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Research Article

Antimicrobial Activity of Quinoa Protein Hydrolysate against *Streptococcus pyogenes* and *Escherichia coli*

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Quinoa seed, as a rich source of protein with strong antioxidant properties, plays an important role in improving consumers' nutrition. This study was aimed at comparing the antimicrobial activity of peptides from quinoa hydrolysed proteins (QHP) on *Streptococcus pyogenes* as a Gram-positive and *Escherichia coli* as a Gram-negative bacterium with gentamicin antibiotic as a positive control. Different enzymatic ratios of pepsin and alcalase (30–90 AU/kg protein) at different temperatures (50–55°C) and times (150–210 min) were used to determine the optimal conditions for peptide hydrolysis with the highest antimicrobial properties. Similar to gentamicin, the maximum growth inhibition zones were 11.88 ± 0.37 mm and 12.49 ± 0.58 mm for *S. pyogenes* and *E. coli*, respectively, with an enzyme/substrate ratio as 60 AU/kg protein, a peptides concentration of 800 μg/ml, and at 50°C for 150 min of hydrolysis. The results showed that QHP has a good inhibitory effect on the bacteria mentioned and can be used as a food preservative.

1. Introduction

The health adverse effects of chemical preservatives including their carcinogenic and teratogenic properties as well as their toxic residues are being proven every day, while the demand for long shelf-life foods is rising [1]. Thus, providing natural alternatives is of utmost importance. In recent years, characterizing natural antioxidants has received special attention, which leads to studies on antioxidant and antibacterial capacity of peptides derived from hydrolysed proteins of a variety of food resources, e.g., soy protein [2], casein [3], egg white protein [4], seeds of river tamarind [5], sesame seed [6], cowpea [7], Okra seed meal [8], and fish proteins [9].

Protein hydrolysis is a beneficial technology for providing high value-added products with antioxidant and antimicrobial activities known as bioactive peptides [10–12]. Bioactive peptides are defined as protein components that are inactive in the core protein structure and exhibit various physicochemical functions after being released by enzymatic hydrolysis [13, 14]. They have a positive impact on body functions and ultimately promote health quality [15–17].

Enzymatic hydrolysis (especially by pepsin and trypsin enzymes) is the most common path to produce potent bioactive peptides [18]. They are known to exert functional operations, i.e., antimicrobial, antioxidant, antithrombosis, and antihypertension properties as well as immune system regulation and mineral binding [13, 19, 20]. Among plant food sources rich in protein are quinoa seeds that are originated from South America and consumed for more than 5,000 years. Due to its botanical characteristics, quinoa is considered as a pseudo-cereal [21, 22].

It is known to be more digestible compared to many grains such as rice probably due to its high fiber content [22, 23]. This plant is well known for adapting to various climates and soils. In recent years, its cultivation in Iran has been started as well [24].

According to studies, quinoa cultivation is beneficial compared to wheat and rice owing to its lower water requirement and salinity tolerance [25]. Wheat, rice, and similar cereals can be replaced by quinoa for children, those who suffer from diabetes, celiac, and also those with special diets. Moreover, due to the fact that quinoa is rich in protein, magnesium, fiber, phosphorus, vitamin B_2, potassium and mineral (e.g., iron) contents and contains essential amino acids lysine and methionine, it can provide the body with complete protein and alleviate malnutrition [10, 21, 26–30]. Quinoa protein has been successfully used in antimicrobial edible coatings as a bio-preservative in food product packages [31, 32]. In addition, a recent study reported that the antimicrobial and antioxidant attributes of fresh burgers incorporated by quinoa peptide-loaded nano-liposomes were significantly improved [33]. Although the antibacterial and antioxidant properties of many local traditional seeds and plants in Iran have been extensively assessed [24, 34]; no much research on the antimicrobial activity of quinoa has been conducted.

Considering the high percentage of protein content in quinoa compared to other cereals (25% more), its hydrolysed protein and resulting bioactive peptides can exhibit high antibacterial activity against the Gram-positive and Gram-negative bacteria.

