Potential Activity of Camel Milk-Amylase and Lactoferrin against Hepatitis C Virus Infectivity in HepG2 and Lymphocytes

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Background and Aims: To exploring which camel milk proteins, have the ability to inhibit and/or blocking the hepatitis C virus (HCV) entry and replication inside the cells system.

Methods: Using human peripheral blood mononuclear cells (PBMCs) and hepG2 cells system, three experiments were setup: 1) cells treated with amylase or lactoferrin then infected with HCV; 2) HCV treated with amylase or lactoferrin then used to infect the cells; and 3) HCV infected cells were treated with amylase or lactoferrin. RNA was extracted, RT-PCR and nested PCR were run, in addition to immune-staining the cells to localize the viral molecule within the cells foci.

Results: Camel milk-amylase and lactoferrin were in vitro tested the ability to inhibit the HCV entry and replication inside the human peripheral blood and hepG2. Amylase could not able to inhibit nor blocking the viral replication. However, lactoferrin demonstrate a clear ability to inhibit the viral entry into both cells system when pre-interact with the virus, but fault to protect the cells before infection. The virus replication inside the cells was completely blocked only when the infected cells were treated with lactoferrin.

Conclusions: Camel lactoferrin was demonstrated a remarked in vitro ability to completely inhibit the HCV entry into PBMC, hepG2 and replication inside those cells system.

Keywords: Camel Milk, Amylase, Lactoferrin, Hepatitis C, Inhibition, In Vitro Assay

Introduction

Hepatitis C virus (HCV) is the major etiologic agent of parenterally transmitted non-A, non-B hepatitis worldwide (1-3). Most infected individuals are unable to eliminate the virus, resulting in a persistent infection in about 80% of cases. Chronically infected patients often develop progressive liver disease, cirrhosis, hepatic failure, and hepatocellular carcinoma (HCC) (4, 5). Because
development of a robust cell culture system for HCV infection has remained elusive, little is known about HCV-host cell interactions and how they influence cell physiology or viral replication. There is no vaccine against HCV until now. The HCV genome carries a single open reading frame (ORF) flanked by non-translated regions and encodes a single polyprotein of about 3010-3033 amino acids. HCV polyprotein is cleaved by both host cell and viral proteases into at least 10 distinct structural and nonstructural protein products. The major structural proteins are a core (C) protein and two envelope proteins called E1, E2 and a short hydrophobic peptide p7 (6).

Egypt considered one of the world’s highest HCV prevalence rates and the subtype 4a is a predominant, but new putative subtypes have been described (7). In addition to the current therapies (IFN-α-2a, -2b, pegylated, ribavirin), there are several traditional medicines used by different Egyptian patients sectors. The most popular one is the camel milk, 50% of the patients shown a marked improvement in general fatigue (8). It has been noted that despite the lack of refrigeration, camel’s milk remain unspoiled for several days; this may be due to the antibacterial activity of certain proteins contained in camel’s milk (FAO). Camel milk contains numerous proteins as immunoglobulins, lactoalbumin, lactoperoxidase, casein, lysozyme, lactoferrin, amylase and other proteins. Lactoferrin is an 80 kDa monomeric glycoprotein present in secretions such as breast milk, saliva, semen and tears. The highest concentration of lactoferrin is found in colostrums (9). Lactoferrin plays an important and multifunctional role in innate and specific host defense alone or with other milk proteins against infection of bacteria, fungi and viruses (10). Lactoferrin acts as an antiviral protein against herpes simplex virus (HSV), human cytomegalovirus (HCMV), HCV, poliovirus, enterovirus 71 (EV71), BK polyomavirus, HIV and HPV (11). Lactoferrin exhibits its antiviral activity early in the infection cycle of HSV (12), HPV-16 (13) and hepatitis B virus (14) through interaction with heparin sulfate on the cell surface seem to block the attachment of the virus. The antiviral activity could also be mediated by a direct interaction between the virus and lactoferrin as seen for poliovirus (15), rotavirus 270, HIV (16), HCV (17), EV71 (18), and BK polyomavirus (13). The current study was analyzed the potential in vitro antiviral activity of both amylase and lactoferrin derived camel milk after purification on normal human lymphocytes and hepatic cell line (hepG2).

