Journal of Food Science and Technology (Iran)

Homepage:www.fsct.modares.ir

Scientific Research

The effect of maltodextrin-gum arabic combined carrier and freeze drying method on the antibacterial activity of lanternfish (*Benthosema pterotum*) bioactive peptides against bacteria causing food spoilage

Soheyl Reyhani Poul[1*], Sakineh Yeganeh[2], Farzaneh Shakerian[3], Faezeh Tork Pahnabi[4], Arash Rakhshani[3]

1- PhD graduate, Department of Processing of Fishery Products, Faculty of Fisheries and Environment, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran

2- Professor, Department of Fisheries, Faculty of Animal Science and Fisheries, Sari Agricultural Sciences and Natural Resources University, Sari, Iran.

3- Postgraduate education student, Department of Fisheries, Faculty of Animal Science and Fisheries, Sari Agricultural Sciences and Natural Resources University, Sari, Iran.

4- Laboratory expert, Caspian Sea Ecology Research Institute, Fisheries Science Research Institute, Agricultural Research Education And Extention Organization, Sari, Iran

ARTICLE INFO

Article History:

Received: 2025/08/19

Review: 2026/02/27

Accepted: 2026/05/26

Keywords:

Lanternfish,

Bioactive peptides,

Nanoencapsulation,

Gum arabic,

Listeria monocytogenes,

MIC

DOI: **10.48311/fsct.2026.84087.0**

*Corresponding Author E-soheylreyhani@gmail.com

ABSTRACT

During the last two decades, research on the use of natural preservatives in food instead of chemical compounds has attracted the attention of researchers due to their safety. Bioactive peptides are one of the natural compounds that have a high potential to prevent food spoilage (oxidative and microbial). The aim of the current research was nanoencapsulation of bioactive peptides produced from lanternfish (*Benthosema pterotum*) (with a molecular weight of less than 3 kDa) using freeze-drying method and maltodextrin-gum arabic combined coating and then evaluating the antibacterial activity of carrier nanocapsules and free peptides against food spoilage bacteria including *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfringens*, *Bacillus cereus*, *Escherichia coli* and *Salmonella typhimurium*. According to the results, nanoencapsulation of bioactive peptides in addition to increasing their antibacterial activity (increasing the diameter values of non-growth halo of bacteria and reducing MIC and MBC), also caused the stability of this property during 60 days of storage of carrier nanocapsules at 4ºC ($p<0.05$). Increasing the concentration of bioactive peptides and nanocapsules carrying them (from 30 to 150 µg/ml) was often associated with increasing the diameter of the non-growth halo of bacteria ($p<0.05$). Among the bacteria, the highest diameter values of non-growth halo and the lowest MIC and MBC were recorded for *Listeria monocytogenes*. Also, the sensitivity of gram-positive bacteria to bioactive peptides and carrier nanocapsules was reported to be higher than gram-negative bacteria. The free peptides on day 60 and the concentration of 500 µg/ml were able to inhibit *Clostridium perfringens* and *Escherichia coli* bacteria, but they could not eliminate these bacteria. This is while the nanoencapsulated form of these peptides (carrier nanocapsules) at the same concentration was able to kill the mentioned bacteria. According to the findings, nanoencapsulation of bioactive peptides using freeze-drying method and combined coating of maltodextrin-gum arabic increases and stabilizes antibacterial activity and, as a result, makes them more effective in food formulations.

1-Introduction

Research on the use of natural compounds of marine origin as preservatives in the food and pharmaceutical industries has made significant progress over the past two decades, and consequently, the industrial production of these compounds has also expanded. Unlike chemical preservatives, these preservatives are not only not harmful to the health of consumers, but also have positive effects on the physiological reactions of the body. Among the natural preservatives extracted from aquatic animals and wastes from their processing, whose preservative properties (antimicrobial and antioxidant activity) have been confirmed in numerous studies and their production (extraction) has been developed, are chitin and chitosan. [1], Fucoidan[2], alginate[3]Various pigments such as astaxanthin[4]and phycocyanin[5]and ... mentioned. Hydrolyzed proteins are another of these compounds.[6]Which are actually the result of hydrolysis (water breakdown) of proteins present in various protein tissues using enzymatic and chemical methods.[7].

