casein protein bands. From these results, a decrease in protein content was observed in response to heating at a temperature of 90°C.

DPPH radical scavenging activity

DPPH is a free radical that becomes a stable molecule by accepting an electron or hydrogen radical. Therefore, it is used as a substrate to estimate the antioxidant activity of caseins. Fig.3 shows the DPPH radical scavenging activity of total caseins and their fractions at different concentrations and heat treatments.

Caseins and their fractions were able to scavenge DPPH radicals in perfect accord with (20) who reported that caseins and their hydrolysates could scavenge DPPH radicals. Thus, Camel casein fractions present higher DPPH scavenging activity than total caseins.

Limited information is available about the effect of heat treatment on the antioxidant activity of camel milk proteins. The effect of heat treatment on the radical scavenging activities of total caseins and casein fractions was also investigated by the determination of the IC50 (The concentration of samples used to inhibit 50% of the initial DPPH scavenging activity). Unheated total caseins, α-CN, and β-CN exhibited the lowest value of IC50 respectively (39, 20, and 17 mg/ml).

The IC50 of the different fractions was reattached before the IC50 of the whole casein; these results suggested the presence of inactive peptides encrypted in the sequence of camel caseinate (21).

The DPPH scavenging activity of total and casein fractions is significantly decreased under the heat treatment of camel milk. The activity was more pronounced in unheated caseins (Fig.3A), α-CN, and β-CN than the heated ones ($p<0.05$) (Fig.3B and C). Ansari et al (2020) reported similar effects on the DPPH activity of raw and pasteurized camel milk.

Significant decreased radical scavenging activity was observed in α-CN and β-CN after heating at 100°C at a concentration of 20 mg/ml. This fact was also observed in the results of FPLC separation of all casein fractions in figure 1, which showed a decrease in the intensity of casein after heating at 100°C.

The significant decrease in radical scavenging activity of heat-treated caseins especially β-CN, may probably be due to the degradation of bioactive components responsible for the antioxidant activities (22).

As camel milk casein fraction is rich in β−CN (65 %

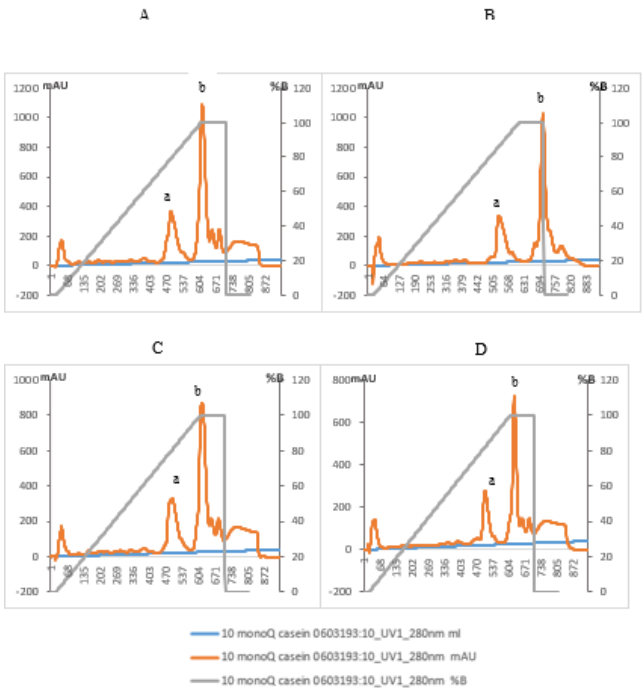

Figure 1. FPLC profiles of different casein fractions under different heat treatments: A) unheated casein (control), B) pasteurized casein at 63°C, C) pasteurized casein at 90°C and D) boiled casein at 100°C.

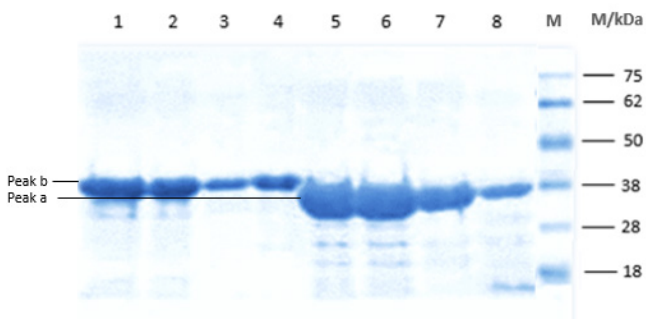

Figure 2. SDS-PAGE profile of camel milk casein fraction under different heat treatments. Lane M=molecular mass markers, α-CN lane (1-4) and β-CN lane (5-8): for unheated, pasteurized at 63°C, pasteurized at 90°C and boiled CN respectively.