Streptococcus pyogenes is known as one of the major pathogens, associated with pharyngitis and deep tissue infections. Although it is not generally considered a food-borne pathogen [35, 36], outbreaks of food-borne pharyngitis have been rarely reported due to poor personal and hand hygiene [36–39]. This Gram-positive bacterium is known to have a significantly long-term survival, i.e., 2 to 88 h depending on the surface type [40, 41].

On the other hand, as a Gram-negative model bacterium, *Escherichia coli* is a well-studied food-borne pathogen, and its survival on surfaces is 2–36 days depending on the surface type [41]. Hence, the present study aimed to prepare quinoa protein hydrolysate and investigate its antimicrobial effect on bacterial strains of *Streptococcus pyogenes* (*S. pyogenes*) and *Escherichia coli (E. coli)* compared to that of gentamicin as the positive control.

2. Materials and Methods

2.1. Chemicals and Reagents. All chemicals and reagents in the analytical grade (purity >99%) were purchased from Merck (Darmstadt, Germany). Quinoa (*Chenopodium quinoa Willd*) seeds (*Santamaria* cultivar) were purchased from the Seed and Plant Improvement Institute (Karaj, Iran).

2.2. Preparation of Bacterial Strains. To evaluate the antimicrobial effect of an active peptide derived from quinoa seeds, standard strains of *S. pyogenes* (PTCC-1447) and *E. coli* (PTCC-1335) were provided by the Pasteur Institute of Iran.

2.3. Sample Preparation. The impurities of quinoa seeds were removed manually, and seeds were ground using a mill (MB 1001B, Magic Bullet Blender, China) in order to obtain the whole quinoa flour (degree of extraction: 96%). The flour was defatted by hexane solvent (1 : 5 ratio) in three stages during 24 h by using an orbital shaker (TM 52E, Fan Azma Gostar, Iran). Thereafter, the suspension was placed in an oven (40°C, 24 h) to separate the solvent residue. The obtained flour was passed through a 0.25 mm mesh sieve and stored in polyethylene bags and kept at −18°C prior to use [12].

2.4. Extraction of Protein. In order to extract proteins, the method described by Chauhan et al. was used with some modifications [42]. Briefly, the defatted quinoa flour was dispersed in a 0.015 M sodium hydroxide solution. The resulting slurry was kept for 24 h at 4°C for a clearer supernatant, and then was centrifuged (Sigma, 6k15, Germany) at 10,000 g, 10°C for 30 min. The supernatant was then filtered (Whatman No. 1), and the filtrate pH value was adjusted to 4.5 by addition of 0.1 N HCl in order to precipitate the proteins. The precipitated proteins were completely isolated by a 30 min centrifugation (Sigma, 6k15) at 10000 g, 10°C.

Thereafter, they were washed with distilled water and lyophilised (freeze dryer, alpha 2, Christ-Germany) to produce quinoa protein concentrate [18, 43–45].

2.5. Protein Hydrolysis. Extracted proteins were digested using pepsin and alcalase enzymes inside a glass container while being mixed by a magnetic stirrer (IKA BH B2, Germany). Protein extract was diluted 5 folds in a sodium phosphate buffer solution while pH was adjusted at 8.0 by a 2 M NaOH solution. Alcalase enzyme was added to the diluted protein samples at pH 8 and the proteins were allowed to be hydrolysed by keeping the condition (pH and temperature) constant. Subsequently, the pH was adjusted to 2.5, and pepsin enzyme was added for proteins to be digested. Afterwards, samples were incubated in boiling water in order to halt the enzyme activity. They were centrifuged after cooling down to room temperature for 15 min, at 8000 g and 10°C. The supernatant was isolated as the protein hydrolysate and passed through a stirred cell ultrafiltration setup (Amicon, U.S.) with the aid of a membrane (molecular weight cutoff of 3000 Da). The obtained permeate from each membrane was lyophilised and kept at −20°C until use [7, 46].

