Antioxidant and Antimicrobial Activities of Enzymatic Hydrolysates of Camel’s Milk Whey Protein and Casein

Abd El-rahim, A. M.*
Dairy Science Department Assiut University.

ABSTRACT
Healthy skimmed camel milk’s, casein and whey proteins precipitated and freeze dried, and were treated with 1% of five proteolytic microbial renin, pepsin, trypsin, collagenase and microbial protease. Hydrolysates were analyzed for pH, degree hydrolysis and scavenging properties. The pH decreased, and the degree of protein hydrolysis increased reaching 16.22, 17.0, 22.12, 22.88, 15.85, 16.23, 15.68, 16.08, 23.75, 24.44 for casein and whey after 8 hours of hydrolysis. The inhibition % of DPPH increased by increasing the proteolysis to 21.4, 22.9, 20.18, 23.2, 24.94 for casein hydrolysate and 34, 31, 30, 30, 28.5 for whey proteins hydrolysates after 8 hours at 37 °C. Whey proteins resulted in higher scavenging properties than casein under the same conditions. Both casein and whey protein hydrolysates had a significant antibacterial activity against undigested or digested protein. The highest antibacterial activity was obtained whey and collagease digested whey protein and microbial protease digested casein characterized with the highest antibacterial activities (180.0, 170.0 and 200.0 mm) for Staphylococcus aureus, Streptococcus pyogenes, and Escherichia coli, respectively. All of the digested enzymes were of antifungal effect against Aspergillus flavus and Aspergillus fumigatus, but of no effect against Aspergillus niger. Casein was found on antifungal against Aspergillus flavus. Only undigested casein, collagease and trypsin digested casein were also found of anti-fungal activity against Aspergillus niger, which recorded 9.0, 8.0 and 8.0, respectively.

Keywords: casein, whey protein, camel milk, antimicrobial, antioxidant.

INTRODUCTION
The one-humped camels (Camelus dromedarius) are well-known producers of milk which differs from bovine milk in the composition and structure of its protein content and are thus of different functional and medicinal properties. Casein fractions of camel milk are α-, β- and κ-CN constitutes about 65, 21 and 3.47%, respectively, of total caseins in human milk as it contains a high amount of β-CN; this can reflect the high digestibility and low sensitivity in infants, since β-CN is more sensitive to gastrointestinal degradation than α-CN (El-Agamy et al. 2009). From the estimated molecular mass CN-CN and α-CN in camel milk using SDS-PAGE technique are 28.6 kD and 35 kD respectively, and are higher than those in bovine milk. The molecular weight of α-lactalbumin from camel milk is 14.6 kDa, and it contains 123 amino acids which is similar to those in cows milk, humans and goats. Peptides derived from milk proteins have been shown to perform various functions such as antioxidant activities, anticancer and anti hypertensive (ACE) activities, opioid activities, mineral binding, growth stimulation and antimicrobial activities (Meisel 2005). Consequently, casein may play important biological functions after decomposition with different proteases. The enzymatic hydrolysis of casein produce specific peptides exert bioactivity which reduce the risk of heart disease, diabetes and cancer (Beg et al. 1985, Mohammad 1993, Farah 1996, Clare and Swaisgood 2000, Rival et al. 2001, Kappeler et al. 2003, Aimutis 2004, Meisel, 2005 Chen et al. (1998) and Chen et al. (1998)

The antioxidant properties of the bio-active peptides are attributed to their composition, structure and hydrophobicity as well as position of amino acid residue, and the molecular weight. The bioactivity of peptides obtained from camel milk casein has not been extensively studied so far, so it needs a lot of research in the future. There is a high degree of protein degradation of camel casein from that in cow’s milk which can be released by enzymatic proteolysis Hernández-Ledesma et al. (2007). Once bioactive peptides are released, they may act as regulatory compounds in the host organism with specific activities such as antihypertensive, antioxidant, antimicrobial or opioid. Search for milk-based bioactive peptides has been focused until now mainly on bovine and to smaller extent on ovine and caprine milk proteins. Therefore, this investigation was conducted to produce casein and whey hydrolysates from camel milk using specific proteolytic enzymes from different sources to estimate the antioxidant and antimicrobial properties to use in further investigation as a food additives or natural food preservatives of the resulting hydrolysates (Chen et al. 1995, Korhonen et al. 2001, Agrawal et al. 2003, Mageed 2005, Hernández-Ledesma et al. 2007, Li et al. 2008, Salami et al. 2008 and Jrad et al. 2014)

