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Hydrolysis improves the inhibition efficacy of bovine lactoferrin against infection by SARS-CoV-2 pseudovirus

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The entry of SARS-CoV-2 into host cells may involve the spike protein cleavage by cathepsin L (CTSL). Certain food proteins such as lactoferrin (Lf) inhibit CTSL. The current study investigated the impact of hydrolysis (0–180 min) by proteinase K on electrophoretic pattern, secondary structure, cathepsin inhibitory and SARS-CoV-2 pseudovirus infectivity inhibitory of bovine Lf. Gel electrophoresis indicated that hydrolysis cut Lf molecules to half lobes (~40 kDa) and produced peptides <18 kDa. Approximation of the secondary structural features through analysis of the second-derivative amide I band collected by infra-red spectroscopy suggested a correlative—causative relationship between cathepsin inhibition and the content of helix-unordered structures in Lf hydrolysate. The half maximal inhibitory concentration (IC50) of Lf hydrolysed for 90 min (H90) against CTSL was about 100 times smaller than that of the Lf hydrolysed for 0 min (H0). H90 had also double activity against SARS-CoV-2 pseudo-types infectivity compared with H0.

1. Introduction

The coronavirus 2019 (COVID-19) pandemic, caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has posed unprecedented health challenges. Coronaviruses (CoVs) are enveloped positive-sense RNA viruses that bind to the human angiotensin-converting enzyme 2 (hACE2), found on the host cell membrane, via their spike glycoproteins (S proteins) (V’kovski, Kratzel, Steiner, Stalder, & Thiel, 2021). SARS-CoV-2 possesses modified S proteins and has a ten-to twenty-fold higher affinity for hACE2 receptors than the SARS-CoV (Xia et al., 2020), resulting in a faster transmission rate. The S protein is composed of two subunits: (a) S1 subunit – containing a receptor-binding domain (RBD) responsible for virus attachment to the ACE2 receptor (Xia et al., 2020); and (b) S2 subunit – containing a fusion peptide responsible for endocytosis (Hoffmann et al., 2020). The entry of SARS-CoV-2 into host cells takes place by two mechanisms: (a) membrane fusion in which transmembrane protease serine 2 (TMPRSS2) primes the S protein and (b) endocytosis where cathepsin L (CTSL), and phosphatidylinositol 3-phosphate 5-kinase are critical (Hoffmann et al., 2020; Ou et al., 2020; Zhao et al., 2021).

CTSL, a cysteine protease found in all tissues and cell types, is a matrix-degrading enzyme. Its principal function is proteolysis of protein antigens generated during pathogen endocytosis (Gomes et al., 2020; Liu et al., 2018). However, the excessive secretion of cysteine cathepsins is associated with a variety of pathological states in humans such as inflammation, apoptosis, tumour cell proliferation, and osteogenesis (Dana & Pathak, 2020; Fonović & Turk, 2014). Since inhibition of cathepsins can lead to the failure reversal (Mittal, Mir, & Chauhan, 2011), cathepsin inhibitory techniques and substances are enthusiastically pursued by researchers. Some studies suggested that CTSL inhibition may be a way to prevent SARS-CoV-2 infection in humans (Liu, Luo, Libby, & Shi, 2020; Ou et al., 2021; Zhao et al., 2021). In this context, synthetic cathepsin inhibitory drugs such as hydroxychloroquine and chloroquine were shown to interfere with the SARS-CoV-2 infection in human cells (Hoffmann et al., 2020; Ou et al., 2021).

Even though the above-mentioned in vitro evidence was not confirmed in human studies (Deng et al., 2022; Hernandez, Roman, Pasupuleti, Barboza, & White, 2020) the mechanistic pathway is worth investigating considering that some plant and animal foods contain cathepsin inhibitory compounds. Examples of the plant-based cathepsin inhibitors are bromelain inhibitor VI, and oryzacystain derived respectively from pineapple stem and rice. They share similar structural features such as (extensive) disulfide...
crosslinks and absence or low contents of $\alpha$-helix structures (Madadlou, 2020). Mammalian milk has also significant levels of cysteine protease inhibitors, the most important one is lactoferrin (Lf) (Ohashi et al., 2003).

