The Identification of a Novel Peptide Derived from Lactoferrin Isolated from Camel Milk with Potential Antimicrobial Activity

Elnaz Khajeh, Majid Jamshidian-Mojaver, Mohsen Naeemipour, Hamidreza Farzin

ABSTRACT

Background and Aim: Antimicrobial peptides have attracted significant attention in recent decades because of their properties, such as rapid bactericidal effects, having a wide spectrum of activity, and a rare development of drug resistance. The purpose of this study was to examine the antibacterial activity of a peptide derived from the lactoferrin isolated from camel milk against Staphylococcus aureus, Pseudomonas aeruginosa, and Acinetobacter baumannii.

Materials and Methods: In the present study, by means of bioinformatics, an antibacterial peptide was potentially identified as candidates in lactoferrin of camel milk, and an appropriate peptide was selected based on defined criteria. The Pepsin-Camel-Lac1 peptide was synthesized. The methyl thiazolyl diphenyl-tetrazolium bromide assay was conducted to examine the toxicity of Pepsin-Camel-Lac1 against a cell line. Three pathogenic bacteria, namely S. aureus, P. aeruginosa, and A. baumannii were analyzed to assess the antibacterial activity of Pepsin-Camel-Lac1 peptide.

Results: The results showed that the newly-identified peptide had no toxicity against the cell line. The minimum inhibitory concentration values of Pepsin-Camel-Lac1 against S. aureus, P. aeruginosa, and A. baumannii were 31.25 µg/mL, 31.25 µg/mL, and 62.5 µg/mL, respectively.

Conclusion: It seems that the growth of S. aureus, P. aeruginosa, and A. baumannii was not affected by Pepsin-Camel-Lac1 treatment in the bacterial culture medium.

Keywords: Milk proteins; Pepsin; Antibacterial activity; Staphylococcus aureus; Pseudomonas aeruginosa; Acinetobacter baumannii
Introduction

Milk contains a large number of proteins, some of which have been well characterized, such as lactoferrin, which exhibits antibacterial activity (1). Two iron-binding proteins, namely lactoferrin, and transferrin, have been shown to have biological activity in the milk of many species (2). The two proteins almost possess similar properties, such as molecular weight (80 kDa) and the ability to bind to iron (3). Lactoferrin is a monomeric glycoprotein with a high affinity to iron (4). The presence of lactoferrin has been confirmed in a large number of mammals, and the amino acid sequence of this protein has been identified in human, pig, horse, camel, cattle, buffalo, goat, and mouse (5). The third structure of lactoferrin has been characterized in the five species of human, cow, buffalo, camel, and horse, all of which have the same structures (6-10). All nutrients found in human milk are also present in camel milk. A number of investigations have recently reported the therapeutic properties of camel milk, such as anti-cancer, anti-diabetic, anti-microbial potential (11-14). Most secretions fluids, such as milk, saliva, tears, and neutrophil granules, contain this glycoprotein (15). In the immune system, lactoferrin is introduced as a part of the innate immune system because of its sensitive position on the mucosal surface as one of the agents existing at the first-line of defense to prevent the entry of microbial agents (16). Camel milk lactoferrin has been identified as a factor against Gram-positive and Gram-negative bacteria, as well as fungi and parasites (17). Proteins and peptides of the milk, such as lactoferrin, lactoperoxidase, and lysozyme, play an influential role in overall antimicrobial activity in milk (18). The milk proteins and peptides can be natural substitutes for antibiotics due to their antibacterial properties (19). Antimicrobial peptides (AMPs) are oligopeptides with varied numbers of amino acids (from 5 to more than 100) (20, 21). Some studies have analyzed the antimicrobial activity of peptides derived from lactoferrin (22-26). Camel milk lactoferrin has antibacterial activity against Escherichia coli, Salmonella typhimurium, Listeria monocytogenes, Staphylococcus aureus and Pseudomonas aeruginosa (11, 27-29). Thanks to bioinformatics tools and knowing the cleavage sites of enzymes, the identification of AMPs in natural sources, such as milk, has been feasible (30-32). Different methods are used to characterize and produce active AMPs (33). These methods are based on proteolysis using bioinformatics software and statistical analyses to assess the role of peptides released from cleaved proteins. In this study, in order to find a novel AMP, different AMPs, derived from camel milk lactoferrin, were designed by bioinformatics approaches, and then, the most appropriate AMP was chosen according to the required criteria. The designed peptide was synthesized and analyzed in terms of the antibacterial activity against P. aeruginosa, A. baumannii, and S. aureus.

Materials and Methods

Bioinformatics Analysis

Selection of Effective Antimicrobial Peptide Derived from Lactoferrin

Camel milk lactoferrin is a glycoprotein that contains 708 amino acids and possesses two domains and two iron-binding sites (34). The strategy used for AMP synthesis from camel milk lactoferrin was in accordance with bioinformatics approaches of a study conducted by Dziuba et al. (32). Based on the cleavage sites identified for various proteolytic enzymes, in-silico studies indicated that pepsin is capable of releasing numerous active peptides from lactoferrin (32, 35, 36). The initial structure of camel milk lactoferrin was obtained from the Uniport database with an ID of Q9TUM0. Next, pepsin was used to hydrolyze the protein, as this enzyme (pH>2) has proteolytic activity and is commonly applied to obtain bioactive peptides (37-39). Four online software tools, namely BIOPEP database, Collection of Antimicrobial Peptides Server, Antimicrobial Peptide Calculator and Predictor and Peptide cutter database were utilized to evaluate the antimicrobial properties of cleaved peptides derived from lactoferrin (40-43). The online server BIOPEP-UWM (www.uwm.edu.pl/biochemia) was utilized since it contains a library of bioactive peptides and comprises of the two essential coordinated parts, namely grouping databases and apparatus for the assessment of proteins as the antecedents of bioactive peptides, which includes the proteolytic procedure plans. Another online database, CAMP (http://www.camp.bicnirrh.res.in), contains information about the conserved sequences, representing some examples and Hidden Markov Models (HMMs) of 1386 AMPs that are categorized into 45 families. On the other hand, the online database APD2 (http://aps.unmc.edu/AP/expectation/forecastmain.php) contains 2684 antimicrobial peptides from six kingdoms (266 from microbes, 4 from archaea, 8 from protists, 13 from parasites, 329 from plants, and 2018 from creatures) (44).