A set of pretreatments were carried out in order to detect the optimum hydrolysis conditions comprising of temperature: 50°C, 55°C; time: 150, 180, 210 min and enzyme/substrate ratio: 30, 60, and 90 Anson unit (AU)/kg protein [47]. The obtained permeate of membranes were lyophilised and maintained at −20°C [12, 46]. Detailed conditions of the hydrolysis process for each treatment are presented in Table 1.

2.6. SDS-Page Analysis. Protein electrophoresis was performed using the SDS-Page technique according to the Laemmli method [48, 49].

An aliquot of 25 μl extracted protein was transferred to a gel electrophoresis system (MSCHOICETRIO, England)

Table 1: Hydrolysis condition of quinoa peptides.

Treatments	Enzyme ratio (AU/kg protein)[B]	Peptide concentration (μg/ml)	Temperature (°C)	Time period (min)
T0[A]	—	—	—	—
T1	30	200	55	210
T2	30	400	55	210
T3	30	800	55	210
T4	60	200	50	150
T5	60	400	50	150
T6	60	800	50	150
T7	60	200	55	180
T8	60	400	55	180
T9	60	800	55	180
T10	90	200	50	150
T11	90	400	50	150
T12	90	800	50	150
T13	90	200	50	180
T14	90	400	50	180
T15	90	800	50	180

[A]T0: control (gentamicin). [B]AU/kg: anson unit per kg.

with the upper gel containing 3.75% acrylamide-bisacrylamide and the lower gel containing 12% acrylamide-bisacrylamide. The gel dimensions were $140 \times 110 \times 1$ mm. The electrophoresis process time was about 3 h (until the bromophenol blue dye reached the lower edge of the gel). The current intensity was 30 mA in a PROTEIN II xi cell (BIO-RAD, USA). After removing gels from the electrophoresis, they were stained according to the Coomassie blue staining protocol.

In the SDS-PAGE technique, the larger the protein, the shorter the distance traveled [46]. Normally, a protein marker, which consists of several peptides with specific molecular weights, is added when loading samples into one of the wells. By comparing the protein sample's movement through the gel with that of protein markers, the weight of the target molecule will be estimated. In the present study, the BIO-RAD protein ladder was used to determine the molecular weight of the subunits of the tested samples [49]. For this section treatments descriptions were as follow: 1: Protein marker, 2: nonhydrolysed protein, 3: [enzyme/substrate ratio (AU/kg protein), temperature (°C); respectively] [30, 50]; 4: [30, 55]; 5: [60, 45]; 6: [60, 55]; 7: [90, 40]; 8: [90, 55].

2.7. Assessment of Antimicrobial Activity (Agar Well Diffusion Method). Bacterial suspensions were prepared from 24 h aged inocula, *i.e.,* Gram-positive *S. pyogenes* and Gram-negative *E. coli*. They were cultured 24 h before the test in Müller–Hinton agar (MHA, Merck, Germany) by using spread plate technique. After creating a well in all samples under sterile conditions, 40 μL from peptide solution was poured in each well by using a 50 μL sampler with 200, 400, and 800 μg/ml peptide concentrations.

For the control sample, gentamicin (5 mg) was poured, and all the cultured samples were incubated at 37°C for 24 h to subsequently determine the diameter of the nongrowth halos by a Vernier Caliper (VWR International Inc., USA) [50]. For each bacterium two replications were performed. In total, six treatments were designated for each bacterium, comprising A: enzyme ratio of 30 AU/kg protein, 55°C and 210 min; B: enzyme ratio of 60 AU/kg protein, 50°C and 150 min; C: enzyme ratio of 60 AU/kg protein, 55°C and 180 min; D: enzyme ratio of 90 AU/kg protein, 50°C and 150 min; E: enzyme ratio of 90 AU/kg protein, 50°C for 180 min and Control: that represented gentamicin as the control treatment.