* Corresponding author.
E-mail address: mo.ali.3@yahoo.com
DOI: 10.21608/jfds.2020.78877
**MATERIALS AND METHODS**

Microbial rennet (EC: 3.4.23.4), pepsin (EC 3.4.23.1), trypsin (EC: 3.4.21.4), collagense (EC:3.4.24.3) and microbial protease (EC: 3.4.21-24) were obtained from Sigma–Aldrich Chemical Co., India (M P Biomedicals, India). 1.1-diphenyl-2-picrylhydrazy1 (DPPH) was obtain from Sigma–Aldrich Chemical Co. India. All solutions, prepared with double-distilled water and kept at 4°C before further use.

Antimicrobial activity of the protein hydrolysate of camel casein and whey protein was determined against three bacterial strains, namely *Staphylococcus aureus, E. coli and Staphylococcus aureus* from Animal & Environmental Hygiene, Fac. of Vet. Medicine, Assiut University, while *Aspergillus fumigatus*, *Aspergillus flavus*and *Aspergillus niger* were isolated from dairy products and identified at plant and microbiology department Fac. of Sci. Assiut University. The organisms were periodically subcultured and maintained in nutrient agar slant at 4°C.

**Casein and whey protein powder preparation:**

The one-humped healthy female Camels (Camelus dromedarius) located Marsa Matrouh farm, milk samples was obtained. The milk samples kept in closed ice box at 5°C, and transferred to the laboratory at the same day of milking. The milk samples were centrifugated at 3000 × g for 10 min at 4°C. The pH of the defatted milk was adjusted to 4.6 using 1.0 N HCl to precipitate the whole caseins. The obtained supernatant was adjusted to pH 7.0 with 1.0 N NaOH and re-centrifugated at 10,000 × g for 30 min at 4°C. The resultant supernatant after is used for precipitation of the whole whey proteins by salting out using ammonium sulphate and dialysis against distilled water to remove the rest of ammonium sulphate (750 g/1 L of liquid whey). Both of the caseins and whey proteins concentrates were freeze-dried and stored in a desiccator until further analyses.

Total nitrogen contents of the camel milk samples were estimated in triplicate using Kjeldahl procedure according to AOAC (1995). Total protein content was calculated as N × 6.38.

Enzymatic hydrolysis of CMCP and CMWP in phosphate buffer at 5% total solid and pH adjusted to (6.5 for microbial rennet, 2.5 for, pepsin and 7.4 for trypsin, collagense and microbial protease) casein and whey protein separated from camel milk were dissolved. The CMCP and CMWP solutions heated in water bath for 5 min to kill the microorganisms, which may produce proteolytic enzymes during the proteolysis process, to denature the indigenous enzymes of milk and denature the proteins, which increases its susceptibility to proteolytic enzymes. The enzyme/substrate ratio (E:S ratio) was kept constant (1:100) for all the enzymes. The hydrolysis was carried out by incubating the samples at 37°C for in stirred water bath and samples were drawn when fresh and after, 2, 4, 6 and 8 hour of incubation. The hydrolyzed sample heated at 85°C for 15 min water bath, then cooled immediately, and centrifuged under cooling at 10,000 rpm for 25 min; and the supernatants were collected and stored at −20°C until further analysis.

The pH of hydrolysate samples was measured using combined glass electrode of Mettler Toledo pH meter (Model FiveEasyTM plus FEP 20, Switzerland). The pH of each sample was measured just before heating to inactivate the residual enzyme.

Degree of proteolysis (DH) of casein and whey protein was estimated by detecting of solubilized protein in 10% (w/v) trichloroacetic acid (TCA), compared to the total protein content of the sample according to Hoyle and Merritt 1994 and Devendra Kumar et al 2016. The DH was calculated according to the equation:

$$\text{DH} (\%) = \frac{[\text{Solubilised protein content in 10% TCA (mg)}]}{[\text{Total protein content (mg)}]} \times 100$$

The ability to scavenge DPPH radical by added antioxidants in samples was estimated according to the method of Brand-Williams et al. (1995) and modified by Devendra Kumar et al (2016).

Scavenging activity (% inhibition) = 100-[(At20/At0) x 100].