Experimental Procedures

Material

S. aureus (1074, ATCC: 6538), P. aeruginosa (1060, ATCC: 27853), and A. baumannii (1003, ATCC: BAA-747) were purchased from the Pasteur Institute of Iran.

Peptide Synthesis

The chosen peptide was named Pepsin-Camel-Lac1 (PCL1) synthesized by MIMOTOPES Pty Ltd, Australia.
In order to prepare the designed peptide, 1 mg of the designed peptide was dissolved in 1 mL of sterile water and used for the experiments.

**Toxicity Assay**

The Methyl thiazolyl diphenyl-tetrazolium bromide (MTT) assay was utilized to assess the toxicity of PCL1 against a human cell line. The assay is based on the decrease of the tetrazolium salt into blue formazan crystals in viable cells (45, 46). The cell line was cultured in the RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 mL penicillin, and 100 mg/mL streptomycin. During the cell culture period, cells were incubated at 37°C in a 95% atmosphere and 5% CO2. When the cells reached 80-90% confluence, the viability of cells was examined and passaged. In this experiment, 100 mL of the cell suspension was added to each well of a 96-well plate, each containing 100 mL of the culture medium. The cells were treated with the pre-defined concentrations of PCL-1 and then incubated for 24 h. After dilution of PCL-1 with sterile water, cells were treated with PCL-1, and then 20 µL of the MTT solution (5 mg/mL) was added to the wells and incubated for 24 hours. After the incubation period, 50 µL of dimethyl sulfoxide (DMSO) was added to the wells to dissolve the purple formazan crystals. Afterward, according to the obtained results, the inhibitory concentration (IC50) of PCL-1 was calculated, in which 50% of the cells underwent cell death. The experiments have been carried out in triplicates, and the values were represented as the percentage of cell viability in comparison with the control cells.

**Preparation of Microbial Suspension Solution**

The lyophilized batches containing bacteria mentioned earlier were transferred to the liquid Mueller Hinton Broth (MHB) and incubated at 37°C for 24 h. In order to determine the antimicrobial effects, a fresh 24-hour culture medium was prepared, then 1 mL of the 24 h microbial suspension was transferred into a tube containing the MHB medium, and the turbidity of the prepared microbial suspension was assessed by McFarland standards using a spectrophotometer at a wavelength of 625 nm. The final suspension was used for the evaluation of drug susceptibility testing, MIC, and cell culture (47).

**Determination of MIC and MBC Values by Micro-Dilution Method**

The MHB medium was used to evaluate the antimicrobial effects of PCL-1 by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods. In general, a sterile flat 96-well plate was used after determining the half-McFarland turbidity. In this method, 50mL of MHB medium was added to well 2 to well 11. Then, 50µL of the PCL-1 peptide was added to well 2 to well 9. Next, 50 µL of the microbial suspension was added to well 1 well 10. Afterward, the plate was incubated for 24 h. Well 1 that contains bacteria were considered as the negative control, while and well 11 containing the MHB medium was considered the positive controls. In order to assess the MBC values, the contents of the wells were cultured on the Mueller Hinton Agar medium. Lack of bacterial growth is indicative of MBC determination (47, 48).

**Agar Well Diffusion Assay**

The antimicrobial activity test was performed using the Agar well diffusion assay (49). This technique is broadly used to assess antimicrobial activity. In this method, the microbial suspension is distributed on the plate surface. Then, a hole is created with a diameter of 6-8 mm in sterile conditions, and then specific concentrations of the PCL-1 peptide were added to the hole and left to release into the pores of Agar. Different concentrations of PCL-1 were prepared as follows; PCL-1 was transferred into 5 sterile microtubes, then 100 mL of sterile water was added into microtube 2 to 5. Next, 100 mL of PCL-1 was added to microtube 1 and was serially transferred into other microtubes until the last microtube. After that, 100µL of the contents of each microtube was isolated and added to the plate agar. After incubating the plates at 37°C for 24h, the results were examined based on the diffused area around the created hole (47).

**Results**

**In-Silico Proteolysis of Lactoferrin**

The sequence of amino acids of lactoferrin was exposed in in-silico proteolysis performed by the BIOPEP database. In-silico proteolysis can be carried out by means of some bioinformatics tools, as we employed the peptide cutter database (https://web.expasy.org/peptide_cutter/). (42, 50). For in-silico protein hydrolysis, a single enzyme was used and the PCL-1 peptide cleavage site was bolded (Table 1). The pepsin enzyme (pH>2) was utilized to create different peptides from lactoferrin, resulted in the production of 50 peptides with the length of 5-21 amino acids. For the statistical prediction of antimicrobial properties, the produced peptides were submitted (Table S1). A short linear peptide, PCL1, met the required criteria, as shown in Figure 1.
Table 1. Results of peptide released from BIOEP database camel milk lactoferrin.

| Enzyme        | No. of cleavages | Positions of cleavage sites                                                                 |
|---------------|------------------|------------------------------------------------------------------------------------------------|
| Pepsin (pH>2) | 188              | 2 3 5 8 9 10 11 13 14 15 16 17 18 27 40 41 62 77 81 82 83 84 87 90 92 100 101 110 111 122 |
|               |                  | 123 124 125 127 128 130 131 138 143 144 149 150 153 155 156 157 163 170 171 188 190 191 |
|               |                  | 207 209 210 211 214 215 217 226 227 233 234 237 246 247 248 265 266 285 286 287 288 289 |
|               |                  | 296 297 304 305 306 307 308 316 318 319 322 324 325 326 327 337 338 339 342 343 349 |
|               |                  | 358 359 365 366 374 375 379 380 401 402 403 404 410 411 412 413 416 418 419 430 441 |
|               |                  | 442 451 453 467 469 470 485 486 492 494 502 504 505 506 520 522 523 542 544 545 548 549 |
|               |                  | 551 560 561 567 568 578 579 582 584 585 587 589 590 591 592 593 608 625 626 629 630 |
|               |                  | 631 636 637 647 648 650 651 657 659 660 666 667 669 670 676 678 679 684 690 691 698 700 |
|               |                  | 704 705 706                                                                                   |

Figure 1. The PCL1 peptide picture consisted of 11 amino acids and has a linear structure.