Bovine Lf is a 78 kDa glycoprotein with an isoelectric point between pH 8 and 9. Lf is rich in cysteine and comprises two highly homologous lobes, representing the N-terminal and C-terminal halves of the polypeptide chain. Each lobe contains an iron-binding site (Moore, Anderson, Groom, Haridas, & Baker, 1997).

Apparently, the iron-binding property have no effect on Lf antiviral activity (Wotring et al., 2022). Lf was shown to inhibit SARS-CoV-endo cytosis by interacting with cell-surface heparan sulphate proteoglycans and thereby blocking the binding of S protein to HEK293E/ACE2-Myc cells (Lang et al., 2011). Lf may also suppress some viral infections by inhibiting cathepsins. While a synthetic peptide targeting the active site of Lf, i.e., lactoferricin was assessed for its antiviral activity against the parent protein. Recently, a well-known proteolytic product of Lf, (Madadlou, 2020), it was hypothesised that (Wotring et al., 2022). Based on the results of a molecular docking proteinase K can be used to generate peptide fragments of half the cutting bovine Lf molecules to the half-molecules may be advan-

Sigma naphtalenesulfonic acid (ANSA) were purchased from (The Netherlands). Proteinase K, pefabloc SC, and 8-anilino-1-

96.4% Lf) was kindly provided by FrieslandCampina Ingredients (Research reagent for anti-SARS-CoV-2 Ab NIBSC code 20/130) was used in pseudo-type viral infectivity assay (Hyseni et al., 2020). Moreover, negative serum, SARS-CoV-2 pseudo-types (full-length S-protein), and HEK 293 T/17 cells (human embryonic kidney 293 cells) (ATCC-CRL 1573) were used in the viral infectivity assay. HEK 293 T/17 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) in high glucose (Euroclone, Pero, Italy) supplemented with 2 mLoL-glutamine (Lanza, Milan, Italy), 100 units mL$^{-1}$ penicillin-streptomycin (Lanza, Milan, Italy), and 10% feta (Euroclone, Pero, Italy) (Hyseni et al., 2020).

2.2. Hydrolysis of Lf

A buffer solution of 100 mmo ammonium bicarbonate (NH$_4$HCO$_3$) in deionised water was prepared and pH was adjusted to 7.00 ± 0.02 using 0.5 mNaOH. A 0.2 mmo solution of Lf in buffer was prepared and the pH was adjusted to 10 ± 0.02, followed by 5 min of slowly shaking at 20 °C. The solution was kept stored for 24 h at 4 °C to warrant complete hydration. Proteinase K and pefabloc SC solutions were prepared in 100 mmo NH$_4$HCO$_3$ buffer (pH 7 ± 0.02) at concentrations of 1 mm and 200 mm, respectively. The Lf solution was incubated with proteinase K at 37 °C in a shaking water bath and the enzyme to Lf mass ratio was kept at 1:100 (Sharma et al., 1999). The hydrolysis pattern was observed at incubation times of 0, 30, 60, 90, 120, and 180 min. Immediately after hydrolysis, samples were cooled to room temperature (20 °C) within an ice bath, and supplemented with pefabloc SC at a pefabloc-to-Lf hydrolysate ratio of 1:50 (for proteinase K inactivation), followed by manual shaking for uniform mixing. The hydrolysates were kept at 20 °C for 5–10 min and stored at −20 °C until further analysis.

2.3. Gel electrophoresis

Sodium dodecyl sulphate-polycrylamide gel electrophoresis (SDS-PAGE) analysis of Lf hydrolysate was performed under non-reducing and reducing conditions. The hydrolysate was diluted threefold to a concentration of 0.07 mm, uniformly mixed with a mixture of SDS-PAGE sample buffer (ratio 2:5, v/v) and treated with either 100 mm ammonium bicarbonate buffer (pH 10 ± 0.01, for non-reducing conditions) or dithiotheitol (for reducing conditions), followed by centrifugation for 1 min at 800 g. The samples were heated in a shaking water bath for 10 min at 70 °C and centrifuged for 1 min at 800 g. Aliquots (20 mL) of samples were loaded on 4–12% bis-Tris precast polycrylamide gels, and Spectram™ multicolour broad range protein ladder was used as the molecular mass marker. After electrophoresis, gels were rinsed with distilled water and stained with Coomassie brilliant blue R-250 for 3 h at 20 °C.