Where: as = absorbency in time t=20 min (At20) and t= 0 min (At0).

Antibacterial activity was conducted for five different enzymes for both casein and whey protein after proteolysis for 6 hours at 37°C at the suitable pH by agar diffusion method adopted by Mounyr et al. (2016) against four bacteria and four molds *Staphylococcus aureus* (MTCC 3160) and *Pseudomonas aeruginosa* (MTCC 424).

Statistical analysis was conducted in triplicate; data were expressed as means with standard deviation.

**RESULTS AND DISCUSSION**

Results presented in Table (1) show slight decrease in pH of both CC and CWP hydrolysates with the advancement of hydrolysis. it was also observed that the deceeding rate depends on the protein substrate and the initial pH. Comparing to pepsin, the rate of pH decrease was lower for microbial protease. Rate of pH decrease was higher in case of whey protein hydrolysate. The decreasing was also higher in the first 4 hours, and then tends to be persistence to some extent.

<table>
<thead>
<tr>
<th>Time / hours.</th>
<th>pH measurements of casein and whey protein at different enzymatic hydrolysis.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MR</td>
</tr>
<tr>
<td></td>
<td>CCH</td>
</tr>
<tr>
<td>0</td>
<td>6.5±0.24</td>
</tr>
<tr>
<td>2</td>
<td>6.4±0.04</td>
</tr>
<tr>
<td>4</td>
<td>6.39±0.01</td>
</tr>
<tr>
<td>6</td>
<td>6.35±0.02</td>
</tr>
<tr>
<td>8</td>
<td>6.35±0.00</td>
</tr>
</tbody>
</table>

MR: microbial rennet
PEP: Pepsin TRY: Trypsin COLL: Collagense
MP: Microbial protease CCH: casein hydrolysate. WPH: whey protein hydrolysate
Change in pH during hydrolysis may not only affect the enzyme structure, but also the occurrence of the changes in the structure or properties of the substrate, which took place in the enzyme-substrate binding and thereby hydrolysis. To avoid sharp change and rapid decline of pH, phosphate buffers of specific pH for each enzyme were used. This decrease might be attributed to release of proteins into hydrolysis medium, which results in the reduction in the pH as reported by Ovisipour et al., 2013, Daroit et al., 2012, and Kumar et al., 2016.

Data presented in Table (2) illustrate the content of soluble peptide released from crude protein during the proteolysis using different five proteolytic enzymes. DH (%) increased with the increase in hydrolysis time, however, after 6h of hydrolysis, the DH% increased slowly and after 8h of hydrolysis it became static. This might be due to the decreased availability of cleavable peptide bonds within the substrate as well as the changing of surrounding medium. Adler-Nissen (1986) attributed the reduction in hydrolysis rate due to the competition between unhydrolysed protein and the peptides being constantly formed during hydrolysis. The reduction of hydrolysis rate in latter hours might also be due to decrease in pH of the medium, which might cause denaturation of protein structure of the enzyme or the disturbances of the ionic character of the substrate, which in turn affect enzyme-substrate binding. The microbial protease treated casein and whey protein showed higher DH% for all time intervals as compared to other enzymes, followed by pepsin with mean values of 23.75 ± 0.05, 22.44 ± 0.07, 22.12 ± 0.09, 22.88 ± 0.00 for casein hydrolysate and whey protein hydrolysate respectively. On the other hand the lowest HD% was obtained by using microbial rennet which recorded 16.25 ± 0.04 and 17.00 ± 1.34 of casein and whey protein hydrolysate after 8 hours of hydrolysis. The highest levels of DH% obtained with both microbial protease and pepsin suggested that this enzyme has more affinity for the substrate and thus more efficient than the others enzymes for the production of protein hydrolysatess of camel milk peptides. Similar results were also reported by Graszkiewicz et al., 2010, Lira et al., 2010, and Kumar et al., 2016. From these data it was noticed that the DH% after 8 hours of hydrolysis did not increase significantly. This might be attributed to enzyme specificity which could not further hydrolyse the remaining bonds within the generated peptides.