Hydrolyzed proteins contain bioactive peptides. Bioactive peptides are specific protein components that typically have 2 to 20 amino acids and a molecular weight of 6,000 daltons and are inactive in the structure of the main protein.But when they are separated from other amino acids in the protein structure by an enzyme or fermentation process, their biological activity begins.[8]Bioactive peptidesIn addition to its effectiveness in the food industry as an emulsifier, foaming agent[9], antimicrobial and antioxidant[10]After consumption by humans, they exert certain physiological effects (such as regulating blood pressure, strengthening the immune system, reducing cholesterol, and having antimicrobial effects) on the body.[8 and 9]. To exert these properties (physiological effects), bioactive peptides must remain intact and active during digestion and absorption (stomach and intestines) and then enter the bloodstream.[11]Since these peptides may be denatured and degraded in acidic and enzymatic conditions (proteolytic enzymes) of the digestive tract, it seems necessary to use methods to protect these peptides. Also, to maintain the preservative properties (antibacterial and antioxidant) of bioactive peptides in foods, it is necessary to use appropriate protective methods. Because each food has specific production conditions (use of high temperatures) and storage, and bioactive peptides that have been used in the formula to increase the shelf life of the food may undergo undesirable structural changes and lose their effectiveness. One of the appropriate methods for protecting active compounds, including bioactive peptides, is their encapsulation (microencapsulation).[12]During this process, a coating is created around the peptides that protects these active compounds from possible changes in adverse conditions. Meanwhile, the coatings (capsules) formed around the peptides in this method release their contents at a controlled rate and under specific conditions.[13].

In the nanoencapsulation process, various carbohydrate compounds (maltodextrin, starch, chitosan, dextrin, sucrose, etc.), cellulose (carboxymethylcellulose, methylcellulose, ethylcellulose, nitrocellulose, etc.), protein (casein, whey protein, gelatin, etc.), lipid (liposome, wax, paraffin, beeswax, diglycerides, monoglycerides, etc.) and... are used to cover (wall) the active compounds (core).[14]Maltodextrin is a carbohydrate coating derived from starch and is obtained from various starch sources such as potatoes, corn, and wheat. Due to its high solubility in water and lack of characteristic odor and color, this material is one of the most important polysaccharide materials used as a core wall or coating during the microencapsulation process.[4 and 5]. Gum arabic is also a polysaccharide that has been widely used in the food industry due to its specific physical and chemical properties (emulsifier, stabilizer, thickener, etc.) and has been of great interest as a wall in the microencapsulation process over the past decade [14 and 15]. Maltodextrin and gum arabic have characteristics such as reasonable price, edibility, high solubility, and low viscosity, which have led to their widespread use as carriers or walls in the

microencapsulation of active adhesive and antioxidant compounds.

The most important bacteria that cause spoilage of various foods include:*Listeria monocytogenes*[1]،*Staphylococcus aureus*[2]، *Clostridium perfringens*[3]، *Bacillus cereus*[4]، *Escherichia coli*[5]and*Salmonella Typhimurium*[6] If the growth and multiplication of these bacteria in food is not prevented, as a result of the chemical interactions carried out by them, proteins and fats are hydrolyzed and as a result spoilage occurs, which often occurs with an undesirable change in taste and smell and ultimately a decrease in the quality of the food. Consumption of such foods causes various poisonings and complications (mostly digestive) in the consumer. In order to prevent such problems, the industry uses preservatives, most of which are chemical in nature and harmful (to the health of consumers). Therefore, the use of compounds such as bioactive peptides for this purpose, which are natural in nature and, in addition to increasing the shelf life of food (by inhibiting the growth of microbes and controlling the oxidation of fats), also exert health-promoting properties on the body [6, 8-10], seems important and largely necessary.

