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Research Article

Effect of Lactoferrin and Its Hydrolysates Prepared with Pepsin and Trypsin on *Escherichia coli* O157:H7

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Abstract: As A Glycoprotein, Lactoferrin (LF) has many biological actions, among which the antibacterial activity is one of the most important effects studied. This study detected the antibacterial activity of bovine LF against *Escherichia coli* O157:H7. The LF hydrolysate (LFH) was acquired with trypsin and pepsin. It was found that the hydrolysates produced with each inhibited bacterial growth although the activities of trypsin hydrolysate were weaker than those of pepsin hydrolysate. Antibacterial activities of all hydrolysates were corresponding to the fraction with a molecular mass greater than 6kDa. Sulfonic acid derivatives were utilized for fractionation of peptides in hydrolysates and the cationic peptides were investigated employing mass spectrometry.

Keywords: Antimicrobial activities, *Escherichia coli* O157:H7, lactoferrin, lactoferrin hydrolysates, pepsin hydrolysate, trypsin hydrolysate

INTRODUCTION

Lactoferrin (LF) as an iron-binding glycoprotein existing in milk, is found to be 0.1 to 0.3 mg/mL and 2 to 5 mg/mL in the milk of mature bovine and colostrum. The protein has the following biological properties including regulating the absorption of metals like iron and the development of animal cells and the antimicrobial activities against yeasts and bacteria (Valenti *et al*., 1998). Harouna *et al*. (2015) found that LF plays its antimicrobial role via iron sequestration as a native molecule. Fungicidal, antiviral and antiprotozoal activities have also been described for lactoferrin (Farnaud and Evans, 2003). In addition to its iron-sequestering capacity, lactoferrin is able to block the microbial metabolism through interacting with carbohydrates of the bacterial cell membrane (Bokkhim *et al*., 2014).

Native proteins and peptides encrypted within them can show bioactivities of milk components (Mandal *et al*., 2014). As a matter of fact, because LF is subjected to many proteases in the gut and then, split into multiple functional fragments, it would be associated with the investigation of the antimicrobial actions and the properties of these hydrolyzed molecules.

Following two outbreaks in Michigan and Oregon, USA, 1982, *Escherichia coli* O157:H7 was first identified as a human pathogen. It was separated from those individuals who had and severe abdominal cramps bloody diarrhea after eating underboiled hamburgers in restaurants. Since then, it has been separated from food products with growing prevalence (Nyachuba, 2010). It is found that *E. coli* O157:H7 generally accesses the food chain of human beings via water or food which is polluted by faeces from animals carrying the bacteria. Numerous methods have been developed to decrease the incidence of *E. coli* O157:H7 in ruminants and therefore to reduce the carriage in human beings. It is important that those strategies will be economical, practical and suitable from the perspective of animal welfare. However so far, no effective strategy has been developed to decrease *E. coli* O157:H7 incidence of ruminants. This situation calls for innovative strategies to effectively decline *E. coli* O157:H7 prevalence and thereby to lower the public health risk.

Although many studies have been reported on the antimicrobial action of pepsin hydrolysates of LF, no studies have been conducted about the antimicrobial effect of LF hydrolysates produced by trypsin or against *E. coli* O157:H7.

This study aimed to assess the antibacterial activities of bovine LF and LF Hydrolysates (LFH) acquired with varying proteolytic enzymes on the pathogen *E. coli* O157:H7 infected through food. Fractionation was performed for the LFH in accordance with their molecular weight to determine the active peptides.

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MATERIALS AND METHODS

Materials: LF with the purity higher than 98% was bought from BiosunBiomart company (Shanghai, China).

E. coli O157:H7 strain was obtained from the China Center of Industrial Culture Collection (CICC, Beijing, China). After a 50-µL culture aliquot of the microorganism was added to tryptic soy yeast extract broth (9 mL), it was incubated at 37°C for 20 h. Then, it was preserved at -30°C after adding 15% glycerol for use as stock cultures.

Preparation of hydrolysates from bovine LF: By adopting the method of Tomita *et al*. (1991), the LFH was generated. The supernatants which were obtained with the two enzymes were lyophilized and preserved at -20°C until use.