2.8. Statistical Analysis. The results were imported into version 20 of SPSS software. Mean and standard deviation were calculated, and one-way analysis of variance (ANOVA) was used to compare the means, and a *t*-test at the level of $P < 0.05$ was performed to detect significant differences between experiments.

3. Results and Discussion

3.1. SDS-Page Analysis. The SDS-Page profiles of quinoa proteins and peptides with different treatments showed different protein bands ranged from less than 6.5 to 100 kDa in the protein profile (Figure 1), which was in accordance with studies conducted by Valenzuela et al. [51]. Brinegar and Goundan showed that all extractable quinoa proteins were in the range of 8 to 100 kDa at pH 8 and assumed that the extracted proteins included all major quinoa proteins [52]. Polypeptide bands with 8 to 10 kDa have been reported to be commonly found in all seeds. Chenopedins are known as major proteins of quinoa, which are among the seed storage proteins belonging to the globulin family. Peptides with 22-23 and 32–39 kDa have been known as basic and acidic subunits of chenopedins, respectively. Polypeptides with a molecular weight of 15 kDa belong to the 2S albumin [48].

During the hydrolysis of quinoa protein, as shown in Figure 1, the bands corresponding to the higher molecular

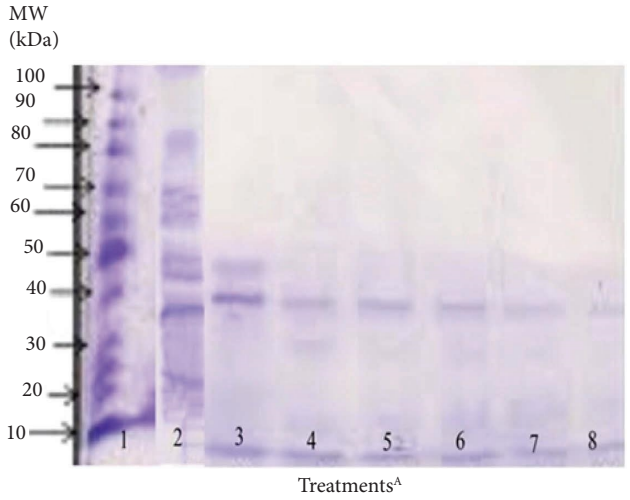

Figure 1: Quinoa protein profile of treatments hydrolysed at different conditions analysed by SDS-PAGE. [A]Treatments descriptions are as follows: 1: protein marker, 2: nonhydrolysed protein, 3 (enzyme/substrate ratio (AU/kg protein) and temperature (°C), respectively): [30, 50]; 4: [30, 55]; 5: [60, 45]; 6: [60, 55]; 7: [90, 40]; 8: [90, 55].

weight peptides were destroyed and solely low molecular weight peptides were observed [51, 53].

According to Figure 1, the molecular weight of T2, *i.e.*, nonhydrolysed protein, was the highest and T8 was the lowest. In the gels used for electrophoresis, smaller molecules move faster and travel longer distances than larger molecules, so that in Figure 1, the more hydrolysis, the lighter the blue colour, indicating more protein hydrolysis. The presence of high molecular weight proteins in this form may be due to incomplete hydrolysis or lack of hydrolysis by the enzymes [18, 54].

3.2. Assessment of Antibacterial Activity. The test results of the growth inhibition zone (mm) against *S. pyogenes* and *E. coli* in different conditions of hydrolysis are shown in Figures 2 and 3, respectively. From the results, it can be deduced that the peptide concentration had a significant influence on the inhibition of bacterial growth. In other words, the mean diameter of growth inhibition zone at 800 μg/ml peptide concentration was significantly greater among all the treatments. As such, the highest inhibitory effect belonged to treatment with the highest concentration (800 μg/ml) against *S. pyogenes* (Figure 2).

For a clearer perception of the influence of the studied treatments (temperature, time, enzyme/substrate ratio, and the concentration of peptides derived from quinoa protein), their role was assessed in individual groups (in-groups) and also between different groups (intergroups). The results of the in-group comparison of treatments showed that only the concentration of peptides had a significant effect on the diameter of the growth halo of pathogens ($P < 0.05$). In other words, under the same conditions of enzyme/substrate ratio, temperature, and time, solely the peptides concentration regulated the degree of inhibition of pathogen growth.

