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Antimicrobial Peptides Derived from Goat's Milk Whey Proteins Obtained by Enzymatic Hydrolysis

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ABSTRACT: In this study the bacterial growth inhibitory activity of peptide fragments produced from **goat's** milk whey proteins by enzymatic hydrolysis using trypsin, ficin and a combination of both was investigated. **Goat's** milk whey proteins were isolated and subjected to enzymatic hydrolysis and peptides were purified by ultrafiltration followed by reverse-phase high-performance liquid chromatography (RP-HPLC). Growth inhibitory activities of hydrolysates ranged from 4.67% to 87.46% for *E.coli* and 3.03% to 98.63% for *B.cereus*. Among all peptide fragments, permeate containing 3kDa peptides produced by trypsin showed maximum inhibition against Gram positive and Gram negativebac bacteria. This fraction was further purified by HPLC. Fourteen peptide fractions were collected and evaluated for their growth inhibitory activities. Two fractions showed the highest growth inhibitory activities with MIC_{50}'s of 383±8 µg/ml and 492±10 µg/ml against *E.coli* and *B.cereus*, respectively. Taken together, the results of this study indicated that it is possible to produce novel antibacterial peptide by enzymatic hydrolysis of **goat's** milk whey proteins which can potentially replace synthetic food preservatives in food industries.

Keywords: *Antimicrobial Activity, Bioactive Peptide, Goat Milk, Whey Proteins*.

Introduction

Whey proteins compromise about 20% of whole milk proteins of mammalian species. Whey proteins are globular molecules with considerable contents of α-helix motifs and their sequences include balanced acidic/basic and hydrophobic/hydrophilic amino acids (Madureira *et al*., 2010). Whey proteins include β-LG, α-LA, bovine serum albumin, immunoglobulins, and several minor proteins which can promote health and prevent diseases due to different nutritional and biological properties (Madureira *et al*., 2007).

Bioactive peptides can be produced from many proteinous sources. These peptides are inactive within the sequence of the parent proteins and exhibit bioactive actions when released by hydrolysis with exogenous enzymes (Gibbs *et al*., 2004) or by gastro-intestinal digestion (Smacchi and Gobbetti, 2000; Meisel and FitzGerald, 2003; Korhonen and Pihlanto, 2003). The size of bioactive peptides is generally between 3–20 amino acid residues.

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Peptides produced from hydrolysis of bovine whey proteins have been found to possess anti-hypertensive, anticancer, antimicrobial, and immunomodulatory activities (Silva and Malcata, 2004). Several antimicrobial peptides have been isolated within the sequences of major bovine whey proteins, namely, α-lactalbumin and β-lactoglobulin as well as lactoferin and lysozyme (Chatterton *et al*., 2006; Brandelli, *et al*., 2015).

Goat is the main supplier of milk for many rural regions of the world. Because of allergy problem presented by cow's milk especially among infants, goat's milk has received considerable attention (Almaas *et al*., 2006; Park, 2009). Compared to cow or human milk, goat milk has distinct biological properties, such as high buffering capacity, distinct alkalinity (Park, 2009; Park & Haenlein, 2006), high digestibility due to the reduced dimensions of casein micelles and smaller size of its fat globules (about 3.49 μm) and higher amounts of medium and short chain fatty acids (caproic, caprilic and capric acid). The latter characteristic apparently accounts for their effect in reduction of cholesterol in human tissues, by limiting cholesterol storage and improving its mobilization (Haenlein, 1992). In addition, goat's milk proteins are more readily digestible, and thus amino acids absorbed more efficiently than those of cow's milk (Park, 2009). Medically, goat's milk is being recommended for neonates when human milk is lacking (Carver, 2003). Furthermore, certain therapeutic properties in human nutrition, such as a better utilization of fat and mineral salts in individuals suffering from malabsorption syndrome, are attributed to goats' milk (Alferez *et al*., 2001).

In recent years, bacterial resistance to the most of the commonly used antibiotics have posed considerable threats to the health of human communities. Therefore, finding new antibiotics with high potential and lower risk of resistance, seems necessary (Ku¨ckelhaus *et al*., 2007). The use of natural antimicrobial peptides has been proposed as novel substituent for synthetic antibiotics at the next decades (Gordon *et al*., 2005; Dashper *et al*., 2007).