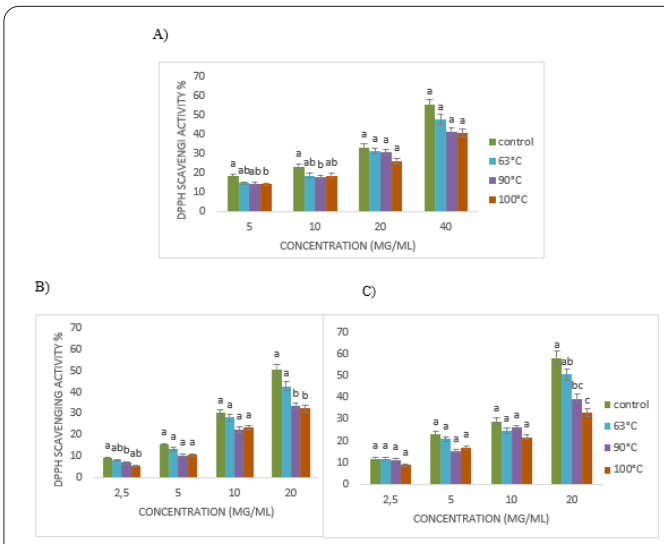

Figure 3. effect of heat treatment in DPPH radical scavenging activity of camel milk caseins: total caseins, (b) α-CN, (c) β-CN. Values with the different lowercase letters at the same concentration are significantly different ($p<0.05$).

of total caseins), this component might be the best source of antioxidant peptides (11). But, Camel β-CN antioxidant activity showed a statical decrease after treatment at 100°C, this can confirm the usefulness of fresh or pasteurized camel milk to preserve its nutritional quality.

ABTS-radical scavenging activity

The radical ABTS is reduced to a colorless product in the presence of antioxidants with hydrogen donating or free radical scavenging (23).

As illustrated in Fig4. (B and C); at the same concentration and heat treatment, β-CN presented a higher ABTS scavenging activity than α-CN. But, this activity has no significant effect of heat treatment, which is in accord with Şanlidere Aloğlu. (24) who didn't find a significant effect on the ABTS scavenging activity of raw, pasteurized, and sterilized bovine milk.

Therefore, Kim et al., (25) showed that alpha cow casein presented a higher ABTS scavenging activity than beta cow casein. This result could be explained by the fact that β-CN is the major fraction present in camel milk while the major cow casein is α-CN.

Based on ABTS scavenging activity results, camel milk caseins may present such elements acting like electron donors that could react with free radicals and convert them to more stable molecules (26).

At all tested concentrations, unheated total caseins, α-CN, and β-CN (Figs.4A and C) showed the highest ABTS scavenging activity. However, the most decrease in this activity was obtained under treatment at 100°C at different concentrations.

Different studies have shown that caseins are the major contributor to the antioxidant capacity of whole milk as they are rich in potential anti-oxidative amino acids like tryptophan, tyrosine, lysine, histidine, and methionine (27-29).

The decrease in the scavenging capacity of ABTS free radicals under the heat treatment suggests that this process contributed to the degradation of some components responsible for the antioxidant activity (22).

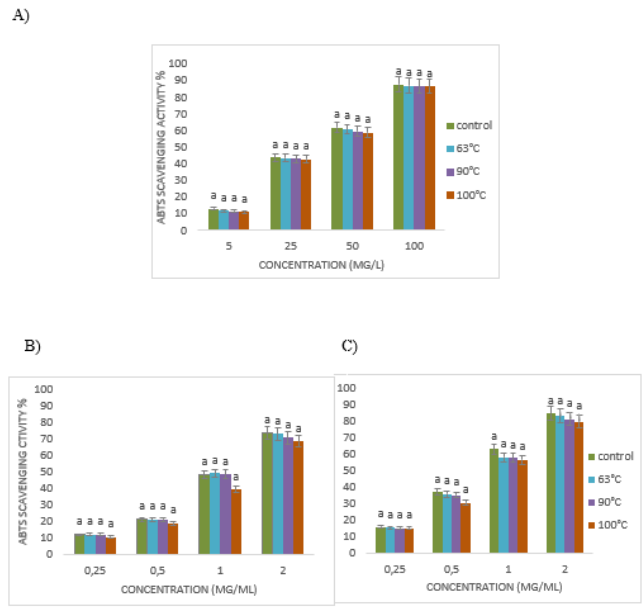

Figure 4. Effect of heat treatment in ABTS radical scavenging activity of camel milk caseins: (A) total caseins, (B) α-CN, (C) β-CN.

Indeed, camel milk caseins scavenged ABTS radicals more than DPPHsimilar to a previous study (Chen et al., 2003). Therefore, casein concentrations in DPPH activity were statically higher than that used in ABTS activity (Fig.3 and Fig.4). The difference in scavenging efficiency between ABTS and DPPH caseins could be due to the solubility and diffusivity of radicals. DPPH is soluble only in alcoholic solution, while ABTS is soluble in both aqueous and hydrophobic organic solution, so it could be easier to reach peptides in an aqueous medium (21).