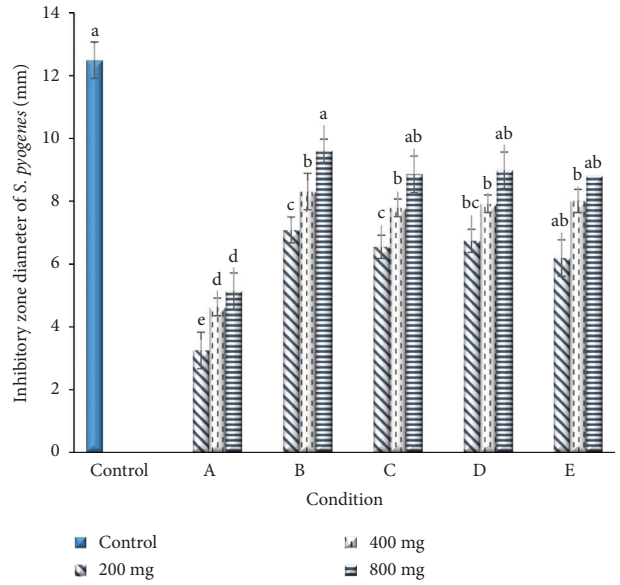

Figure 2: Growth inhibition zone of *S. pyogenes* in different conditions. Control: gentamicin, A: enzyme ratio of 30 AU/kg protein, 55°C and 210 min; B: enzyme ratio of 60 AU/kg protein, 50°C and 150 min; C: enzyme ratio of 60 AU/kg protein, 55°C and 180 min; D: enzyme ratio of 90 AU/kg protein, 50°C and 150 min; E: enzyme ratio of 90 AU/kg protein, 50°C for 180 min and control: represented gentamicin as the control treatment.

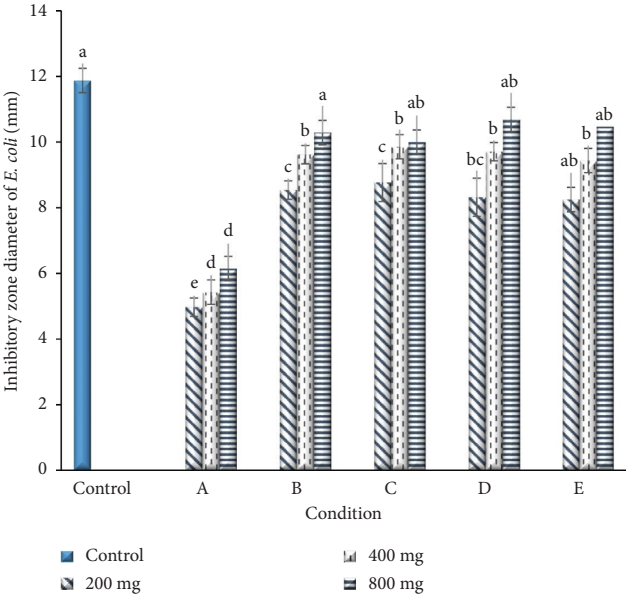

Figure 3: Growth inhibition zone of *E. coli* in different conditions. Control: gentamicin, A: enzyme ratio of 30 AU/kg protein, 55°C and 210 min; B: enzyme ratio of 60 AU/kg protein, 50°C and 150 min; C: enzyme ratio of 60 AU/kg protein, 55°C and 180 min; D: enzyme ratio of 90 AU/kg protein, 50°C and 150 min; E: enzyme ratio of 90 AU/kg protein, 50°C for 180 min and control: represented gentamicin as the control treatment.