Marine resources used to produce hydrolyzed proteins (bioactive peptides) must be abundant, cheap, and have very low marketability in order to make the product economically viable. For this reason, the use of wastes as well as aquatic organisms with very low marketability are a priority. The large family of lanternfishes or myctophytes[7]About 70% of mesopelagic fish constitute the open waters. In fact, lanternfishes are among the fish that have the highest distribution, population and diversity among all vertebrates; so that their biomass is estimated at 550 to 650 million tons. Various species of this family have a huge and untouched (virgin) biomass in the Iranian waters of the Oman Sea; so that they occupy 90% of the fish of this sea and are considered as bycatch[16]Among the different species of lanternfish, the dominant species is*Betosoma petrotum*[8]It is known as lanternfish and the largest stock of the Myctophyte family in the Oman Sea and East China Sea belongs to this species. The lanternfish family, despite being rich in high-quality protein and abundant minerals,[17]Due to the high levels of ascorbic acid in their fat and their very small size (1 to 5 cm), they are often not edible for humans (in fact, less than 10 of the 249 species of lanternfish are edible for humans). Their high nutritional value, lack of direct human consumption, high abundance, and of course their cheapness and easy access have all made the lanternfish family a suitable and desirable source for the production of fishmeal and the provision of food for livestock, poultry, and aquatic animals.[18]Technological advances in the field of aquatic processing have made it possible to use these fish species to produce bioactive compounds with key applications in the food and pharmaceutical industries, so that their proteins can also enter the human diet.[17].

The aim of the present study is to produce bioactive peptides from lanternfish by enzymatic method (Protamax) and then nanoencapsulate these peptides using freeze-drying method and combined coating of maltodextrin and gum arabic. In the next step, the antibacterial activity of free form peptides and carrier nanocapsules against Gram-positive and Gram-negative species causing food spoilage will be evaluated (and compared).

2-Materials and methods

2.1- Preparation of substrate, enzyme and bacteria

1- *Listeria monocytogenes*
2 -*Staphylococcus aureus*
3- *Clostridium perfringens*
4 -*Bacillus cereus*
5- *Escherichia coli*
6 -*Salmonella typhoid fever*
7 -Myctophidae
8 -*Benthosema pterotum*

Lanternfish were purchased from local fishermen in Bandar Abbas after the trawler docked. The fish were then packed in a 5 kg box containing ice and transported by plane to Tehran as soon as possible and then to the laboratory, where they were kept in a freezer at -18°C until the start of the experiments. The enzyme used in this study was the (commercial) microbial enzyme Protamax extracted from bacteria.*Bacillus subtilis*[9]It was from the Novozyme company representative.[10]Denmark and stored at 4°C until the start of the experiment. In this study, gram-positive bacteria including*Listeria monocytogenes*(ATCC 7644)، *Staphylococcus aureus*(PTCC 1112)، *Clostridium Perfringens*(PTCC 1766)، *Bacillus Cereus* (PTCC 1247) and gram-negative*Escherichia coli*(PTCC 1330) and*Salmonella Typhimurium*(PTCC 1609) were purchased from the Scientific and Industrial Research Organization of Iran.

2.2- Production of hydrolyzed protein

In order to prepare the substrate, a certain amount of lanternfish was removed from the freezer and thawed by placing it in a refrigerator (4±1°C) for 24 hours. Then, the sample was washed with cold water and finally ground completely. In order to initiate the hydrolysis reaction,First, 100 grams of sample (spiny lanternfish) was placed in a 500 ml Erlenmeyer flask. Then 200 ml of phosphate buffer with 7.4% pH was added.pH was added to the Erlenmeyer flask. In the next step, the Erlenmeyer flask was placed in a water bath at 85°C for 20 minutes to inactivate the internal enzymes of the tissue. After this time, the Erlenmeyer flask was allowed to cool to room temperature. In the next step, Protamax enzyme (activity level: 1.5 Anson units per milliliter of enzyme) was added to the corresponding Erlenmeyer flask in an amount of 30 Anson units (per kilogram of protein substrate). The Erlenmeyer flask was immediately transferred to a shaking incubator at 50°C and incubated for 90 minutes in these conditions to complete the protein hydrolysis process. After these times, in order to stop the hydrolysis reaction, the Erlenmeyer flask was placed at 95°C for 15 minutes. After this time and the Erlenmeyer flask was cooled, the contents of the Erlenmeyer flask were stirred at 10°C for 20 minutes with a rotation ofg8000 centrifuge and the supernatant was dried using a freeze dryer [19 and 20]. It should be noted that the hydrolysis conditions (temperature, time, enzyme amount andpH) Optimization and then optimal limits were considered.