Materials and Methods

Camel milk processing
Ten liters of Arabian camel (Camelus dromedaries) milk was purchased from (ALKHIR Farm, Giza, Egypt), collected from about 200 camels and transferred to our laboratory in about 200 mL frozen aliquots. To prevent microbial growth and reduce protease activity 0.2% sodium azide, 5 mM EDTA, 5 mM PMSF were added to the milk before processing. The milk was skimmed and casein removed as previously described (8).

Lactoferrin purification
Casein free, skimmed milk was used for amylase and lactoferrin purification. The skimmed milk was diluted with 50 mM Tris-HCl buffer, pH 8.0 then applied directly to CM-Sephadex G50 column, this protocol was detailed elsewhere (8).

Camel milk β-amylase purification and characterization
Casein free, skimmed milk was used for β-amylase purification; it was diluted with 50 mM tris-HCl buffer, pH 7.0 then applied directly to a DEAE-Sepharose column equilibrated with 50 mM tris-HCl buffer, pH 7.0 as mentioned elsewhere. In brief, the exchanged material was eluted with a step wise gradient ranging from 0.0 to 0.4 M NaCl prepared in the same buffer at a flow rate of 4 ml/min and 5 ml fractions. DEAE-Sepharose fractions were concentrated separately by amicon ultrafiltration cell (30 KDa MWCO) and applied to a Sephadex G-100 column equilibrated with the same buffer and developed at a flow rate of 10 ml/h and 1 ml fractions.

Protein determination
Protein was determined either by measuring the absorbance at 280 nm or by the method of Bradford (1976) (19) using bovine serum albumin as a standard.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis
For examining the homogeneity and molecular weight of purified proteins, they were subjected to electrophoresis, with the use of homogenized 12% SDS polyacrylamide gel. After the electrophoresis, the gel was stained and distained based on previous protocols (20).
Infected serum samples

For infection experiments, we utilized HCV positive serum samples of genotype 4 as described by Ohno et al. (18).

Human blood lymphocytes separation

Peripheral blood cells (PBMCs) were isolated, as reported by Lohr et al. (21). Briefly, peripheral blood samples were diluted with 5 volumes of a freshly prepared RBC lysis buffer (38.8 mmol/L NH4Cl, 2.5 mmol/L K2HCO3, 1 mmol/L EDTA, pH 8.0), incubated at room temperature for 10 min and centrifuged at 1500 rpm for 5 min. The nucleated cells were precipitated in the bottom of the tube.

Cytotoxic effect of lactoferrin and amylase

The cytotoxic effect of purified cLf or amylase on human separated (PBMCs) and HepG2 was examined by the counting of viable cells after trypan blue treatment. PBMCs cells (2.5×10⁵) and HepG2 (10⁵) were plated in two 24-well microtiter plates in duplicate and cultured for two days at 37°C before cLf and amylase treatment, then the medium was refreshed with new RPMI-1640 supplemented medium containing 4.0 mg/mL of cLf or amylase. The cells and cLf or amylase were incubated for 90 min at 37°C and washed three times with 1 mL of PBS. The cells were maintained with 1 mL of fresh medium for seven days at 37°C. After one week in culture, the cells were collected and suspended in medium, and the total number of viable cells was counted after trypan blue treatment. We also examined the viability of cells which were cultured for one day with medium containing 2.0 mg/mL of cLf or amylase as previously described (8).

Inhibition potential of the cLf and amylase on HCV

To examine the interaction of cLf or amylase with the human PBMCs (2.5×10⁵) and HepG2 (10⁵) cells were plated in two 24-well microtiter plates. cLf or amylase were added to the leukocytes and HepG2 cells (50 mL of RPMI-1640 supplemented medium) at a final concentration of 1.0 mg/mL for each and incubated for 60 min at 37°C. Free cLf or amylase was removed by washing three times with 1 mL of PBS. After addition of 50 ml of medium containing 1 mL of serum (8.3×10⁶ copies/mL, genotype 4a), the cells were incubated for 90 min at 37°C. The cells were washed three times with PBS and cultured for seven days at 37°C. To examine the interaction of cLf or amylase with HCV, 1 mL of infected serum and cLf or amylase (final concentration of 1.0 mg/mL) was pre-incubated in 50 mL of medium for 1 h at 4°C, and then the mixture of HCV and cLf or amylase was added to leukocytes and HepG2 cells cultured as described above, and incubated for 90 min at 37°C. The cells were washed three times with 1 mL of PBS and further cultured for 7 days at 37°C, followed by total RNA extraction.