Using intergroup comparison of treatments, the effect of time and temperature and enzyme/substrate ratio on inhibition of bacterial growth was detected, and the results

indicated that an increase in enzyme ratio and temperature can lead to a higher rate of pathogen growth inhibition. Furthermore, in A, B, C, and D treatments (Figure 2), with increasing the peptide concentration, the diameter of the growth inhibition zone against *S. pyogenes* gradually increased, so that in the stated conditions, at a concentration of 800 μg/ml, the highest and in concentration of 200 μg/ml the lowest diameter of growth inhibition zone were observed. In general, treatments B, C, and D at concentrations of 800 μg/ml and treatment E, at concentrations of 200 μg/ml and 800 μg/ml exhibited the greatest effect against in diameter of growth inhibition zone against *S. pyogenes* being equal to that of control (gentamicin antibiotic). The lowest antimicrobial activity was attributed to treatment A at 200 μg/ml concentration.

As illustrated in Figure 3 in each of the studied conditions, by increasing the peptide concentration, the diameter of the growth inhibition zone against *E. coli* increased. As such, the highest diameter of the growth inhibition zone against *E. coli* was at 800 μg/ml of concentration, and the lowest diameter of growth inhibition zone was at 200 μg/ml. However, this increase in antimicrobial activity due to higher peptide concentration was not statistically significant for treatments D and E. Generally, E and D treatments at all the three concentrations and B treatment at 800 μg/ml concentration showed the greatest effect on the diameter of the growth inhibition zone against *E. coli*, so that there was no significant difference between these samples and our positive control (gentamicin antibiotic). Nevertheless, the lowest antimicrobial activity was observed in treatment A at concentrations of 200 and 400 μg/ml (Figure 3).

The correlation between inhibitory effect and peptide concentration is unambiguously explained by bioactivity attributes of peptides as discussed earlier [10–12, 31–33]. Electrostatic interaction of peptides with the negatively charged molecules on the microbial cell membrane is known as the key mechanism of action of antimicrobial activity in peptides [55].

These results are consistent with those of Salehi et al., which investigated the antibacterial properties of the synthetic peptide D28 on *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains [56]. Their results indicated that the synthesized peptide was only effective against *S. aureus*. In addition, according to their report, enhancing the antibacterial activity of peptides through dimerization depends highly on the methods of dimerization and the bacterial strain [56]. In another study, the antioxidant and antimicrobial properties of quinoa seeds in Korea was compared to those of quinoa seeds cultivated in the United States and Peru [57], and the highest antioxidant activity and total phenolic compounds belonged to the quinoa seeds cultivated in South Korea. In addition, quinoa seed extract showed high potency in DPPH free radical scavenging which tallies with the report by Mahdavi-Yekta et al. [44]. They also investigated the antimicrobial properties of quinoa seed extract by disk diffusion method, and in contrast to our study, reported a very low influence against food-borne pathogens [57]. The positive inhibitory effect of quinoa seed in the present study is mainly attributed to its bioactive peptides [58–60].

4. Conclusions

Results of the present study showed that quinoa is a good source to produce bioactive peptides with antimicrobial properties through enzymatic hydrolysis and that time, temperature, and enzyme/substrate ratio are indeed effective parameters in optimal production of peptides. The results also indicated that QHP had the ability to compete with gentamicin as a control treatment in terms of growth inhibition of *E. coli* and *S. pyogenes*. The highest growth inhibitions against *E. coli* and *S. pyogenes* were obtained at a concentration of 800 μg quinoa peptide per ml. As such, antimicrobial compounds of QHP can be exploited in formulating food products and packages in order to enhance the product's shelf life and maintain its quality during preservation. Nevertheless, further assessment is needed to evaluate the extent of the inhibitory effect of QHP either as an incorporated ingredient or a coating component for food packages against major food-borne pathogens, e.g., *Staphylococcus aureus, Salmonella* Typhimurium*, Campylobacter jejuni, Listeria monocytogenes, and Bacillus cereus* in different food products.

Data Availability

The data used to support the findings of this study are included within the article.

Disclosure

The research was performed as part of the employment of the authors.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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