2.3- Degree of hydrolysis and protein recovery process

After the hydrolysis process is complete, the trichloroacetic acid solution[11](Merck, Germany) 20% was added to the supernatant in equal proportions and the resulting solution was centrifuged.g6700 was centrifuged at 4°C for 20 minutes. Then, the nitrogen in the new supernatant was measured by the biuret method. Finally, the degree of hydrolysis was calculated as a percentage from the ratio of nitrogen in 10% trichloroacetic acid solution to total nitrogen in the sample.. Protein recovery was obtained as a percentage using the ratio [grams of hydrolyzed protein × amount of hydrolyzed protein nitrogen] over [grams of substrate × amount of substrate nitrogen] [21].

2.4- Approximate analysis of substrate and hydrolyzed powders

To determine the moisture content, 1 gram of the sample was heated at 105°C (oven,Fanazma like, BM 55() was dried until constant weight was reached. The moisture content of the sample was calculated based on the difference between the initial weight and the secondary weight. After placing the sample in an oven (FM.2.8) At a temperature of 600 degrees Celsius and the difference in weight, the ash content was determined. The Kjeldahl and Soxhlet extraction methods were used to evaluate the fat and nitrogen content, respectively. The crude protein of

9- *Bacillus subtilis*
10- Novozyme
11 -TCA

the sample was measured by a factor of 6.25 in the nitrogen content. [22].

2.5- Isolation of peptides

In order to separate peptides from ultrafiltration with two Amicon filters of different molecular weights (MWCO), 3 and 30 kDa were used. Then, three fractions with molecular weights less than 3 kDa, 3 to 30 kDa and more than 30 kDa were separated. First, the crude hydrolyzed protein was filtered using an Amicon 30 kDa filter at room temperature for 10 minutes at a speed ofg7500 centrifuges (Sigma، at16-2, Spain). Then, the hydrolyzed protein <30 kDa was re-filtered using an Amicon 3 kDa filter at room temperature for 20 minutes at a speed ofgThe supernatant was centrifuged at 7500 rpm. Thus, peptides less than 3 kDa, between 3 and 30 kDa, and greater than 30 kDa were separated [23]. Since, based on the pre-test results, peptides with a molecular weight of less than 3 kDa showed better performance than the other two weights, this weight was chosen for further operations.

2-6- Nanoencapsulation of bioactive peptides

For nanoencapsulation of bioactive peptides, a combined coating of maltodextrin (Sigma, Germany) and gum arabic (Sigma, Germany) was used.The ratio was 1:1 and the ratio of coatings to core was 4:1. First, a homogeneous suspension of maltodextrin in distilled water (4 g/50 ml) was prepared. Then, to prepare the second coating, a suspension ofGum ArabicA homogeneous suspension was obtained by preparing it in distilled water (4 g/50 ml) and placing it on a magnetic stirrer at 45°C for 30 minutes. The solution containingGum Arabic(after reducing the temperature to 25 degrees) was added to the solution containing maltodextrin and kept at 4 degrees for 24 hours to increase water absorption. In the final stage, 2 grams of bioactive peptides (with a molecular weight of less than 3 kilodaltons) were added to the solution containing the coatings and after dissolving, an ultrasound device was used to produce nanocapsules.(Hilscher, UP200, Germany)With a wavelength of 40 kHz for 15 minutes and 6 cycles (each cycle time 30 seconds and a rest time of 15 seconds between cycles,Temperature 25 degrees Celsius) was used. To further reduce the particle size and increase the efficiency of nanoencapsulated particles, a high-speed homogenizer (Ultratorax,Ika, Italy) at 10,000 rpm for 15 minutes. The resulting solution was frozen at -18°C and dried using a freeze dryer.(Vaco 2 Zirbus, Germany)It was dried at a pressure of 0.051 mbar and a temperature of -50°C [24-26].

2-7- Evaluation of physical properties of nanocapsules

Average particle size and particle size distribution indices After diluting the samples 10-fold with phosphate buffered saline[12]By dynamic light scattering method[13]Using a Zetasizer device (NanoSizer, 3000, made in England, companyMalvern, 90 degree angle and special cell with a width of 0.01 m) were measured. In order to measure the zeta potential of the surface of the nanocapsules, a zeta sizer device was used. In this method, the nanocapsules were dissolved using 50 mM phosphate buffer (= 7.4).pH) were diluted 10 times. Zeta potential evaluation at an angle of 173°C and a wavelength of 633 nm of helium-Tungsten (at 25°C) was performed[13]. The efficiency of the nano-encapsulation process was also determined using the method.Yan et al. (2014)It was determined [25].