Physicochemical Properties of AMPs

The physicochemical properties of antimicrobial peptides designed by the BIOPEP database were characterized using free web tools and the algorithms for the chosen AMP are shown (Table S1). The molecular weights of created peptides were in a range of 446 to 2493 Da (Table S1). Among 50 peptides created, 26 peptides had a pI value of less than 7, while the rest has above 7. It is now known that the molecular charge and hydrophobicity play a significant role in the functionality of AMPs (51-53). Among 50 peptides derived from lactoferrin, 14 peptides had a negative charge, 25 possess a positive charge, and 11 were neutral. In the present study, only positive AMPs were selected for further analyses. Because cationic peptides constitute a significant percentage of AMPs, as a result of their higher affinity to bind to negatively charged cell membranes of bacteria and eventually disrupt the bilayer lipid structure (54, 55). Amphipathic property is another marked parameter to choose efficient AMPs since these structures allow them to bind to hydrophilic regions; hence AMPs with both hydrophilic and hydrophobic domains have a higher priority to be used for antibacterial tests (56). The amino acid composition also plays a role in designing the antibacterial peptides. Gram-positive and Gram-negative antimicrobial peptides have a similar ratio of cysteine and lysine residues (57). As shown in Table 1, the PCL1 peptide composed of 63% hydrophobic region, 27% hydrophilic region, and 9% charged amino acids; Also, it contains 18% lysine and 9% cysteine.

Table 2. Physical and chemical characteristics of the Pepsin-Camel-Lac1 peptide.