Intracellular HCV immunostaining assay

The intracellular HCV immunostaining assay was performed in 24-well microtiter plate. cLf or amylase (1 mg/mL) was incubated with infected serum for 1 h at 4°C, then the mixture of HCV and cLf or amylase was added to HepG2 cells cultured as described above, and incubated for 90 min at 37°C. The cells were washed three times with 1 mL of PBS each and further cultured for 7 days at 37°C. After 7 days, blocking buffer (2% bovine serum albumin or gelatin in PBS) was added for one hour at room temperature. Then the cells were fixed with paraformaldehyde and permeabilized with 0.5% Triton X-100. Purified primary antibody (22) was diluted to 1:200 prior to use and then incubated for 1 h at room temperature. Horseradish peroxidase-conjugated anti-human IgG diluted 1:2000 with 2% bovine serum albumin or gelatin-PBS were added to the cell monolayer and incubated for 1 hour at room temperature. The reaction was developed with DAB peroxidase substrate and stopped after 10 min of incubation with distilled water. Positive and negative controls were included. The distinct colored foci were examined and some positive and negative fields were pictured by phase contrast microscope (Olympus IX70).

HCV replication inhibition potential of cLf and amylase

Human PBMCs and HepG2 cells were washed twice in RPMI 1640 media. The PBMCs and HepG2 cells were suspended at 2×10⁶ cells/mL in RPMI-1640 culture media. The cells were left to adhere on the four 24-well plates for 24 h at 37°C, then infected with HCV-infected serum (8.3×10⁶ copies) in RPMI 1640 media and incubated for 48 h at 37°C. The cLf or amylase was added at concentrations of 0.25, 0.5, 0.75, 1.0 and 1.25 mg/mL. Positive and negative control cultures were included. After 96 h of incubation at 37°C, two plates were used for total RNA extraction. Another two plates were used for adding another dose of the test proteins. The cells were incubated for another 96 h at 37°C, followed by total RNA extraction.

RNA extraction from PBMCs and HepG2 cells

RNA was isolated from PBMCs and HepG2 cells as described by Lohr et al. (21). Briefly, cells were
precipitated and washed in the same buffer to remove adherent viral particles before lysis in 4 mol/L guanidinium isothiocyanate containing 25 mmol/L sodium citrate, 0.5% sarcosyl and 0.1 mol/L β-mercaptoethanol and 100 µL sodium acetate. The lysed cells were centrifuged at 12000 rpm for 10 min at 4°C. The aqueous layer was collected and mixed with equal volume of isopropanol. After incubation at 20°C overnight, RNA was precipitated by centrifugation at 12000 rpm for 30 min at 4°C and the precipitate RNA was washed twice with 70% ethanol.