Antibacterial activity assays: The antibacterial activities were detected using the approached employed by Balouiri *et al*. (2016). The LF assayed were found to have final concentrations of 0.5, 1 and 2 mg/mL and those of LFH were 0.1, 0.5 and 1mg/mL. LF and LFH solutions (100 µL) were dropped into the wells of a microtitre plate and immediately mixed with 100 mL bacterial suspension afterwards. After incubating the plates at 37°C, the authors measured the absorbance at 620 nm after 8 and 24 h and read after shaking. Each well was seeded in duplicate.

Fractionation of bovine LFH by ultrafiltration: LFH solutions (concentration: 2 mg/mL) were ultrafiltrated with 6kDa cut off filters (Millipore). Fractions higher and lower than 6kDa were obtained and sterilised through filtering with 0.22 µmporesize to check their activities against *E. coli* O157:H7.

Sodium dodecylsulphate-polyacrylamide gel electrophoresis: Whole and fractionated LFH were analysed by Sodium Dodecylsulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE analysis of peptides was carried out using the methods in previous research (Schagger and Von Jagow, 1987). The authors employed two different molecular mass markers in the gels.

Mass spectrometry analysis: The authors isolated peptides from LFH utilizing the triple Quad 3500 mass spectrometry system (AB Sciex 3500, AB Sciex Pte. Ltd, China). The peptides was identified in reference to the method of Kuddus *et al*. (2016).

Statistical analysis: The experiments were carried out for three times using freshly prepared samples. Microsoft Excel 2010 was applied to calculate mean and standard deviations based on all of the data obtained in the experiments. Statistical evaluation was conducted for the data by ANOVA on the basis of Tukey test using the SPSS 19.0 package.

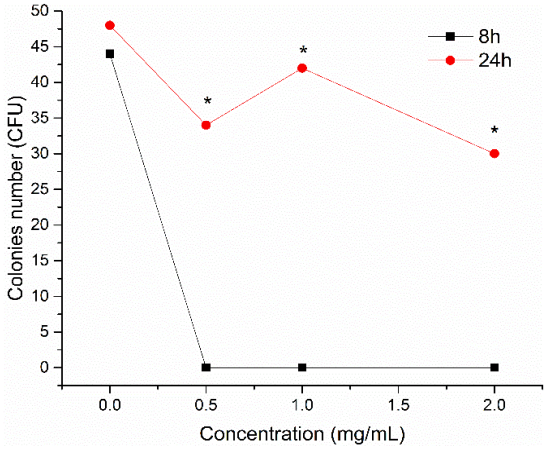


Fig. 1: Effect of the concentration of lactoferrin on the growth of Escherichia coli O157:H7

Values are the mean ± standard deviation from three replicates of three independent experiments. An asterisk indicates significant differences ($p<0.05$) with respect to the control for each time of incubation

RESULTS

Activity of bovine LF and its hydrolysates against *E. coli* O157:H7: LF (0.5 mg/mL) was shown to have inhibition effects on *E. coli* O157:H7 at 8 and 24 h of incubation (Fig. 1). The highest reduction was found to be 9 log cycles at the concentration of 2 mg/mL after 24 h and no microorganisms were observed after incubation for 8 h. At 8 and 24 h, significantly different antibacterial activities were observed relative to the control for all the tested concentrations. For all the concentrations, the number of colonies increased significantly after 24 h, in comparison with those at 8 h. Figure 2 shows that all of the LFH attained with the two enzymes exhibited inhibitory effects from 0.1 mg/mL with dissimilar statistical significances relying on the enzyme. The highest decrease (35 log cycles) relative to the control was found with pepsin LFH (1 mg/mL) after incubation for 24 h. With the same incubation time and concentration, trypsin LFH reduced the bacterial counts 14 log cycles. The most significant effects of trypsin and pepsin LFH were found with all concentrations detected and incubation for 8 h. The antibacterial activities of pepsin LFH at 24 h were found to be significant from 0.1 to 1 mg/mL. While, that of trypsin LFH at 24 h increased gradually, with the highest inhibitory activity far weaker than that of pepsin LFH at the same time of incubation. All of the LFH and the concentrations detected showed obvious discrepancy between the bacterial counts at 8 and 24 h.