The antimicrobial peptides are defined as peptide fragments comprised of less than 50 amino acid residues with a molecular mass of less than 10 kDa. The majority of amino acid residues are hydrophobic in the nature. These types of bioactive peptides are typically produced using an enzymatic hydrolysis under in vitro conditions (Bulet *et al.*, 2004). However, while reports on bioactive peptides of human and bovine milk and/or their products are abundant, the milk of other species, including goat, has not been explored sufficiently (De Gobba, 2014; Atanasova and Ivanova, 2010; Eriksen, *et al*., 2008; Park, 2009).

In this communication we describe production and isolation of peptides derived from ficin and trypsin hydrolysis of goat milk whey proteins and demonstrate their activities against representative gram positive and negative bacteria.

Materials and Methods

Goat's milk was purchased from local goat producers, trypsin was obtained from Merck Chemical Company, Germany and ficin was purchased from Sigma Aldrich Chemicals, USA. *Escherichia coli* ATCC 8739, *Bacillus cereus* ATCC 11788 were obtained from the Persian Type Culture Collection (PTCC), Tehran, Iran. Culture media and o-phataldehyde were obtained from Merck Chemical Company, Germany. All other chemicals used were of analytical grade.

- *Isolation of whey proteins*

Goat's milk was warmed to 37˚C and skimmed immediately by centrifugation (5000 ×g, 15 min). Whey proteins were separated from caseins by precipitating of

caseins using 1.0 M HCl at the pH of 4.6 followed by centrifugation (5860×g, 60 min, 4˚C). The supernatant was then lyophilized and stored at -20˚C until use.

- *Determination of free amino groups*

Determination of free amino groups was carried out by o-phthaldialdehyde (OPA) assay to measure the degree of proteolysis (Church *et al*., 1983). To evaluate proteolysis of whey proteins as the substrates, an aliquot (usually 10–50µL containing 5–100µg proteins) was added directly to 1.0 mL of OPA reagent (25 mL of 100 mM sodium tetra hydroborate, 2.5 mL 20% SDS solution (w/w), 40 mg of OPA, dissolved in 1 mL methanol, and 100 mL of β-mercaptoethanol, adjusted to a final volume of 50 mL with distilled water, prepared fresh daily). The solutions were mixed and incubated for 2 min at room temperature and the absorbance at 340 nm was recorded by spectrophotometer (Analytikjena, Spekol 1500, Germany). Leucine was used as the standard amino acid. The number of free -NH_2 groups per mg protein was calculated based on the protein concentration of each sample determined by the Bradford method (Bradford, 1976).

- *Proteolysis by trypsin and ficin*

Whey proteins solutions were prepared by dissolving whey proteins powder in 50 mM phosphate buffer, pH 7.8 to give a final concentration 6 mg/ml. Trypsin was added to the sample at enzyme/substrate ratio of 1/12.5, w/w followed by incubation at 37˚C for 6 hours (Salami *et al*., 2008**)**. In ficin-treated samples, 100 mg freeze dried whey proteins was dissolved in 10 mL 0.01 M phosphate buffer (pH 7.6, 37˚C) followed by adding solutions as below; 1.0 mL 0.25 M L-cystein, 1.0 mL 0.25 M EDTA, 1.0 mL 0.25 M NaOH, 3.0 mL 1.0 M phosphate buffer, pH 7.0 and 4 mL distilled water (Liener and Friedenson, 1970). Immediately ficin was added to the solution at enzyme/substrate ratio of 1/10, w/w and incubated at 37˚C for 3 hours (Pierro, *et al*., 2014). Finally, the proteinases were inactivated by heating at 90˚C for 15 min. The hydrolysates were then lyophilized. In another set of experiment, whey protein sample was first hydrolyzed by trypsin as described above and after inactivation of trypsin, hydrolysis was continued by ficin. A control sample of whey proteins in which no enzyme was added was included. All the experiments were performed in triplicate order.

- *Ultrafiltration and HPLC*

Samples obtained from whey proteins hydrolysis were fractionated by ultrafiltration (UF) membranes (Sartorius, vivaspin 20 with cutoff of 10, 5 and 3 kDa). Samples from UF fractionation were analyzed for their growth inhibitory activities. The sample with highest growth inhibitory activities was further fractionated using a KNAUER HPLC system in reversed phase (RP-HPLC) mode on an analytical C18 column using a linear gradient of 5–50% eluent B (0.1% trifluoroacetic (TFA) in acetonitrile) in eluent A (0.1% TFA in distilled water ,v/v), with a flow rate of 1.0 mL/min for 60 min. The absorbance of the elution peaks was monitored at 220 nm using a UV detector (Asoodeh *et al*., 2012a). Major peaks were collected, lyophilized and evaluated for their growth inhibitory activity.