| Position of cleavage site | Enzyme       | Peptide sequence | Peptide length [aa] | Peptide mass [Da] | Seq. ID | Charge | pI    |
|--------------------------|--------------|------------------|---------------------|------------------|--------|--------|-------|
| 430                      | Pepsin (pH>2)| >30              | 11                  | 1069.371         | 30     | 8.22   | +1    |
| Sequence ID | Name of cleaving enzyme(s) | Resulting peptide sequence | Peptide length [aa] | Peptide mass [Da] | Position of cleavage site | pl | Charge |
|-------------|----------------------------|----------------------------|---------------------|------------------|---------------------------|----|--------|
| 1           | Pepsin (pH>2)              | >1 AASKKSVRW               | 9                   | 1032.211         | 27                        | 11.12 | +3     |
| 2           | Pepsin (pH>2)              | >2 CTTPAESSKCAQ            | 13                  | 1312.432         | 40                        | 6.25  | 0      |
| 3           | Pepsin (pH>2)              | >3 QRRMKKVRGPSVTCVKTSRF   | 21                  | 2493.033         | 62                        | 12.2  | +8     |
| 4           | Pepsin (pH>2)              | >4 ECIQAISTEKADAVT         | 15                  | 1578.755         | 77                        | 4.14  | -2     |
| 5           | Pepsin (pH>2)              | >5 LRPIAEV                | 8                   | 868.044          | 100                       | 6.25  | 0      |
| 6           | Pepsin (pH>2)              | >6 GTENNPQTH              | 9                   | 996.989          | 110                       | 5.5   | -1     |
| 7           | Pepsin (pH>2)              | >7 YAYAIKKGTN             | 11                  | 1135.329         | 122                       | 9.72  | +2     |
| 8           | Pepsin (pH>2)              | >8 KSCHTGL               | 7                   | 744.864          | 138                       | 8.22  | +1     |
| 9           | Pepsin (pH>2)              | >9 GRSAG                  | 5                   | 446.464          | 143                       | 10    | +1     |
| 10          | Pepsin (pH>2)              | >10 NIPMG                | 5                   | 530.640          | 149                       | 5.5   | 0      |
| 11          | Pepsin (pH>2)              | >11 TGPEPEP              | 6                   | 596.638          | 163                       | 3.81  | -1     |
| 12          | Pepsin (pH>2)              | >12 LQKAVAK               | 7                   | 756.944          | 170                       | 10    | +2     |
| 13          | Pepsin (pH>2)              | >13 FSASCVPVDGKEYPNL      | 17                  | 1829.073         | 188                       | 4.47  | -1     |
| 14          | Pepsin (pH>2)              | >14 CAGTGENCASSQQEP       | 16                  | 1584.711         | 207                       | 10    | -1     |
| 15          | Pepsin (pH>2)              | >15 LQDGAGDVA            | 9                   | 844.877          | 226                       | 3.53  | -2     |
| 16          | Pepsin (pH>2)              | >16 VKDSTV               | 6                   | 647.726          | 233                       | 6.25  | 0      |
| 17          | Pepsin (pH>2)              | >17 PAKADRDQY            | 9                   | 1063.135         | 246                       | 6.25  | 0      |
| 18          | Pepsin (pH>2)              | >18 LCPNTRPVDASQECH      | 17                  | 1912.125         | 265                       | 7.09  | 0      |
| 19          | Pepsin (pH>2)              | >19 ARVPUSHAVVARSVNGKEDL | 19                  | 2005.265         | 285                       | 9.06  | +1     |
| 20          | Pepsin (pH>2)              | >20 LVKAEK               | 7                   | 814.980          | 296                       | 8.88  | +1     |
| 21          | Pepsin (pH>2)              | >21 GRGXPASA            | 7                   | 671.754          | 304                       | 11.12 | +2     |
| 22          | Pepsin (pH>2)              | >22 GSPAGQKD          | 8                   | 758.786          | 316                       | 6.25  | 0      |
| 23          | Pepsin (pH>2)              | >23 RIPSKIDSG          | 9                   | 972.109          | 336                       | 8.88  | +1     |
| 24          | Pepsin (pH>2)              | >24 ITAIRG             | 6                   | 629.757          | 349                       | 10    | +1     |
| 25          | Pepsin (pH>2)              | >25 LRETAEEVE          | 9                   | 1017.104         | 358                       | 4.14  | -2     |
| 26          | Pepsin (pH>2)              | >26 RRAQVV              | 6                   | 727.865          | 365                       | 11.97 | +2     |
| 27          | Pepsin (pH>2)              | >27 RRAQVV             | 8                   | 807.830          | 374                       | 11.97 | +2     |
| 28          | Pepsin (pH>2)              | >28 SROSNQSVCATATTEDCIA | 21                  | 2171.339         | 401                       | 4.47  | -1     |
| 29          | Pepsin (pH>2)              | >29 KGEADA             | 6                   | 589.603          | 410                       | 4.47  | -1     |
| 30          | Pepsin (pH>2)              | >30 IAGKGLVPVL        | 11                  | 1069.371         | 430                       | 8.22  | +1     |
| 31          | Pepsin (pH>2)              | >31 AESQSPRESS          | 11                  | 1106.068         | 441                       | 3.53  | -2     |
| 32          | Pepsin (pH>2)              | >32 DCVHRPVKG         | 9                   | 1010.179         | 451                       | 8.22  | +1     |
| Sequence ID | Name of cleaving enzyme(s) | Resulting peptide sequence | Peptide length [aa] | Peptide mass [Da] | Position of cleavage site | pI | Charge |
|-------------|---------------------------|----------------------------|--------------------|-----------------|--------------------------|----|--------|
| 33          | Pepsin (pH>2)             | >33 AVAVVRKANDKITW          | 14                 | 1570.855        | 467                      | 10 | +2     |
| 34          | Pepsin (pH>2)             | >34 RGKKSCHTAVDRTAG         | 15                 | 1586.789        | 485                      | 10 | +3     |
| 35          | Pepsin (pH>2)             | >35 NIPMGP                 | 6                  | 627.756         | 492                      | 5.5| 0      |
| 36          | Pepsin (pH>2)             | >36 KNTDCSRF               | 8                  | 970.068         | 502                      | 8.2| +1     |
| 37          | Pepsin (pH>2)             | >37 SQSCAPGSDPRSKL         | 14                 | 1432.571        | 520                      | 8.2| +1     |
| 38          | Pepsin (pH>2)             | >38 CAGNEEGQNKCVNSSERY     | 19                 | 2085.207        | 542                      | 4.7| -1     |
| 39          | Pepsin (pH>2)             | >39 LAENVGDVA              | 9                  | 886.957         | 560                      | 3.5| -2     |
| 40          | Pepsin (pH>2)             | >40 VKDVT                   | 6                  | 659.781         | 567                      | 6.2| 0      |
| 41          | Pepsin (pH>2)             | >41 DNTDGKNTEQ             | 10                 | 1121.082        | 578                      | 4.1| -2     |
| 42          | Pepsin (pH>2)             | >42 NGTRKPVTEAESCHL        | 15                 | 1641.819        | 608                      | 7.1| 0      |
| 43          | Pepsin (pH>2)             | >43 PVAPNHAUVSRIDKVCH      | 17                 | 1810.089        | 625                      | 9.0| +1     |
| 44          | Pepsin (pH>2)             | >44 GRNGQDCPGK              | 10                 | 1031.111        | 647                      | 8.2| +1     |
| 45          | Pepsin (pH>2)             | >45 QSKTKN                 | 6                  | 704.781         | 657                      | 10 | +2     |
| 46          | Pepsin (pH>2)             | >46 NDNTEC                  | 6                  | 694.671         | 666                      | 3.5| -2     |
| 47          | Pepsin (pH>2)             | >47 QGKTTY                  | 6                  | 696.758         | 676                      | 8.8| +1     |
| 48          | Pepsin (pH>2)             | >48 LGPQY                  | 5                  | 576.650         | 684                      | 5.5| 0      |
| 49          | Pepsin (pH>2)             | >49 VTAIAK                 | 6                  | 601.744         | 690                      | 8.8| +1     |
| 50          | Pepsin (pH>2)             | >50 RRCSTSP                | 7                  | 805.907         | 698                      | 10 | +2     |