Effects of the fractionation on the LFH activity: To measure the molecular range of the peptides taking part

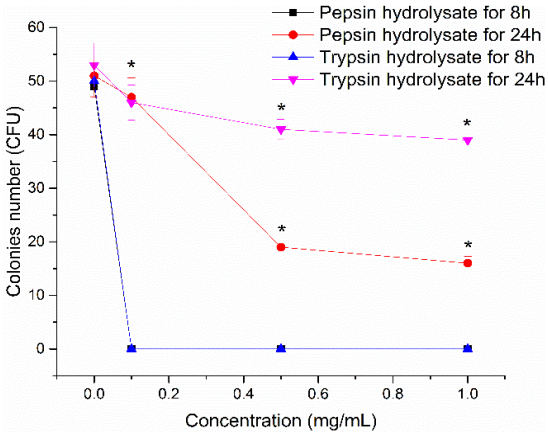


Fig. 2: Activity of LFH obtained with pepsin and trypsin against Escherichia coli O157:H7

Values are the mean ± standard deviation from three replicates of three independent experiments. An asterisk indicates significant differences ($p<0.05$) with respect to the control for each time of incubation

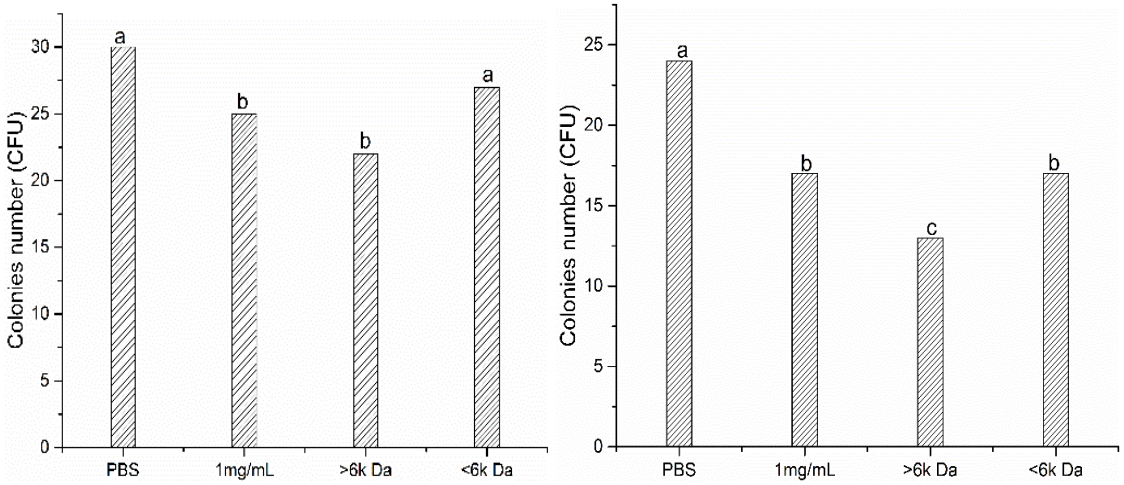
in the inhibitory effects of the LFH against *E. coli* O157:H7, 6kDa cut off filters was adopted for the fractionation of LFH. It can be seen from the results in Fig. 3 that the peptides showing antimicrobial activities were basically in the fraction higher than 6kDa and stronger activities were found in this fraction of trypsin LFH compared with the corresponding unfractionated hydrolysate at 24 h. No microorganisms were found after 8 h of incubation (data not shown). In significant inhibitory activities were observed in the fraction from pepsin LFH containing peptides below 6kDa in comparison with the control.

SDS-PAGE of LFH: SDS-PAGE was carried out to study the patterns of peptides attained from LF with each enzyme (Fig. 4). The results showed that the electrophoretic patterns of peptides varied for different enzymes. The proportion of small peptides attained with pepsin LFH was smaller than with the other LFH and all the peptides acquired utilizing this enzyme contained molecular masses lower than 14kDa. Where as peptides attained using trypsin exhibited a larger molecular mass. Both of the LFH did not show remaining whole native LF. SDS-PAGE was also conducted to investigate the fractionated LFH. The fraction higher than 6kDa exhibited similar pattern of peptides to the corresponding unfractionated hydrolysate in all cases. However, no bands were found in the fractions lower than 6kDa for they contained peptides lower than 3k Da that migrated to the bottom of the gel.

Mass spectrometry analysis of peptides: The peptides attained using trypsin and pepsin were subjected to by mass spectrometry analysis (Fig. 5). It can be seen from Fig. 5a that a major peak appeared in the region of 580Da in the spectra of peptides acquired with pepsin LFH, while other minor peaks lower than 1kDa were also observed. Figure 5b shows that the pattern of peptides released from LF with trypsin contained peptides with molecular masses ranging from 20 Da to 1k Da.