PCR of genomic and anti-genomic RNA strands of HCV

Reverse transcription-nested PCR was carried out according to Lohr et al. (21), with few modifications. Retrotranscription was performed in 25 µL reaction mixture containing 20 U of AMV reverse transcriptase (Clontech, USA) with either 400 ng of total HepG2 or PBMCs cells RNA, 40 U of RNAsin (Clontech, USA), a final concentration of 0.2 mmol/L from each dNTP (Promega, Madison, WI, USA) and 50 pmol of the reverse primer 1CH (for plus strand) or 50 pmol of the forward primer 2CH (for minus strand). The reaction was incubated at 42°C for 60 min and denatured at 98 for 10 min. Amplification of the highly conserved 5'-UTR sequences was done using two rounds of PCR with two pairs of nested primers. First round amplification was done in 50 µL reaction mixture, containing 50 pmol from each of 2CH forward primer and P2 reverse primer, 0.2 mmol/L from each dNTP, 10 µL from RT reaction mixture as template and 2 U of Taq DNA polymerase (Promega, USA) in a 1× buffer supplied with the enzyme. The thermal cycling protocol was as follows: 1 min at 94°C, 1 min at 55°C and 1 min at 72°C for 30 cycles. The second round amplification was done similar to the first round, except for use of the nested reverse primer D2 and forward primer F2 at 50 pmol each. A fragment of 174 bp was identified in positive samples. Primer sequences were as follows: 1CH: 5′-ggtgcacggtctacgagacctc-3′, 2CH: 5′-aactactgtcttcacgcagaa-3′, P2: 5′-tgccacgctacgctagctcagc-3′, D2: 5′-actcggctagcagtctcgcg-3′ and F2: 5′-gtgcagcctccaggaccc-3′. To control false detection of negative-strand HCV RNA (38, 39) and known variations in PCR efficiency, specific control assays and rigorous standardization of the reaction were employed: (1) cDNA synthesis without RNA templates to exclude product contamination; (2) cDNA synthesis without RTase to exclude Taq polymerase RTase activity; (3) cDNA synthesis and PCR step done with only the reverse or forward primer to confirm no contamination from mixed primers. These controls were consistently negative. In addition, cDNA synthesis was carried out using only one primer present followed by heat inactivation of RTase activity at 95°C for 1 h, in an attempt to diminish false detection of negative-strand prior to the addition of the second primer.

Results

Camel lactoferrin purification

Ten liters of Arabian camel milk was defatted and decaseinated. The camel lactoferrin (cLf) was purified from skimmed milk by loaded to CM-Sephadex column. The lactoferrin was eluted at 0.05-0.4 M NaCl, the first was a large peak. Only one band was visualized on SDS polyacrylamide gel of the protein eluted at ionic strength 250-400 mM of NaCl (Fig. 1).

Camel milk β-amylase purification

The purification of β-amylase is summarized in Table 1. The chromatogram of DEAE-Sepharose (Fig. 2) was showed a number of protein peaks, the second peak eluted at ionic strength 200 mM was contained most amylase activity. Amylase DEAE-Sepharose fractions with highest activities were concentrated and applied on Sephadex G-100 column (Fig. 3). The enzyme activity was estimated only in second peak which shown a consistent with the protein peak. The electrophoretic behavior of different purification steps was examined. Only one band was visualized on SDS polyacrylamide gel after
Sephadex G-100 step, which indicated the homogeneity of the final preparation of β-amylase (Fig. 1).

Molecular weight

The molecular mass of camel lactoferrin and amylase were estimated to be 80, 63 KDa (Fig. 1) using SDS polyacrylamide gel electrophoresis, respectively.

Cytotoxic effect of cLf and amylase

To avoid any possibility that the elimination of the HCV was caused by a reduced viability of PBMCs or HepG2 cells, we examined the cytotoxic effects of the cLf and amylase on PBMCs and HepG2 cell line. The PBMCs (2.5×10⁵) and HepG2 (10⁵) were treated with 4 mg/ml of cLf or amylase for 24 h or 7 days. Cell viability was observed in comparison with the untreated PBMCs and HepG2 cultures. Neither 4 nor 2 mg/mL had any effects on the viability of the PBMCs or HepG2 or cytotoxic effects after 24 hour and 7 days of incubation periods, respectively (data not shown).

Inhibition potential of the cLf and amylase

The camel lactoferrin was able to completely inhibit the HCV entry into PBMC and hepG2. However, the purified camel milk β-amylase could not able to block the HCV entry. Two sets of cells, PBMCs (2.5×10⁵) or HepG2 (10⁵) were cultured, in duplicate, as described under materials and methods. One of the cultures was treated with cLf 1 mg/ml or amylase 1 mg/mL for 60min, and then infected with HCV for 90 min; both proteins could not protect the cells from HCV entry (Fig. 4). The other cultures were inoculated with HCV infected sera pretreated with cLf (1 mg/mL) or amylase (1mg/mL) for 60 min. The inoculated cells were cultured for seven days. The RT-nested PCR amplified the 174 bp of 5 end of HCV non coding sequence in comparison to the positive and negative control (Fig. 4 & 5). The band of 174 bp was not amplified in case of cLf while it was detected with amylase treatment.