Table S 2. Predict Antimicrobial Peptides.
|   | Results with Support Vector Machine (SVM) classifier | Results with Random Forest Classifier | Results with Artificial Neural Network (ANN) classifier | Results with Discriminant Analysis classifier |
|---|---------------------------------------------------|---------------------------------------|------------------------------------------------------|-------------------------------------------------|
| 15 | AMP 0.929                                         | NAMP 0.354                            | NAMP                                                 | NAMP 0.001                                       |
| 16 | NAMP 0.008                                         | NAMP 0.347                            | NAMP                                                 | NAMP 0.000                                       |
| 17 | NAMP 0.011                                         | AMP 0.5275                            | NAMP                                                 | NAMP 0.001                                       |
| 18 | NAMP 0.063                                         | NAMP 0.083                            | NAMP                                                 | NAMP 0.004                                       |
| 19 | NAMP 0.345                                         | NAMP 0.17                             | NAMP                                                 | NAMP 0.036                                       |
| 20 | NAMP 0.002                                         | NAMP 0.297                            | NAMP                                                 | NAMP 0.004                                       |
| 21 | NAMP 0.001                                         | NAMP 0.3515                           | NAMP                                                 | NAMP 0.001                                       |
| 22 | NAMP 0.000                                         | NAMP 0.3385                           | NAMP                                                 | NAMP 0.000                                       |
| 23 | NAMP 0.093                                         | NAMP 0.265                            | NAMP                                                 | NAMP 0.009                                       |
| 24 | NAMP 0.000                                         | NAMP 0.4965                           | AMP                                                 | NAMP 0.263                                       |
| 25 | NAMP 0.001                                         | NAMP 0.2205                           | NAMP                                                 | NAMP 0.000                                       |
| 26 | NAMP 0.008                                         | NAMP 0.455                            | NAMP                                                 | NAMP 0.019                                       |
| 27 | NAMP 0.025                                         | NAMP 0.378                            | NAMP                                                 | NAMP 0.000                                       |
| 28 | NAMP 0.033                                         | NAMP 0.1255                           | NAMP                                                 | NAMP 0.002                                       |
| 29 | NAMP 0.216                                         | NAMP 0.455                            | NAMP                                                 | NAMP 0.000                                       |
| 30 | NAMP 0.146                                         | AMP 0.5805                            | AMP                                                 | AMP 0.949                                        |
| 31 | NAMP 0.387                                         | NAMP 0.364                            | NAMP                                                 | NAMP 0.000                                       |
| 32 | NAMP 0.003                                         | NAMP 0.3025                           | AMP                                                 | NAMP 0.000                                       |
| 33 | NAMP 0.399                                         | AMP 0.567                            | AMP                                                 | NAMP 0.326                                       |
| 34 | NAMP 0.188                                         | NAMP 0.186                            | NAMP                                                 | NAMP 0.003                                       |
| 35 | NAMP 0.005                                         | NAMP 0.4345                           | AMP                                                 | NAMP 0.003                                       |
| 36 | NAMP 0.004                                         | NAMP 0.3385                           | NAMP                                                 | NAMP 0.187                                       |
| 37 | NAMP 0.008                                         | NAMP 0.1685                           | NAMP                                                 | NAMP 0.003                                       |
| 38 | NAMP 0.178                                         | NAMP 0.2445                           | NAMP                                                 | NAMP 0.039                                       |
| 39 | AMP 0.593                                          | NAMP 0.3495                           | NAMP                                                 | NAMP 0.001                                       |
| 40 | NAMP 0.007                                         | NAMP 0.443                            | NAMP                                                 | NAMP 0.000                                       |
| 41 | NAMP 0.026                                         | AMP 0.627                            | NAMP                                                 | NAMP 0.000                                       |
| 42 | NAMP 0.060                                         | NAMP 0.0225                           | NAMP                                                 | NAMP 0.001                                       |
| 43 | NAMP 0.256                                         | NAMP 0.0655                           | NAMP                                                 | NAMP 0.014                                       |
| 44 | NAMP 0.002                                         | NAMP 0.2105                           | NAMP                                                 | NAMP 0.001                                       |
| 45 | AMP 0.999                                         | AMP 0.501                            | AMP                                                 | NAMP 0.063                                       |
| 46 | AMP 0.984                                         | NAMP 0.336                            | NAMP                                                 | NAMP 0.017                                       |
| 47 | AMP 0.760                                         | NAMP 0.3345                           | NAMP                                                 | NAMP 0.002                                       |
| 48 | NAMP 0.052                                         | NAMP 0.294                            | AMP                                                 | NAMP 0.041                                       |
| 49 | NAMP 0.000                                         | AMP 0.503                            | AMP                                                 | AMP 0.169                                        |
| 50 | AMP 0.999                                         | NAMP 0.4025                           | NAMP                                                 | NAMP 0.086                                       |
Prediction of the Antimicrobial Activity of Produced Peptides During In-Silico Proteolysis from Lactoferrin

The in-silico proteolysis of lactoferrin leads to the generation of short peptides. Among the created peptides, the PCL-1 peptide shows anti-bacterial, anti-viral, and anti-tumor activity due to its high positive charge and ability to interact with negatively charged substrates, such as glycosaminoglycan, lipopolysaccharide, heparan, phosphatidylserine, and nucleic acids (58, 59). The design of peptides varies depending on the biological activity of these macromolecules (22). Many peptides have a wide range of activity, such as the antibacterial and anti-cancer properties (60). For example, the design of AMPs derived from the lactoferrin protein has been conducted using the CAMP tools. To this aim, Four software tools have been employed, namely Random Forest, Support Vector Machines, Artificial Neural Network, and Discriminant Analysis (41). In this analysis, the resultant score was in excess of 0.45; therefore, the PCL1 peptide was listed as AMPs, and a positive classification was obtained for at least two statistical methods (Table 3).

| Sequence       | SVM classifier | RF Classifier | ANN classifier | DA classifier |
|----------------|----------------|---------------|----------------|---------------|
|                | Class          | AMP Probability | Class          | AMP Probability | Class | AMP Probability |
| IAGKCGLVPVL    | NAMP           | 0.146 AMP      | 0.5805 AMP     | 0.949 AMP      |       |                |

Four statistical models in the CAMP database: SVM, RF, ANN and DA classifiers that used for proteolysis and prediction peptides.

Toxicity Assay

In order to achieve a linear regression equation of the concentrations of PCL-1 versus the growth inhibition, Microsoft Excel software was used, and the values were expressed as the means and standard deviation of the means of three separate experiments. The below equation was used to measure the growth curve and draw a plot:

\[ \text{Growth inhibition} = \frac{\text{control OD} - \text{sample OD}}{\text{control OD}} \times 100 \] (61)

Finally, the IC50 values of specimens, representing a concentration of the peptide by which 50% of bacteria are eliminated, were obtained from the growth curve. The SPSS software version 16 was used for the analysis of the obtained data, and the difference between various treatments was analyzed by one-way analysis of variance (one-way ANOVA) followed by Tukey’s post hoc test (Table 4). The MTT results showed that PCL-1 has no significant effect on the viability of the cell line used (Figure 2).