Table 2. Degree of hydrolysis (mean±SE) of CMCP and CMWP:

<table>
<thead>
<tr>
<th>Time / hours.</th>
<th>MR</th>
<th>PEP</th>
<th>TRY</th>
<th>COLL</th>
<th>MP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CH</td>
<td>WPH</td>
<td></td>
<td>CH</td>
<td>WPH</td>
</tr>
<tr>
<td>0.00</td>
<td>0.45±0.01</td>
<td>0.21±0.04</td>
<td>0.45±0.01</td>
<td>0.21±0.04</td>
<td>0.45±0.01</td>
</tr>
<tr>
<td>2.00</td>
<td>8.22±0.27</td>
<td>9.14±0.28</td>
<td>11.8±0.03</td>
<td>12.04±0.88</td>
<td>7.87±0.07</td>
</tr>
<tr>
<td>4.00</td>
<td>13.99±0.0414</td>
<td>16.1±0.04</td>
<td>18.6±0.04</td>
<td>13.3±0.04</td>
<td>13.24±0.04</td>
</tr>
<tr>
<td>6.00</td>
<td>15.55±1.0216</td>
<td>21.1±0.9021</td>
<td>11.1±0.0322</td>
<td>0.3±0.33</td>
<td>14.99±0.1215</td>
</tr>
<tr>
<td>8.00</td>
<td>16.25±2.0417</td>
<td>0.0±1.3422</td>
<td>12±0.0922</td>
<td>1.88±0.00</td>
<td>15.85±1.0416</td>
</tr>
</tbody>
</table>

Data presented in Figs. (1 and 2) by using different proteolytic enzymes in hydrolysis lyophilized camel whey protein isolate and camel casein, to evaluate the antioxidant properties through using DPPH scavenger. It could be observed that there was a significant increase in DPPH activity with the progress in hydrolysis time, and a positive relationship between hydrolysis time and DPPH activity could be noticed. Both of camel casein and camel whey protein hydrolysates produced by all of the examined 5 enzymes resulted in an increase in the DPPH-scavenging activity up to 6h of hydrolysis period.

Fig. 1. DPPH-scavenging activity of camel whey protein in different enzymatic hydrolysis.

Fig. 2. DPPH-scavenging activity of camel casein hydrolysate at different enzymatic hydrolysis.

As compared to other 4 enzymes, the pepsin produced hydrolysates which had higher antioxidant activity at 4h of hydrolysis and it remained higher up to 8h h of hydrolysis, except for the microbial rennet which exhibited higher value of DPPH activity after 6 hours of hydrolysis time. However, the camel casein hydrolysates produced by all enzymes showed slight or no increase in DPPH-scavenging activity. While in case of camel whey protein as shown in Fig (2) the collagenase had the higher antioxidant properties (DPPH scavenging activity %) flowed by pepsin enzyme, and the trypsin enzyme was the lowest antioxidant properties.
All enzymes used in this study showed no antioxidant properties after 7 hours of hydrolysis time. From the DPPH-scavenging activity, it could be hypothesised that both hydrolysed camel casein and camel whey protein contain some electron donating substances that could interact with free radicals, making them more stable molecules and stopping the radical chain reaction. The increase in DPPH radical scavenging activity of camel milk protein hydrolysates was in agreement with results obtained by Mao et al 2011, Thiansilakul et al 2007 and Khantaphanta et al 2011).

**Antimicrobial activity:**

Five different proteolytic enzymes used to produce casein and whey protein hydrolysates which examined for antimicrobial activity against *Staphylococcus aureus*, *Streptococcus pyogenes* and *Escherichia coli*.

Data presented in Figs. (3) and Table (3) show that both casein hydrolysate and whey protein hydrolysate were of positive effect of antibacterial activity with the three examined bacterial strains.

Fig. 3. Antibacterial activity of camel casein and whey protein hydrolysate at different enzymatic hydrolysis.

### Table 3. Antibacterial Activity of five different enzymes digested protein hydrolysates of CMWP and CMCP.

<table>
<thead>
<tr>
<th>Bacterial inhibition zone in mm.</th>
<th>Control</th>
<th>MR</th>
<th>PEP</th>
<th>TRY</th>
<th>COLL</th>
<th>MP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>CH</td>
<td>10.0</td>
<td>18.0</td>
<td>9.0</td>
<td>10.0</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>WPH</td>
<td>11.0</td>
<td>11.0</td>
<td>9.0</td>
<td>13.0</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>CH</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
<td>9.0</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>WPH</td>
<td>13.0</td>
<td>13.0</td>
<td>13.0</td>
<td>12.0</td>
<td>12.0</td>
</tr>
</tbody>
</table>