2.4. Cathepsin inhibitory assay

The activity of human cathepsins L, B, H, and K as influenced by the Lf hydrolysate was measured using fluorometric inhibitor screening kits. The assay uses the ability of active human cathepsin to cleave a synthetic substrate 7-amino-4-trifluoromethylcoumarin (AFC) and release a fluorescent component that could be detected and measured using a fluorescence microplate reader. Cleavage of substrate is decreased in the presence of a cathepsin inhibitor, resulting in reduced or no formation of AFC fluorescence. All cathepsin assays were performed in accordance with the detailed protocols outlined in the Abcam booklet (www.abcam.com).

For measuring the inhibitory efficacy on CTSL, a CTSL reagent aliquot was made by mixing 5 mL dithiotheitol (DTT) with CTSL...
reagent. According to the preliminary experiments, the inhibitor compound was prepared by dissolving Lf hydrolysate (0–180 min hydrolysis time in replicates) in CTSL assay buffer at final concentrations of 1.45 \( \times 10^{-6} \), 6.25 \( \times 10^{-7} \), 2.50 \( \times 10^{-6} \), 7.50 \( \times 10^{-3} \), and 2.50 \( \times 10^{-4} \) M. For measuring the inhibitory efficacy on other cathepsins (B, H, and K), a slightly different protocol was applied, i.e., DTT was not used. The difference in protocol was according to the manufacture (Abcam) guidelines. The wavelengths required to detect the activity of cathepsins B, H, and K [excitation/emission wavelengths \( \lambda_{ex}/\lambda_{em} = 400/505 \text{ nm} \)] were also slightly different from those needed for cathepsin L \( \lambda_{ex}/\lambda_{em} = 405/505 \text{ nm} \). The duration of reading for cathepsin L was 30 min, while that for cathepsin B, H, and K was 120 min.

The activities of all cathepsins were monitored every 3 min in VersaMax microplate reader (Company: VersaMax™, Software: SoftMax Pro, Wageningen, the Netherlands) at 37 °C at previously stated fluorescence intensities (I) in a kinetic mode. The samples were measured in triplicates to account for standard errors. Irreversible inhibitors that fully inhibit cathepsin activity at the tested concentration had a relative fluorescence unit (RFU) = 0, and the percent relative inhibition was 100%.

2.5. Fourier-transform infra-red spectroscopy

For spectra acquisition, the FTIR measurements were taken with a Bruker Invenio-S FTIR spectrometer (Bruker Optik GmbH, Germany) and data acquisition was controlled by OPUS 8.5 software. The spectra were recorded in transmission mode from 4000 cm\(^{-1}\) to 400 cm\(^{-1}\) with a resolution of 4 cm\(^{-1}\), an aperture of 6.0 mm, and a total of 60 scans. The background and buffer were collected before each sample scanning and subtracted at the same time, while 1% SDS and Milli-Q water were used to clean the cell.

For spectra analysis, the spectra were smoothed and normalised, and second-derivative spectra were obtained by Savitsky-Golay derivative method for a nine data point window using OPUS 8.5 software. Then the second derivative curves were cut from 1700 cm\(^{-1}\)–1600 cm\(^{-1}\) (amide I) and analysed by fitting Gaussian curve using OriginPro 2021 software (OriginLab Cooperation, Northampton, US). The quantification of secondary structures was performed by calculating each peak area as a percentage of the sum of all peak areas.

2.6. SARS-CoV-2 pseudo-type infectivity assay

2.6.1. Production of SARS-CoV-2 pseudo-types

The protocol described by Hyseni et al. (2020) was used to generate lentiviral-based pseudo-types containing the surface (S) glycoprotein of wild-type SARS-CoV-2 virus.