![Figure 2. MTT cell line with PCL1 peptide with sig= 0.00](Image)

Table 3. The characteristics of PCL1 peptide released during in-silico proteolysis and prediction.

| Sequence       | Class | SVM classifier | Class | RF Classifier | Class | ANN classifier | Class | DA classifier | Class | AMP Probability |
|----------------|-------|----------------|-------|---------------|-------|----------------|-------|---------------|-------|-----------------|
| IAGKCGLVPVL    | NAMP  | 0.146 AMP      | AMP   | 0.5805 AMP    | AMP   | 0.949 AMP      |       |               |       |                 |

Table 4. Results of analysis using SPSS technology of mixed PCL1 peptide levels on cell lines.

|                      | Sum of Squares | df | Mean Square | F     | Sig   |
|----------------------|----------------|----|-------------|-------|-------|
| Between Groups       | 1.500          | 5  | 0.3         | 8.498 | 0.001 |
| Within Groups        | 0.424          | 12 | 0.035       |       |       |
| Total                | 1.924          | 17 |             |       |       |
Determination of MIC and MBC Values by Micro-Dilution Method

The MIC values of bacterial strains were calculated by the micro-dilution method. After two hours of incubation, the turbidity of samples was read at wavelengths of 490 and 630nm using an ELISA reader. The well in which bacterial growth inhibition occurred was regarded as the MIC values and used to determine the MBC value. Following the incubation period, the first dilution in which bacteria did not grow on the agar plate was regarded as the MBC value. According to Figure 3, the MIC values for *S. aureus* in response to PCL-1 was reported to be 31.25µg/mL, while the MBC value was 500µg/mL (Figure 3A).

The MIC and MBC values for *P. aeruginosa* were 31.25 µg/mL and 125 µg/mL, respectively (Figure 3B). In addition, the values of MIC and MBC for *A. baumannii* were 62.5µg/mL and 500µg/mL, respectively (Figure 3C).

![Figure 3](image.png)

**Figure 3.** MIC and MBC analyze outcomes for the three bacterial strains that were tested in A, B, and C for *S. aureus, P. aeruginosa, and A. baumannii.*

Agar Well Diffusion Assay

The diameter created around the holes in agar indicates the effect of PCL-1 on the inhibition growth of bacterial strains. The results demonstrated that PCL-1 had no significant effect on the growth inhibition of the three bacterial strains when compared with the control group (Figure 4).
Discussion

In an in-silico proteolysis conducted by Dziuba et al., 11 new peptides with potential antimicrobial activity were picked from all the digested peptides in the milk protein (32). Jahani et al. demonstrated that lactoferrin to be effective as an antibacterial against Gram-positive (Staphylococcus epidermidis, Bacillus cereus) and Gram-negative (Campylobacter jejuni, Salmonella spp.) bacteria. However, it was more effective on the Gram-positive instead of the Gram-negative bacteria (62). In line with the investigation of peptides derived from milk lactoferrin through the milk fermentation and assessment of their antibacterial effects, Korheonan et al. reported that milk proteins contain a rich source of peptides that could be potentially released and activated. For example, when these peptides are activated during gastrointestinal digestion or milk fermentation, they could be involved in modulatory processes in living systems (33). Bushra Niaz et al. reported that due to the association of lactoferrin with enterobacterial lipopolysaccharide (LPS), it acts as a permeabilizing agent of Gram-negative bacteria and destabilizes the bacterial membrane and thus enhance bacterial permeability. Lactoferrin is not an effective antibacterial agent of its own, but allows those peptides that have been derived from it, whose hydrophobicity impose a limit on their effectiveness, to enter the bacterial membrane as an antibacterial agent (63). As a result of direct interaction between protein or lactoferrin-derived peptides, Farnaud and Evans identified that it possesses bactericidal activities (25). Cameleers believe that camel milk is more resistant to spoilage than the milk of other animals, and it has a longer shelf life. The effect of lactoperoxidase can be one of the significant factors leading to camel milk resistance to spoilage. In this work, antimicrobial peptides were identified from camel milk lactoferrin using bioinformatics; then, the PCL-1 peptide was selected, and the activity of this peptide was evaluated in-vitro. In the first step, the hydrolysis of lactoferrin was performed using the pepsin enzyme using the BIOPEP database by which 50 peptides were produced containing 5–21 amino acids. The physicochemical properties of the synthesized peptides were investigated using online tools, such as the APD2 and Peptide cutter databases, to select appropriate AMPs. The general properties of the generated peptides, including molecular weight, charge, and PI values, were analyzed to select the appropriate antimicrobial peptides (Table S1). Among the created peptides, two peptides met the initial criteria, but since the presence of lysine and cysteine amino acids in AMPs is an advantage, the peptide with an ID of 30 (PCL-1 peptide) was selected for further analyses (Table 1). Four tools of the CAMP database (ANN, DA, RF, SVM) were employed to determine the antibacterial activity of the created peptides in this study. The PCL-1 peptide was classified as AMPs since it had parameters with scores of higher than 0.45, including RF: 0.5805 and DA: 0.949 (Table 3). After synthesizing the PCL-1 peptide, the viability of bacteria in response to PCL-1, MBC, MIC, agar well diffusion was assessed in order to determine the antimicrobial activity, as well as toxicity against the human cell line. The MTT assay showed that this peptide had no toxicity against the human cell line. Our findings also showed the MIC and MBC values for S. aureus, P. aeruginosa, and A. baumannii were 31.25/500, 21.25/125, and 62.5/500, respectively. The agar well diffusion assay showed that the analyzed bacteria were resistant to the PCL-1 peptide. The experimental results of this study showed that the PCL-1 peptide had no antimicrobial properties.
Conclusion

There are naturally occurring bioactive peptides in fermented dairy products, such as yogurt, butter, milk, and cheese, which are inactivated in primary sources, and they could be activated by digestion of milk, milk fermentation, exposure to proteolytic starter media, or hydrolysis using proteolytic enzymes. Milk-derived peptides exhibit super-beneficial activity, even through oral consumption. In this study, pepsin-digested peptides in milk lactoferrin were identified by bioinformatics tools. After the synthesis of PCL-1, the MTT assay was applied to assess the toxicity of the selected peptide against the human cell line. The results showed that the chosen peptide had no toxicity against the human cell line or bacterial strains.