**Fungal inhibition zone in mm.**

<table>
<thead>
<tr>
<th>MR: microbial rennet</th>
<th>PEP: Pepsin</th>
<th>TRY: Trypsin</th>
<th>COLL: Collagenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus niger</td>
<td>9.0</td>
<td>8.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>10.0</td>
<td>19.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>10.0</td>
<td>14.0</td>
<td>11.0</td>
</tr>
</tbody>
</table>

The undigested whey protein resulted in a halo of 18mm against *S. aureus*, followed by the collagenase digested whey protein hydrolysate with a halo of 17.0mm against *Street. pyogenes*. While in case of casein hydrolysates it
was observed that, the undigested camel casein and microbial protease digested casein were of higher antibacterial effects with a halo of 20.0 mm against *Escherichia coli* flowed by pepsin digested casein with a halo of (15.0 mm). From these results it could be clouded that, both undigested casein and undigested whey protein had the highest antibacterial effects than the enzymatic digested proteins. A slight decreasing in the antibacterial activity of digest proteins may be attributed to the degradation of native milk protein polypeptide structure which had a high antibacterial activity. These findings were in accordance with Memarpoor-Yazdi *et al.* 2012 and Najafian and Babji, 2012 who stated that, the amino acid sequence is closely related to the antimicrobial activity against both Gram-positive and Gram-negative microorganisms.

Regarding to the antifungal activity of both casein and whey protein hydrolystes Table (3) and Fig (4) showed that both of casein hydrolysate and undigested casein had no effect against *Aspergillus flavus*, while the whey protein whether digested or hydrolysates had a significant antifungal activity properties. The undigested whey protein had the highest antifungal activity with measured halo (19.0 mm) followed by digested microbial protease whey protein with halo of 12.0 mm. From Table (3) it could also be noticed significant antifungal effects of both casein and whey protein against *Aspergillus fumigatus*, collagenase digested casein, which recorded the largest halo diameter of 18.0 mm, while the smallest halo diameter of 7.0 mm. was recorded for microbial rennin digested whey protein. Lastly, the antifungal properties of both casein and whey protein hydrolysates against *Aspergillus niger* was noticed as a positive effects only in case of undigested casein, collagenase digested casein and trypsin digested casein with halo diameter of 9.0, 8.0 and 8.0 mm respectively. The protein hydrolysates which cotain cationic acids amino acids in their composition will show higher antimicrobial activity.

**Fig. 4. Antifungal activity of camel casein and whey protein hydrolysate at different enzymatic hydrolysis**

**REFERENCES**


خصائص المادة الأساسية والمضادة للميكروبات لبروتينات شرخ وكازين لبن الجمال لتعزيز جودة пицев المлечم

علي محمد عبد الرحيم

قسم الابرار - جامعة أسيوط

في هذه الدراسة تم تزويد الزهور الملونة من الحمض الهيدروجيني والكلوريد الأسياني، وتم توفير الكازين والشرخ لاستخدامه كتعويضات للبروتينات بشكل منفصل. تم تجربة أنواع مختلفة من الكازين والشرخ في أنابيب خلايا المكروبات (Aspergillus niger, Aspergillus flavus, Escherichia coli, Staphylococcus aureus) وعقارية (Streptococcus refrigeratus, Staphylococcus aureus) وميكروبات الفم (Bifidobacterium longum, Lactobacillus acidophilus). تم تجربة أنواع مختلفة من البروتينات المحترقة (casein, milk protein hydrolysates) في هذا الدراسة. تم تضمين النتائج المستخدمة في هذا الدراسة في البحث السابق لدراسة خصائص المواد الفعالة للكازين والشرخ. ربطت النتائج أن كازين لبن الجمال كان له أثر في تقليل نشاط البكتيريا وتعزيز عدد الخلايا. بزيادة درجة الحرارة وكمية البروتين المحترق، تزيد الكازين والشرخ في محتوى البروتينات الحيوية. ملاحظة: هذه البكسل هي نص كبير يحتوي على مصادر مرجعية.

Abd El-rahim, A. M.


الخواص المضادة للأكاسيد والمضادة للميكروبات لبروتينات شرخ وكازين لبن الجمال المحلة إزمiëنا