- *Determination of protein content*

Protein concentration was determined by Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as standard (Marshall and Williams, 1993); each measurement was carried out in triplicate order and the results shown are the average of the data from three experiments.

- *Growth inhibitory activity*

E coli, and *B. cereus* were the indicator microorganisms used to test antibacterial activities. The bacteria were grown to approximately 1.5×10^8cfu/mL. The lyophilized whey protein powders were dissolved in distilled water to give a final peptide content of 1 mg/mL (Pritchard *et al*, 2010). Three reaction mixture were prepared as follows: Sample 1: solution containing 50 µL of peptide fraction, 10µl of bacterial culture and 50 µL of Tryptic Soy Broth (TSB). Negative control: TSB alone. Positive control: bacterial culture and TSB. The assay was carried out in sterile 96-well plates and after 24 hour incubation at 37 °C absorbance (A) at 600 nm was measured using a microplate reader (BioTek, Powerwave XS2, USA). Percent inhibition after 24 hour of incubation was calculated according to (Casey *et al*., 2004).

$$\%\ \text{Antimicrobial activity} = 100 - \left(\frac{\text{A sample} - \text{A negative control}}{\text{A positive control} - \text{A negative control}} \times 100\right)$$

- *etermination of minimum inhibitory concentration (MIC)*

Determination of MIC was performed by the method of Asoodeh et al., (2012b) with some modifications. To a 96 well microplate, 100 µl of TSB medium and 100 µl of 4mg/ml peptide solution were poured into microplate; then 100 µl of one well was poured to another well and dilution continued to 8 well to reach a protein concentration of 15.6 µg/ml. 10 µl of bacterial suspension (1×10^7 CFU/ml) were added to each well to yield a final count of (1×10^6 CFU/ml). Absorbance at 600 nm was measured after 24 hour at 37 °C using a microplate reader (BioTek, Powerwave XS2, USA). MIC_{50} value was defined as the lowest peptide concentration at which inhibition of bacterial growth was decreased to 50%.

- *Statistical analysis*

To test for the significant differences of biological activities in peptides derived from hydrolysis of whey proteins, data were subjected to analysis of variance (ANOVA) using SPSS 22 software. To describe the results, mean, standard deviation and analysis of variance (ANOVA) were used.

$P<0.05$ was considered as a level of significance.

Results and Discussion

Goat's milk whey proteins were hydrolyzed with trypsin, ficin, and a combination of both enzymes. The hydrolysates were fractionated according to their size with UF membranes with molecular mass cutoff of 10, 5 and 3 kDa.

- *Proteolysis of goat milk proteins*

Degree of hydrolysis can be determined by the number of free amino groups (Church *et al.* 1983). The number of free amino groups and growth inhibitory activity of hydrolysed and unhydrolysed whole goat whey proteins is presented in Table 1. In all the samples, the number of free amino groups significantly increased after hydrolysis. The number of free amino groups in un-hydrolyzed sample was 71.81±3.39 µmol/mg protein and increased to 384.00±13.74 µmol/mg protein in trypsin hydrolysis, 385.50±7.46 µmol/mg protein in ficin hydrolysis and 744.90±19.65 µmol/mg protein when both enzymes were present. These results indicated that both enzymes caused extensive proteolysis, although hydrolysis by both enzymes produced more free amino groups.

- *Determination of growth inhibitory activity*

Goat's milk whey proteins, and their hydrolysates, inhibit the growth of *E. coli* and *B.cereus* (Table 1). The controlled hydrolysis performed with proteolytic enzymes used in this study enhanced the

growth inhibitory activities of goat's milk whey proteins.

The growth inhibitory activity of peptide fractions (>10 kDa, 5-10 kDa, 3-5 kDa, and <3 kDa) obtained from goat's milk whey proteins hydrolyzed by ficin and trypsin and a combination of both showed that hydrolysis resulted in significant increase of growth inhibitory activity (Tables 2 and 3). The highest inhibition of growth of *E.coli* (87.46±2.04%) and *B.cereus* (98.63±0.56%) was present in the <3 kDa UF filtrate of trypsin-hydrolyzed goat whey proteins.