Abbreviations

Antimicrobial peptides: AMPs, Collection of Antimicrobial Peptides: CAMP, Antimicrobial Peptide Calculator and Predictor: APD2, Pepsin-Camel-Lac1: PCL1, Methyl thiazolyl diphenyl-tetrazolium bromide: MTT, Mueller Hinton Broth: MHB, Minimum inhibitory concentration: MIC, Minimum bactericidal concentration: MBC, Agar well diffusion assay: AWDA, Random Forest: RF, Support Vector Machines: SVM, Artificial Neural Network: ANN, Discriminant Analysis: DA.

Acknowledgment

I want to thank all those who have supported me in this work. It is worth mentioning that this essay is a thesis by Ms. Elnaz’s study at Islamic Azad University, branch of Sabzevar, Iran.

Ethics Approval and Consent to Participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for Publication

All authors gave their consent for publication.

Conflict of Interest

The authors declared no conflict of interest.

Reference

1. Bullen J, Rogers HJ, Leigh L. Iron-binding proteins in milk and resistance to Escherichia coli infection in infants. Br Med J. 1972;1(5792):69-75. [DOI:10.1136/bmj.1.5792.69]
2. Hoshino A, Hisayasu S, Shimada T. Complete sequence analysis of rat transferrin and expression of transferrin but not lactoferrin in the digestive glands. Comparative Biochemistry and Physiology Part B: J. Biochem. Mol. Biol. 1996;113(3):491-7. [DOI:10.1016/0305-0491(95)02068-3]
3. Baker EN, Baker HM, Kidd RD. Lactoferrin and transferrin: functional variations on a common structural framework. Biochem. Cell Biol. 2002;80(1):27-34. [DOI:10.1139/o01-153]
4. Legrand D, Mazurier J. A critical review of the roles of host lactoferrin in immunity. Biometals. 2010;23(3):365-76. [DOI:10.1007/s10534-010-9297-1]
5. Baker E, Baker H. Lactoferrin. Cell. Mol. Life Sci. 2005;62(22):2531. [DOI:10.1007/s00018-005-5368-9]
6. Anderson BF, Baker HM, Norris GE, Rice DW, Baker EN. Structure of human lactoferrin: Crystallographic structure analysis and refinement at 2.8 Å resolution. J. Mol. Biol. 1989;209(4):711-34. [DOI:10.1016/0022-2836(89)90602-5]
7. Moore SA, Anderson BF, Groom CR, Haridas M, Baker EN. Three-dimensional structure of diferric bovine lactoferrin at 2.8 Å resolution. J. Mol. Biol. 1997;274(2):222-36. [DOI:10.1006/jmbi.1997.1386]
8. Karthikeyan S, Paramasivam M, Yadav S, Srinivasan A, Singh TP. Structure of buffalo lactoferrin at 2.5 Å resolution using crystals grown at 303 K shows different orientations of the N and C lobes. Acta Crystallogr., Sect. D: Biol. Crystallogr. 1999;55(11):1805-13. [DOI:10.1107/S0907444999010951]
9. Sharma AK, Paramasivam M, Srinivasan A, Yadav M, Singh TP. Three-dimensional structure of mare diferric lactoferrin at 2.6 Å resolution. J. Mol. Biol. 1999;289(2):303-17. [DOI:10.1006/jmbi.1999.2767]
10. Khan JA, Kumar P, Paramasivam M, Yadav RS, Sahani MS, Sharma S, et al. Camel lactoferrin, a transferrin-cum-lactoferrin: crystal structure of camel apolactoferrin at 2.6 Å resolution and structural basis of its dual role. J. Mol. Biol. 2001;309(3):751-61. [DOI:10.1006/jmbi.2001.4692]
11. Conesa C, Sánchez L, Rota C, Pérez M-D, Calvo M, Farnaud S, et al. Isolation of lactoferrin from milk of different species: calorimetric and antimicrobial studies. Comparative Biochemistry and Physiology Part B: J. Biochem. Mol. Biol. 2008;150(1):131-9. [DOI:10.1016/j.cbpb.2008.02.006]

Year 15, Issue 3 (May & June 2021)
12. Magjeed NA. Corrective effect of milk camel on some cancer biomarkers in blood of rats intoxicated with aflatoxin B1. J. Saudi Chem. Soc. 2005;9:253-63.

13. Agrawal RP, Saran S, Sharma P, Gupta RP, Kocher DK, Sahani MS. Effect of camel milk on residual β-cell function in recent onset type 1 diabetes. Diabetes Res. Clin. Pract. 2007;77(3):494-5. [DOI:10.1016/j.diabres.2007.01.012]

14. Quan S, Tsuda H, Miyamoto T. Angiotensin I-converting enzyme inhibitory peptides in skim milk fermented with lactobacillus helveticus 130b4 from camel milk in Inner Mongolia, China. J. Sci. Food Agric. 2008;88(15):2688-92. [DOI:10.1002/jsfa.3394]

15. Yen C-C, Shen C-J, Hsu W-H, Chang Y-H, Lin H-T, Chen H-L, et al. Lactoferrin: an iron-binding antimicrobial protein against Escherichia coli infection. Biometals. 2011;24(4):585-94. [DOI:10.1007/s10534-011-9423-8]

16. Claren D, Swaisgood H. Bioactive milk peptides: a prospectus. J. Dairy Sci.2000;83(6):1187-95. [DOI:10.3168/jds.S0022-0302(00)74983-6]

17. Mills S, Ross R, Hill C, Fitzgerald G, Stanton C. Milk intelligence: Mining milk for bioactive substances associated with human health. Int. Dairy J. 2011;21(6):377-401. [DOI:10.1016/j.idairyj.2010.12.011]

18. Martin E, Ganz T, Lehrer RI. Defensins and other endogenous peptide antibiotics of vertebrates. J. Leukocyte Biol. 1995;58(2):128-36. [DOI:10.1002/jlb.58.2.128]

19. Wang Z, Wang G. APD: the antimicrobial peptide database. Nucleic Acids Res. 2004;32(suppl_1):DS90-D2. [DOI:10.1093/nar/gkh025]