2.6.2. SARS-CoV-2 pseudo-type-based neutralisation assay

The bovine Lf hydrolysate samples were evaluated for their efficacy to neutralise the SARS-CoV-2 pseudovirus internalisation into HEK 293 T/17 cells (human embryonic kidney 293 cells). A control positive with 100% neutralisation activity (NIBSC serum, code 20/130) and a control negative serum with 0% neutralisation activity (NIBSC serum, code 20/130) and a control negative serum with 0% neutralisation activity (NIBSC serum, code 20/130) and a control negative serum with 0% neutralisation activity (NIBSC serum, code 20/130) were most probably corresponds to apo-Lf half molecule, whereas the latter was most likely monoferric Lf half molecule (Sharma et al., 1999). According to the manufacturer, the Lf preparation used in the current study had an iron saturation level of only 10% and, hence, generation of a thicker Lf solution.

Non-reducing SDS-PAGE indicates that hydrolysation significantly influenced the electrophoretic pattern of Lf. As a consequence of hydrolysation even for the shortest time, i.e., 30 min, the band at ~80 kDa under both conditions. The band corresponds to intact Lf. Also, a thin band at around 200 kDa was observed and attributed to protein aggregates (Goulding, O’Regan, Bovetto, O’Brien, & O’Mahony, 2021). Moreover, under the non-reducing condition, several thin bands between 22 and 58 kDa were observed (Fig. 1a). These bands are likely minor amounts of other milk proteins co-puriﬁed with Lf (Lönnander, Du, & Jiang, 2021) and/or large peptide fragments that were generated promptly by proteinase K despite its immediate inhibition after addition to the Lf solution.

Non-reducing SDS-PAGE indicates that hydrolysation significantly influenced the electrophoretic pattern of Lf. As a consequence of hydrolysation even for the shortest time, i.e., 30 min, the band at ~80 kDa almost completely disappeared (Fig. 1a) and two bands at 38 kDa and 43 kDa emerged. The band at 38 kDa was comparatively thinner than that at 43 kDa. The former probably corresponds to apo-Lf half molecule, whereas the latter was the most likely monoferric Lf half molecule (Sharma et al., 1999). According to the manufacturer, the Lf preparation used in the current study had an iron saturation level of only 10% and, hence, generation of a thicker band for apo-Lf half-molecule was expected. The production of Lf half molecules due to hydrolysation confirme...
bands corresponding to peptides with molecular masses below 15 kDa were observed under reducing condition (Fig. 1b). In agreement with our observation on LF half molecules, Sharma et al. (1999) did not detect the band corresponding to LF half molecules (~40 kDa) under reducing SDS-PAGE when they run hydrolysis of (buffalo) LF at a high proteasease K-to-substrate ratio. However, when they applied an enzyme-to-substrate ratio comparable with that used by Sharma et al. (1999), the bands corresponding to peptides with molecular masses below 15 kDa were observed under reducing condition (Fig. 1b). Nonetheless, when they prolonged the hydrolysis process to 60 min and 90 min (~12 kDa) became faint by hydrolysis for 60 min (H60), and 90 min (H90), respectively. H90 had a comparable inhibitory on CTSL and H120 and H180 had even a lower CTSL inhibitory than H0. For other kinds of cathepsins, similar results were obtained by hydrolysis for >120 min. The cathepsin B and H inhibitory of H120 and H180 was lower than that of H0 and the inhibitory property of H120 and H180 on cathepsins K was different from H0 (Table 1). For all types of cathepsins, the IC\textsubscript{50} of the LF hydrolysate was lowest at H90 and close to that of the synthetic cathepsin inhibitory compound, Z-Phe-Phe-fluoromethyl ketone (Table 1). The CTSL IC\textsubscript{50} of the synthetic inhibitor was in the same order of magnitude (only 2.4 fold smaller) compared with H90.