DISCUSSION

In recent years, great attentions have been paid on bioactive components obtained from food, particularly those showing antimicrobial activities. As a matter of fact, LF has been widely used as a supplement of



(a) (b)

Fig. 3: Effect of the fractionation with 6kDa cut off filters of the hydrolysates obtained from LF with pepsin (a) and trypsin (b) against Escherichia coli O157:H7, after 24h of incubation at 37ºC; Values are the mean ± standard deviation from three replicates of three independent experiments. Letters (a, b, c) indicate statistically significant differences ($p<0.05$)

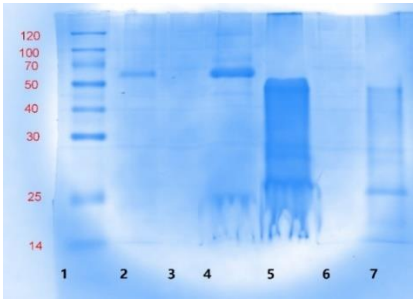
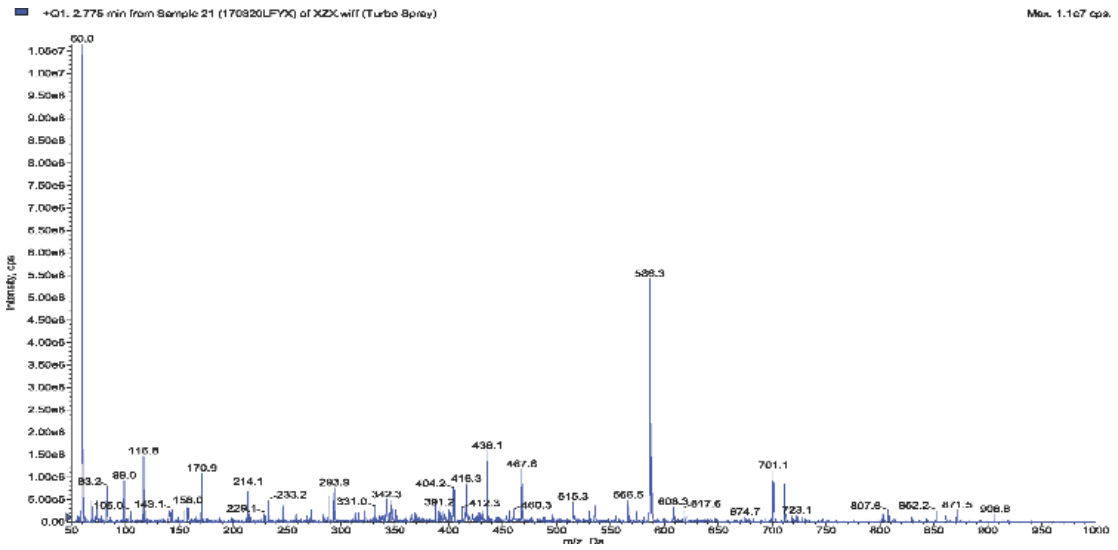
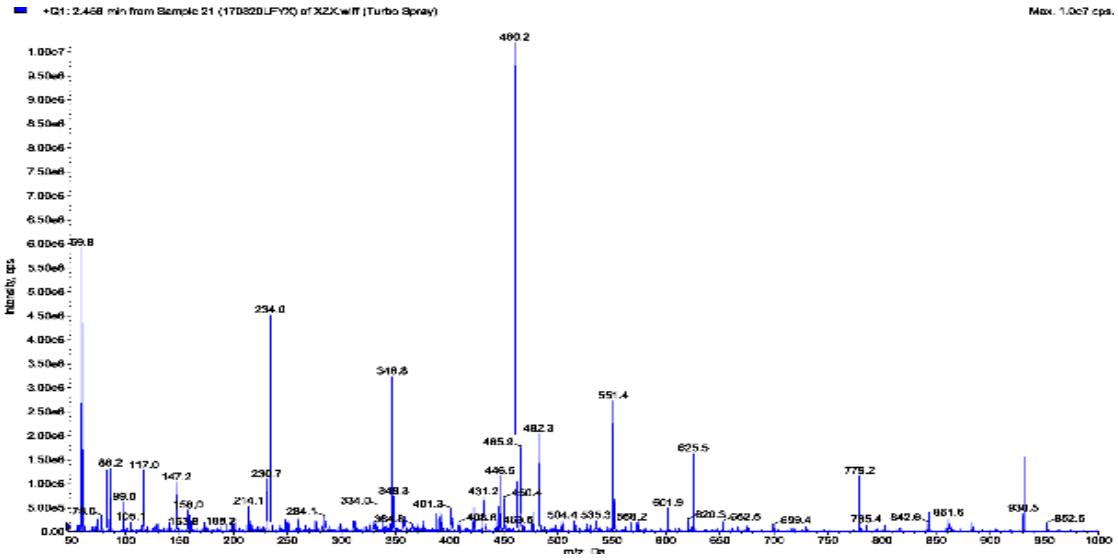