Antimicrobial peptide sequence usually are composed of 2 to 20 amino acid; therefore, a peptide with a smaller size that might pass through the membrane of the bacteria more conveniently, should have a higher antimicrobial activity (Korhonen and Pihlanto, 2006). Higher growth inhibitory activity of trypsin hydrolysates than ficin demonstrates that hydrolysis pattern of these enzymes are different and they produce peptides with different sizes and sequences.

Goat whey proteins hydrolysates produced by trypsin with MW<3 kDa that showed the highest growth inhibitory activity against *E.coli* and *B.cereus* and was selected for further fractionation. This sample was injected to RP-HPLC as described in Material and Methods (Figure 1). RP-HPLC fractions were analyzed for growth inhibitory activity and their MIC_{50}s' were determined (Figure 2). Fraction 9 had the lowest MIC_{50} (383±8 µg/ml) against *E.coli* and Fraction 11 exhibited the highest growth inhibitory activity against *B.cereus* (492±10 µg/ml).

The different growth inhibitory activities of fractions obtained after ultrafiltration and RP-HPLC suggested that not only the size but also the amino acid composition of peptide fractions are particularly important for their activity (Salami, 2010). Different mechanisms have been forwarded to explain the antimicrobial effects of small peptides. The variability in amino acid composition, sequence and physiochemical properties mostly account for the difference in the antimicrobial activities of these peptides (Benkerroum, 2010). Majority of antimicrobial peptides are cationic and thereby produce amphipathic α-helices and β-sheet structures which can selectively interact with anionic bacterial membranes and changing the permeability, resulting in bacterial death (Hancock and Sahl, 2006).

Considering the nature of RP-HPLC stationary phase, one might expect more polar peptides elute in the beginning and more hydrophobic one elute at the end of chromatography. According to Figures 1 and 2, the peptide fraction exhibiting highest activity against *E. coli* and *B. cereous* eluted in the middle of chromatography and are therefore intermediate with respect to hydrophobicity. As discussed above, peptides having both hydrophobic and charged (polar) residues harbor excellent growth inhibitory activity.

Table 1. Growth inhibitory activity of whole goat whey proteins hydrolysates

Enzyme treatment	**Free amino groups (µmole/mg protein)**	**growth inhibitory activity against *E.coli* (%)**	**growth inhibitory activity against *B.cereus* (%)**
Unhydrolysed Sample	71.81±3.39 [a]	30.14±1.54 [a]	43.49±2.94 [a]
Trypsin	384.00±13.74 [b]	76.48±4.35 [d]	82.53±3.34 [d]
Ficin	385.50±7.46 [b]	42.96±3.83 [b]	62.63±2.32 [b]
Trypsin and Ficin	744.90±19.65 [c]	68.75±3.03 [c]	69.22±3.61 [c]

[*] Small letters superscripts indicate significant differences in columns (P<0.05).

Table 2. Growth inhibitory activity (%) of peptides obtained from UF fractionation of the products from different enzyme treatments of goat milk whey proteins against *E.coli*

Enzyme treatment	Growth inhibitory activity (%)			
	>10 kDa	5-10 kDa	3-5 kDa	<3 kDa
Trypsin	12.48 ± 0.63^{aA}	66.25 ± 1.10^{aB}	26.73 ± 0.91^{aC}	87.46 ± 2.04^{aD}*
Ficin	35.33 ± 0.62^{bA}	66.07 ± 1.65^{aB}	31.46 ± 0.71^{bC}	20.69 ± 1.42^{bD}
Trypsin and Ficin	4.67 ± 0.45^{cA}	21.62 ± 0.84^{bB}	29.22 ± 0.47^{cC}	74.89 ± 1.79^{cD}

* Small letters superscripts indicate significant differences in columns (P<0.05). Capital letter superscripts indicate significant differences in rows (P<0.05).