In an attempt to explore the cause that explains the highest cathepsin inhibitory activity of H90 among all the hydrolysate samples, we assessed the secondary structure of LF as in Sharma et al. (1999) attributed to dissimilarities in the iron saturation level of the LFs used in the two studies. The iron saturation level of the LF used in the present study was 10%, whereas that of the LF used by Sharma et al. (1999) was 80%. It is known that iron binding decreases the molecular stability of LF (Baker & Baker, 2004) and hence, iron-depleted (apo-lactoferrin) LF has a looser structure than iron-saturated (holo-lactoferrin) LF, causing differences in their proteolytic degradability (Bokkhim, Tran, Bansal, Grøndahl, & Bhandari, 2014). The anti-cathepsin activity of bovine LF hydrolysate samples was measured (Fig. 2) and the results are reported in Table 1. There was statistically no significant difference between the inhibitory efficacy of the samples H0 and H30 against any kind of cathepsins. This suggests that short-term hydrolysis and generation of the half molecules (Fig. 1a) did not affect the cathepsin inhibitory property of LF. Prolongation of the hydrolysis process to 60 min and 90 min caused a significant increase in the cathepsin inhibitory property of LF. When compared with H0, the CTSL IC\textsubscript{50} of LF decreased ~10.5 and ~9.2 folds due to hydrolysis for 60 min (H60), and 90 min (H90), respectively. H90 had a comparable inhibitory on CTSL and cathepsin B, but lesser inhibitory on two other cathepsins assessed in the present study (Table 1). Non-reducing SDS-PAGE indicated that the band corresponding to peptides with molecular masses of ~12 kDa became faint by hydrolysis ≥60 min (Fig. 1a). Nonetheless, it could not (completely) explain the increase in cathepsin inhibitory property of LF by hydrolysis up to 90 min. Interestingly the LF cathepsin inhibitory activity dramatically decreased by further prolonging the hydrolysis to ≥120 min. H120 and H0 had similar CTSL inhibitory, and H180 had even a lower CTSL inhibitory than H0. For other kinds of cathepsins, similar results were obtained by hydrolysis for ≥120 min. The cathepsin B and H inhibitory of H120 and H180 was lower than that of H0 and the inhibitory property of H120 and H180 on cathepsins K was different from H0 (Table 1). For all types of cathepsins, the IC\textsubscript{50} of the LF hydrolysate was lowest at H90 and close to that of the synthetic cathepsin inhibitory compound, Z-Phe-Phe-fluoromethyl ketone (Table 1). The CTSL IC\textsubscript{50} of the synthetic inhibitor was in the same order of magnitude (only 2.4 fold smaller) compared with H90.

In an attempt to explore the cause that explains the highest cathepsin inhibitory activity of H90 among all the hydrolysate samples, we assessed the secondary structure of LF as influenced by hydrolysis was studied by FTIR spectroscopy. The amide I band (1600–1700 cm\textsuperscript{-1}) caused by Ca\textsubscript{\textomega} stretching (about 80%), coupled with in-phase N–H bending (Yang, Yang, Kong, Dong, & Yu, 2015) is primarily used for secondary structure studies. Therefore, the amide I band was derivatised and the structural features were determined. The allocation of the bands in the second-derivative amide I spectra to the structural features, i.e., unordered-helical structures (1646–1666 cm\textsuperscript{-1}), β-sheets, and β-turns was done according to Yang et al. (2015). It was not possible for us to separate the bands standing for unordered and helical (including α-helix) structures. Hence, a collective proportion of these two structures was determined.

Low α-helix content is a characteristic feature of many cysteine protease inhibitors such as bromelain inhibitor VI (Hatano, Kojima, Tanokura, & Takahashi, 1995), and the cytotoxic T-lymphocyte antigen-2β (Delaria et al., 1994). Indeed, LF was employed in the present study because if its low α-helix content (Madadlou, 2020). Therefore, the percente proportion of unordered-helical structures was measured and is reported in Table 1. The data indicate that H90 had the lowest content (i.e., percentile proportion) of unordered-helical structures amongst all the hydrolysate samples.
Even though the percentile proportion of helical structures could not be individually determined, the data suggested an association between the highest cathepsin inhibitory and the lowest unordered-helical structures in H90. The data in Table 1 also indicated that extending the hydrolysis duration to 120 min increased the percentile proportion of unordered-helical structures. We assumed that some peptides which were generated by extended (≥120 min) hydrolysis aggregated and formed structures with higher helix contents. This assumption is supported with SDS-PAGE results. The most massive band identified under reducing SDS-PAGE, i.e., 15 kDa (Fig. 1b) became thicker when hydrolysis duration extended to 180 min. In agreement, other researchers have suggested an increase in α-helix content during protein/peptide aggregation (Ghosh et al., 2015). The increase in unordered-helical structures content due to extended hydrolysis coincided with low cathepsin inhibitory of H120 and H180 (Table 1).