Assay of HCV immunostaining in HepG2 cell line

Immunostaining assays have been widely used to evaluate the neutralizing antibody responses to viruses that can form foci in infected cells. After 7 days of infection and cell permeabilization, detection of the HCV foci was carried out using primary antibody and a peroxidase anti-human IgG-antibody. The reaction was developed with DAB horseradish peroxidase substrate. The viral foci were stained by brownish color, making them easy to see under light microscopy. Figure 6 indicated that the viral foci were not detected in case of cLf pretreated...
Camel amylase activity against HCV. Amylase was tested for block the HCV virus entry into PBMCs and HepG2, through direct interaction between the HCV molecules and amylase (lane 4) or through interaction of amylase with cultured cells (lane 5). DNA ladder (lane 1), negative control (lane 2), and amplified 174 bp of HCV from used positive control (lane 3). Amplified products were resolved in 3% agarose gel/ethidium bromide staining.

**Figure 5.** Camel amylase activity against HCV.

**Figure 6.** Typical pictures of HCV-infected HepG2 cells (A) and PBMCs (B) under a light microscope (X20). Pictures A1 and B1 are positive control, pictures A2 and B2 are negative control, pictures A3 and B3 shows antiviral activity of cLf and pictures A4 and B4 shows no viral effect of amylase against HCV. The foci are pointed with arrows.

**Figure 7.** In vitro camel lactoferrin activity against HCV-infected hepG2 cells. Lane 1 contains the molecular weight marker ladder, lane 2 is negative control, lane 3 is positive control and lanes (4-7) shows the effect of cLf different concentrations (0.25, 0.5, 0.75, 1.0 and 1.25 mg/mL) on HCV-infected HepG2 after 4 days of treatment.

**Figure 8.** In vitro camel amylase activity against HCV-infected hepG2 cells. DNA ladder (lane 1), positive control (lane 2), negative control (lane 3) and lanes (4-7) shows the effect of amylase different concentrations (0.25, 0.5, 0.75, 1.0 and 1.25 mg/mL, respectively) on HCV-infected HepG2 after 4 days of treatment.

**Discussion**

Drinking milk is a practice that dates back to the domestication of animals in prehistoric times, and has taken advantage of the extensive nutritional value of that natural product - not only to the young born, but also to the child, the adult and the elderly. Milk possesses a protein system constituted by two major families of proteins: caseins (insoluble) and

with HCV-infected serum, while detected with amylase treatment.

**Effect of cLf and amylase on replication of HCV**

cLf and camel milk amylase at concentrations 0.25, 0.5, 0.75, 1.0 and 1.25 mg/mL were investigated for their in vitro ability to inhibit the viral replication inside the infected PBMC and hepG2. Inhibition of viral replication was detected by amplification of viral RNA segments using the RT-PCR technique. cLf could inhibit the HCV replication only at concentrations of 1.0 and 1.25 mg/mL after 96 h has shown in Figure 7. However, the β-amylase did not inhibit HCV replication at any concentrations used (0.25, 0.5, 0.75, 1.0 and 1.25) after 96 h (Fig. 8).
whey proteins (soluble). Whey proteins are globular molecules with a substantial content of $\alpha$-helix motifs, in which the acidic/basic and hydrophobic/hydrophilic amino acids are distributed in a fairly balanced way along their polypeptide chains (23). Whey proteins include lactoglobulin, lactoalbumin, immunoglobulins, lactoferrin, amylase and lactoperoxidase, together with other minor components.

In this study, we investigated the potential activity of camel milk-amylase and lactoferrin against hepatitis C virus infectivity in HepG2 and PBMCs. Results shown that camel milk lactoferrin inhibits, in vitro, the HCV entry into human PBMCs and HepG2 cell lines through direct interaction with virus molecules rather than interaction with cells. While camel milk $\beta$-amylase did not have any blocking activity against HCV, in vitro, either direct interaction of $\beta$-amylase with virus molecules nor interaction with cells. This inhibitory activity of cLf seems congruent with the previous study, which used cLf to inhibit HCV (genotype 4) entry into human PBMCs (22). This study confirmed the HCV entry by RT-nested-PCR and the indirect intracellular immunostaining of HCV E1 with the flowcytometry (22). While our study used RT-nested-PCR and the intracellular immunostaining of HCV assay. Intracellular immunostaining assays have been widely used to evaluate neutralizing antibody responses against a range of non cytopathic viruses (24). Intracellular immunostaining assay is a simple, specific and reproducible cell culture based on neutralizing anti-HCV antibodies by viral foci. The inhibitory activity of cLf seems agree with the previous studies (25, 26) using human and bovine lactoferrin to inhibit HCV (genotype1) entry into the PH5CH8 cell-line. These studies were evaluated the HCV entry by RT-nested-PCR only.