Table 3. Growth inhibitory activity (%) of peptides obtained from UF fractionation of the products from different enzyme treatments of goat milk whey proteins against *B.cereus*

Enzyme treatment	Growth inhibitory activity (%)			
	>10 kDa	5-10 kDa	3-5 kDa	<3 kDa
Trypsin	16.03 ± 0.91^{aA}	46.81 ± 0.56^{aB}	72.68 ± 0.86^{aC}	98.63 ± 0.56^{aD}*
Ficin	10.23 ± 0.47^{bA}	5.56 ± 0.19^{bB}	75.41 ± 0.67^{bC}	42.72 ± 0.60^{bD}
Trypsin and Ficin	3.03 ± 0.33^{cA}	14.46 ± 0.83^{cB}	16.88 ± 0.93^{cB}	67.66 ± 1.46^{cC}

* Small letters superscripts indicate significant differences in columns (P<0.05). Capital letters superscripts indicate significant differences in rows (P<0.05).

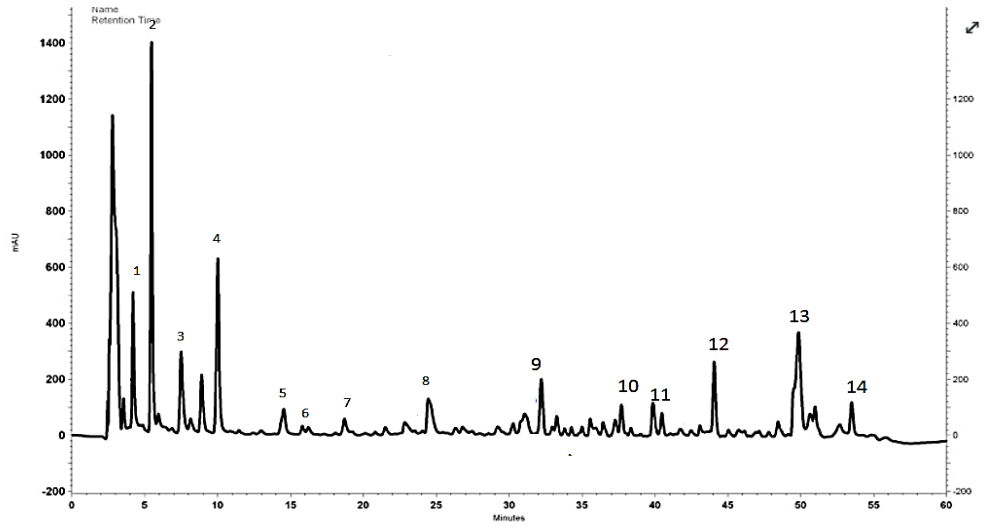

Fig. 1. Chromatograms of RP-HPLC for goat whey proteins hydrolyzed by trypsin passed through ultrafilter membrane with cutoff of < 3 kDa.

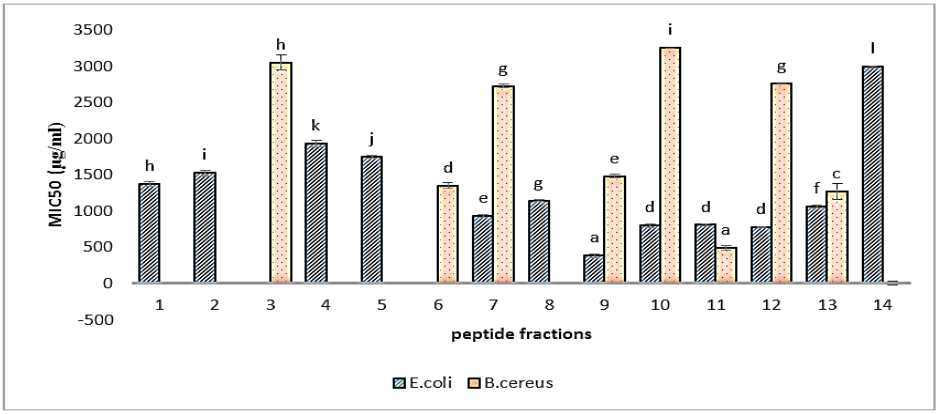

Fig. 2. MIC_{50} (µg/ml) of fractions obtained from RP-HPLC of trypsin-treated goat whey proteins (<3 kDa).

Conclusion

This study has shown that the enzymatic hydrolysis of **goat's** milk whey proteins improves the antimicrobial properties and offers an interesting opportunity for food application. **Goat's** whey proteins and their hydrolysates, especially those obtained by treatment with trypsin, could be considered as suitable natural preservatives in the food industry and could become ingredients of functional foods.

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