3.3. SARS-CoV-2 pseudo-type infectivity assay

The efficacy of the bovine LF hydrolysate samples in inhibition of SARS CoV-2 pseudo-type infectivity was assessed according to the method of (Hyseni et al., 2020) and the results are reported in Table 1. The IC₅₀ value of the non-hydrolysed LF (i.e., hydrolysed for 0 min, H0) and the LF hydrolysed for the longest duration (H180) was approximately 4.5 µM. This disagrees with an earlier report where IC₅₀ values less than 1 µM were reported for lactoferrin
against SARS-CoV-2 in various cell systems (Mirabelli et al., 2021). The disagreement is probably associated with the different methods used for the viral infectivity assessment in the present study and the referenced research (Mirabelli et al., 2021). Moreover, it is noteworthy that in the present study SARS-CoV-2 pseudotypes were used, whereas Mirabelli et al. (2021) used SARS-CoV-2.

The IC₅₀ value against SARS-CoV-2 pseudo-types of the sample H90 was ≈2 fold smaller than that of H₀ (Table 1), therefore the sample H90 showed a two times increased capacity to prevent the cell infection. The infectivity assay included entry of SARS-CoV-2 pseudo-types into target cells (HEK 293 T/17 cells) and involved the S protein engagement with the ACE2 receptor and priming of the S protein by TMPRSS2 (Hyseni et al., 2020). Although we could not employ a protocol to particularly study the role of CTSL in the pseudo-types entry to the target cells, the infectivity results are in accordance with CTSL inhibitory efficacy of the hydrolysate samples (Table 1).

The higher infectivity inhibitory of H90 compared with the other samples could be associated with its higher CTSL inhibitory, thus proving the relevance of the mechanism. However, the difference between the IC₅₀ values of H₀ and H90 against CTSL was approximately 90 times (see Table 1), which is enormously higher than the difference between the IC₅₀ values of the samples against SARS-CoV-2 pseudovirus. This may indicate that the virus finds other ways to enter in the cells. In this respect, the inhibition of phosphatidylinositol 3-phosphate 5-kinase, TMPRSS2 or blocking two pore channel subtype 2 (Ou et al., 2020) together with CTSL inhibition might be more effective at preventing virus endocytosis. It is noteworthy that in the present study cathepsin inhibitory activity was measured using screening assay kits (abcam.com), not by cell cultures and hence, the CTSL inhibitory measured might not fairly indicate cathepsin inhibitory efficacy in cell studies.

4. Conclusion

Our data showed that hydrolysis by proteinase K can be used to modulate the cathepsin inhibitory of bovine LF. The LF sample hydrolysed for 90 min, i.e., H90 showed comparable CTSL and cathepsin B inhibitory and a lower inhibitory activity on cathepsins H and K. This is a positive feature as it may provide a venue for preferential inhibition of CTSL and cathepsin B by LF without hazardously disturbing the functionality of other cathepsins. Such a preferential-selective inhibitory can be useful in treatment of diseases associated with over-expression of certain cathepsins.

A relationship was observed between the CTSL inhibitory and secondary structure of bovine LF hydrolysates. A lower content of helical-unordered structures coincided with higher CTSL inhibitory. The literature supports a causative relationship between the CTSL inhibitory and helical-unordered structures coincided with higher CTSL inhibitory.

In accordance with CTSL inhibitory, LF hydrolysed for 90 min (H90) had a higher SARS-CoV-2 pseudovirus infectivity inhibitory than the LF hydrolysed for either shorter (0, 30, 60 min) or longer (120, 180 min) durations. However, we noticed that hydrolysis for 90 min caused ≈90-fold reduction in the IC₅₀ of LF against CTSL, whereas it caused only a ≈2-fold reduction in LF IC₅₀ against SARS-CoV-2 pseudovirus infectivity.

It is concluded that either inhibition of CTSL did not take place effectively in the pseudo-type virus experiments (when compared with CTLS inhibitor screening kit) or strategies other than (or together with) CTSL inhibition should be sought and developed for the infectivity inhibition.

Declaration of competing interest

None.

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