Fig. 4: SDS-PAGE of lactoferrin hydrolysates fractionated with 6k Da cut off. Lane 1, low molecular mass marker; lane 2, pepsin fraction higher than 6k Da; lane 3, pepsin fraction lower than 6k Da; lane 4, hydrolysate with pepsin at 1mg/mL; lane 5, trypsin fraction higher than 6k Da; lane 6, trypsin fraction lower than 6k Da; lane 7, hydrolysate with trypsin at 1mg/mL



(a)



(b)

Fig. 5: Mass spectrometry of the peptides from LFH with pepsin (a) and trypsin (b)

functional and infant products attributed to its various functions, including immuno modulatory and antibacterial activities. Although LF is normally added to products as a whole protein, the peptides discharged from it after enzymatic hydrolysis have been investigated extensively during the last two decades because of its strengthened antibacterial activities (Bruni *et al*., 2016).

In existing research, except for trypsin LFH, it has been found that LFH of all concentrations shows higher antibacterial activities than LF with the same concentration. This result does not absolutely confirm with previous findings reported for *E. coli* O157 by Rybarczyk *et al*. (2016), who found that trypsin LFH was more active than pepsin LFH. However, it is worth noting that the activities of enzymes, the protein sources and the methods used for evaluating bacterial growth are not identical in various investigations. In other relevant studies referenced, the reduction of bacterial growth was determined via absorbance measurement, while our study evaluated it through bacterial enumeration. The authors believe that the measurement of the bacterial counts has closer relation with the actual viability of bacteria.

The antibacterial activities of the LFH acquired using all the enzymes are mainly related to the fraction higher than 6kDa. As for the LFH attained using pepsin, the antimicrobial activity mainly depends on lactoferricin, as it has been identified a peak close to its molecular mass in the MALDI-TOF-MS analysis of cationic peptides (Conesa *et al*., 2010). Hoek *et al*. (1997) reported that a fragment derived from lactoferricin with a molecular mass of 1718 Da presents weak activities against *L. monocytogenes*.

In the LFH attained with pepsin, a peptide (molecularmass around 2.4kDa) was identified with peptides showing antibacterial activities against various microorganisms in existing research (Recio and Visser, 1999). Activities were also found in the lower molecular mass fraction acquired through ultrafiltration using 6kDa cut off centrifugal filters.

Moreover, many peptides within a wide molecular mass range have been attained in the mass spectra of the LFH obtained with trypsin. For trypsin LFH, Elbarbary *et al*. (2010) described peptides with a molecular mass of 950 Da around presenting activities against *E. coli* and *Bacillus subtilis*. Considering their close molecular mass, some of the peptides determined in the mass spectral analysis of the trypsin LFH were probably related to those put forth by Elbarbary *et al*. (2010).

CONCLUSION

LF hydrolysis with different proteolytic enzymes produces antimicrobial peptides activities against *E. coli* O157 with very low concentrations. These results show that it is promising to use LFH as a functional ingredient or as a food preservative in the future. However, further research is expected to be carried out to clarify the activities of LFH in a complex food system. This enables people to obtain protein fractions with strong antimicrobial effects and add them in food products. Therefore, LF is supposed to become a promising approach to relieve colonization pressure on farms and to prevent *E. coli* O157:H7 pollution of food and, hence to lessen human illness associated with *E. coli* O157:H7.

Conflict of interest statement: The authors declare that there are no conflicts of interest.

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