20. Yamauchi K, Tomita M, Giehl T, Ellison RR. Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. Infect. Immun. 1993;61(2):719-28. [DOI:10.1128/iai.61.2.719-728.1993]
The Identification of a Novel Peptide Derived from...
55. Zhang L, Rozek A, Hancock RE. Interaction of cationic antimicrobial peptides with model membranes. J. Biol. Chem. 2001;276(38):35714-22. [DOI:10.1074/jbc.M104925200]

56. Jenssen H, Hamill P, Hancock RE. Peptide antimicrobial agents. Clin. Microbiol. Rev. 2006;19(3):491-511. [DOI:10.1128/CMR.00056-05]

57. Wang G, Mishra B. The importance of amino acid composition in natural AMPs: an evolutionary, structural, and functional perspective. Front. Immunol. 2012;2:221. [DOI:10.3389/fimmu.2012.00221]

58. Conneely OM. Antiinflammatory activities of lactoferrin. J. Am. Coll. Nutr. 2001;20(sup5):389S-95S. [DOI:10.1080/07315724.2001.10719173]

59. Utsugi T, Schroit AJ, Connor J, Bucana CD, Fidler IJ. Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes. Cancer Res. 1991;51(11):3062-6. [DOI:10.1023/B:BIOM.0000027691.86757.e2]

60. Orsi N. The antimicrobial activity of lactoferrin: current status and perspectives. Biometals. 2004;17(3):189-96. [DOI:10.1023/B:BIOM.0000027691.86757.e2]

61. Wang J, Liu H, Zhao J, Gao H, Zhou L, Liu Z, et al. Antimicrobial and antioxidant activities of the root bark essential oil of Periploca sepium and its main component 2-hydroxy-4-methoxybenzaldehyde. Molecules. 2010;15(8):5807-17. [DOI:10.3390/molecules15085807]

62. Jahani S, Shakiba A, Jahani L. The Antimicrobial effect of lactoferrin on Gram-negative and Gram-positive bacteria. Int. J. Infect. 2015;2(3). [DOI:10.17795/iji27594]

63. Drago-Serrano ME, De La Garza-Amaya M, Luna JS, Campos-Rodriguez R. Lactoferrin-lipopolysaccharide (LPS) binding as key to antibacterial and antiendotoxic effects. Int. Immunopharmacol. 2012;12(1):1-9. [DOI:10.1016/j.intimp.2011.11.002]
شناسایی یک پپتید جدید به دست‌آمده از لاکتوفرین جوشانه از شیر شتر با فعالیت بالقوه
 ضد میکروبی

النز خواجه۱، محسن نعیمی پور۲، محمد رضا فرژین۲

۱ گروه بیوتکولوژی، واحد سپزور، دانشگاه آزاد اسلامی، سپزور، ایران
۲ گروه تحقیقات بیوتکولوژی و دامپزشکی، مؤسسه تحقیقات سرم و واکسن‌سازی رازی، شهر شمال شرق کشور، مشهد، ایران
۳ مرکز تحقیقات سلولی و موادکولی دانشگاه علوم پزشکی سپزور، سپزور، ایران

چکیده
زمینه و اهداف پپتید‌های ضد میکروبی در بهبود صحت بدن به عنوان یکی از اثرات سریع در مقایسه با داروهای ضد باکتری، داشتند. سعی در بررسی اثرات تقویت‌کننده و تقویت‌کننده مولکول‌های سلولی از لاکتوفرین از شیر شتر، به‌عنوان یک پپتید، در مقایسه با داروهای ضد باکتری، بررسی شده است.

مواد و روش کار: در مطالعه حاضر، با استفاده از بیوتکولوژی، چندین پپتید ضد باکتری به‌عنوان کاندیداهای لاکتوفرین شیر شتر نشان داد. سپس، یک پپتید مناسب بر اساس موارد میکروبی بیولوژیکی تعیین شد. پس از تعیین مولکول‌های نشان‌دهنده میکروب‌های سالم، سپس با روش میکروبی با استفاده از Pepsin-Camel-Lac1 تولید شد. سپس با روش MTT، خاصیت آنتی‌توموری گردید. سپس با استفاده از Pepsin-Camel-Lac1، بیماری‌های استافیلوکوکوس و سودوموناس آئرژینوزا، استافیلواکوکوس و سودوموناس آئرژینوزا، شایعه‌ی بیماری‌های فیزیولاجیکی، تجزیه و تحلیل شدند.

پایان‌نامه: نتایج حاصل که پپتید نشان‌دهنده میکروب‌های سالم، سپس با روش MTT، خاصیت آنتی‌توموری گردید. سپس با استفاده از Pepsin-Camel-Lac1، بیماری‌های استافیلوکوکوس و سودوموناس آئرژینوزا، استافیلواکوکوس و سودوموناس آئرژینوزا، شایعه‌ی بیماری‌های فیزیولاجیکی، تجزیه و تحلیل شدند.

یافته‌ها: نتایج حاصل که پپتید نشان‌دهنده میکروب‌های سالم، سپس با روش MTT، خاصیت آنتی‌توموری گردید. سپس با استفاده از Pepsin-Camel-Lac1، بیماری‌های استافیلوکوکوس و سودوموناس آئرژینوزا، استافیلواکوکوس و سودوموناس آئرژینوزا، شایعه‌ی بیماری‌های فیزیولاجیکی، تجزیه و تحلیل شدند.

نویسنده مسئول: مجید جمشیدیان مجاور، گروه تحقیقات بیوتکولوژی و دامپزشکی، مؤسسه تحقیقات سرم و واکسن‌سازی رازی، شهر شمال شرق کشور، مشهد، ایران
ایمیل: m.jamshidian@rvsri.ac.ir

کلید واژه‌ها: پروتئین‌های سیر، پپتید، فعالیت ضد باکتری‌ای، استافیلوکوکوس و سودوموناس آئرژینوزا

گرایهٔ گروه میکروب‌شناسی پزشکی ایران دسترسی آزاد کی بهبودی توزیع و نشر برای انتشار فیش‌تراشی با نکردن آزاد است.