The results indicated that cLf inhibit HCV replication at concentrations of 1.0 and 1.25 mg/mL after 8 days. $\beta$-amylase could not inhibit HCV replication at any concentrations of 0.25, 0.5, 0.75, 1.0 and 1.25 after 4 days. LF is an iron-chelating, monomeric glycoprotein, characterized by a molecular weight of 80 kDa, to which two carbohydrate groups are attached. The major mechanisms by which LF is believed to exert its antiviral activity are LF interacts with viruses, but not with cells infected thereby; however, it can interact with the host cells before they are infected with hepatitis B virus (HBV) for which interaction with the cell surface proteins blocks viral adhesion to the cells themselves (14). Bovine lactoferrin (bLf) inhibits the viral entry into hepatocytes and lymphocyte cells via neutralizing the virion and blocking the invasion of the cell thereby. In the case of HIV-1, succinylation of that protein slightly increases the extent of inhibition of the HIV-1 reverse transcriptase (27). In vivo experiments involving HIV-infected individuals have indicated that bLf inhibits strongly that enzyme, but only slightly protease and integrase (28).

Camel lactoferrin has three unique characteristics which characterize it from lactoferrin of other species: i) the predicated glycosylation sites are entirely different in cLf, ii) some critical residues such as Pro418, Leu423, Lys433, Gln651, Gly629, Lys637, Arg652, and Pro592 related to domain movement in the protein are different in cLf from those found in other lactoferrins, indicating the possibility of specific structural differences, iii) most importantly, cLf loses 50% iron of its bound iron at pH 6.5 and the remaining 50% is released at pH 4.0-2.0. Its proteolyticly generated N- and C-lobes showed that the C-lobe lost iron at pH 6.5, while the N-lobe lost it only at pH less than 4.0, which indicating a striking difference in the iron release mechanism from the two lobes. These data demonstrate that the cLf behaves as half lactoferrin “iron binding protein” and half transferring “iron-transporter protein”, unlike other lactoferrins and transferrins (29).

Previous studies have suggested that patients with chronic HCV infections have elevated levels of serum markers of iron stores (ferritin, transferrin-iron saturation, or iron). The presence of elevated body iron stores and, in particular, elevated hepatic iron levels, is one of the strongest predictors of resistance to interferon treatment for HCV. Hepatic iron overload increases the production of reactive oxygen species, which may lead to lipid peroxidation, steatosis and depletion of glutathione stores, accelerated liver damage and developing hepatocellular carcinoma (30, 31). A recent study showed that the therapy with bovine lactoferrin lead to lipid peroxidation inhibition (32). In accordance, the camel milk lactoferrin maintains a dual function: 1) it inhibits lipid peroxidation and 2) it regulates the hepatic iron content through its ability to bind and transport the iron at various pHs. The current results demonstrated the marked viral inhibition and/or blocking in two different cells systems by camel lactoferrin, which may confirm the nutraceutical capacity of camel milk. On the other hand, the data subside the anti-HCV activity from the camel milk amylase.

Conclusion, we demonstrated, for the first time, that the purified camel milk lactoferrin of 80 KDa has remarked inhibitory effect on HCV (genotype 4a) entry into hepG2 as well as PBMCs. This
inhibition takes place by direct interaction with viral molecules and rather than with the host cells. cLf inhibit the viral replication in the HCV-infected PBMC and HepG2 cells, while β-amylase could not at all. This finding may attribute to the dual function of cLf as both an iron binding protein and an iron transporter protein. Further studies are ongoing to identify other proteins and peptides of camel milk, which may have anti-HCV activity.

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