Ferric reducing antioxidant power

The reducing power of casein and its fractions at different concentrations was illustrated in Fig.3. This activity is based on the ability of a composite to reduce the Fe^{3+} ferricyanide complex to the ferrous form (Fe2+). The antioxidant activity of all used samples was proportional to the concentration. The reducing power of caseins can be explained by the possibility of their contribution as an electron donor, reducing the peroxidation of lipids and suggesting that it likely contributes to the antioxidant activity (30).

Pasteurization and boiling did not have a significant effect on the reducing power of both caseins and their fractions (p>0, 05). As shown in Fig.5, using different concentrations, casein samples treated at 100°C revealed the lowest reducing power. Similar results were reported by Khan *et al*., (6) who studied the reducing power of pasteurized and boiled cow and buffalo milk.

However, Cervato et al (32) demonstrated that alpha cow caseins showed the most significant inhibitory action against Fe-induced peroxidation.

Although, a UHT treated bovine milk showed an increase in antioxidant activity as a result of severe heat treatment which may be explained by the degradation of natural antioxidants and the formation of novel oxidized compounds (brown melanoidins) via the Maillard reaction (17, 27). Therefore, these results highlight the important combination time temperature of heat treatment, which could explain the non-significant effect of heating on the antioxidant properties of milk. Maillard reaction product could compensate for the loss of organic antioxidant properties but not the loss of the nutritional value of milk

Indeed, the results of different radical scavenging methods used to evaluate the antioxidant activity revealed that the agent in caseins could inhibit the formation of free radicals. In addition, the presence of these components could reduce the Fe^{3+} (ferricyanide complex) to the ferrous form (Fe^{2+}). The antioxidant activity of camel casein samples used in this study may be explained by the presence of hydrogen donation, the power to scavenge the free radicals and inhibit the formation of peroxide.

Different antioxidant peptides have been isolated from the hydrolysates of various proteins by scavenging the free radicals could consequently prevent lipid peroxidation (33, 34).

Otherwise, the mechanisms of action of these peptides have not been completely revealed. Preliminary research suggested that these mechanisms are related to the amino acid composition; such as histidine, lysine, glycine, and valine (35), sequence/structure, hydrophobicity, and physicochemical properties of the amino acids, of the peptides (27).

In this study, the effect of heat treatment on the an-

tioxidant activity of camel milk casein was evidenced; the antioxidant activities of total camel caseins and their fractions under heat treatment were not usually stable and the high temperature may be the cause of the decrease of antioxidant activity.

Throughout the different methods used in the present study, pasteurized milk for 30min at 63°C presented the highest antioxidant potential. Therefore it could be a suitable alternative to preserve the antioxidant properties of camel milk casein for a longer time.

Besides, camel milk casein could be used as a natural source of antioxidant activities. It encourages the utilization of camel milk caseins as antioxidant agents for food products to enhance their functionalities and shelf life.

Further studies are required to purify and identify the bioactive peptides with the highest antioxidant activity in camel milk caseins to be used as a natural antioxidant element in functional nutriment formulation.

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Interest conflict

The authors declare no conflict of interest.

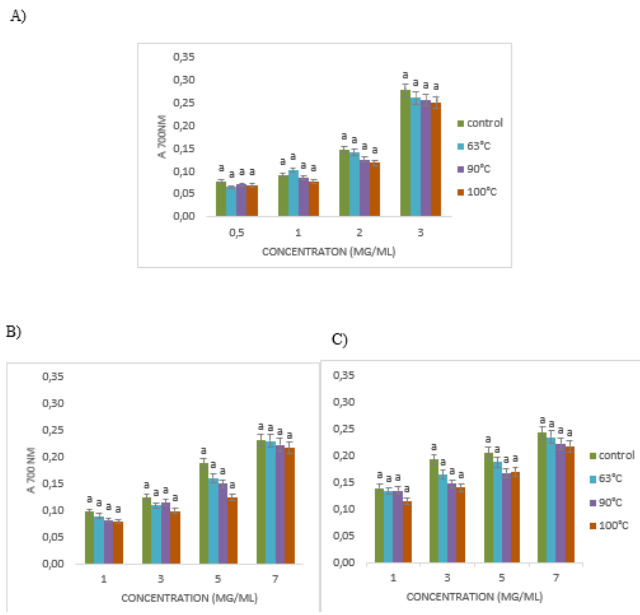

Figure 5. Effect of heat treatment on ferric reducing power of camel milk caseins: total caseins, (B) α-CN, (C) β-CN.

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