2-8- Cultivation conditions of the bacteria used

Culture medium used to prepare bacteria, bovine brain and heart infusion broth[14](Biolab India). After the initial bacterial culture, the samples were incubated at two temperatures of 37 and 30°C for Gram-positive bacteria (*Listeria*

12 -PBS

13- Dynamic Light Scattering

14 -Brain Heart Infusion Broth

monocytogenes،*Staphylococcus aureus*، *Clostridium Perfringens* and*Bacillus Cereus*) and gram-negative (*Escherichia coli*and*Salmonella Typhimurium*) were incubated for 18 hours. In the next step, to obtain bacterial sediment, the culture media containing the bacterial suspension were centrifuged at 5000 rpm for 15 minutes, and after removing the supernatant and adding phosphate buffer to the remaining sediment, the centrifugation process was repeated two more times. In each step, phosphate buffer was used to remove the effects of the initial broth medium. Then, some phosphate buffer was added to the remaining sediment and collected with a standard 0.5 McFarland tube (equivalent to $10^8\times$ 1.5 colony forming units per milliliter[15]) were compared. Finally, by diluting the samples, a dilution of $10^6\times1$ was used for subsequent experiments [26-28].

2-9- Evaluation of antimicrobial activity of bioactive peptides and carrier nanocapsules

2-9-1- Agar medium diffusion method

In this method, first, a suspension of bacteria is plated on Mueller Hinton agar culture medium.[16] (Sahand Chemical Research Institute) was cultured. In the next step, 6 mm diameter wells were placed on the surface of the medium and 50 ml of concentrations of 30, 60, 90, 120 and 150 mg/ml were added.Bioactive peptides and their carrier nanocapsulesThe samples were then incubated at 35 and 30°C for 24 hours. After incubation, the diameter of the zone of no growth around the well was measured using a vernier caliper with an accuracy of 0.2 mm and reported in millimeters. From the antibiotic doxycycline[17]30 mg (antibody Teb) was used as a positive control.[26-28].

2-9-2- Dilution method in the tube[18]

In this method, the minimum inhibitor concentration[19]and the minimum bactericidal concentration[20] Bioactive peptides and their carrier nanocapsules It was determined using dilutions in test tubes. The dilutions used of bioactive peptides and their carrier nanocapsules, 50, 100, 200, 400 and 500 µg/mg were added to the test tube along with one milliliter of bovine heart and brain infusion broth medium (Biolab India). In the next step, 100 µl of 24-hour culture of the indicator bacteria were added to the tubes and incubated at 30 and 37°C. To examine the resulting changes, the optical absorbance of the samples was read at a wavelength of 600 nm. Distilled water and doxycycline were also considered as negative and positive controls. The concentration of the microbial suspension that showed the lowest optical absorbance was used asMinimum inhibitor concentrationAnd the concentration of the suspension that did not produce colony growth in the culture medium after 24 hours of cultivation was also determined as the minimum bactericidal concentration. [26-28].

2-10- Statistical analysis

The present study was conducted in a completely randomized design and one-way analysis of variance was used to analyze the data.[21]and Duncan test (at 95% confidence level) were used. Statistical software was used.$SPSS_{22}$For data analysis and softwareExcel (Version 16) It was used to draw tables and figures.

3-Results

3-1- Characteristics of the hydrolysis process and the chemical composition of the substrate and hydrolyzed powder

Table 1 presents information on the hydrolysis process and the chemical composition of the substrate and hydrolyzed proteins. According to this table, the hydrolyzed powder contained about 93%

15- CFU/ml
16 -Muller-Hinton Agar
17 -Doxycycline
18- Microdilution test
19- Minimal Inhibitory Concentration (MIC)
20- Minimal Bactericidal Concentration (MBC)
21- One way Anova

protein and less than 0.3% fat..Also, the degree of hydrolysis of the process[22] (DH) 86/1±53/21 and protein recovery[23] (PR) 24/1±92.67 percent was recorded.

Table 1. Analysis of the hydrolysis process, substrate and fish protein hydrolysate (%)

	Protein	Fat	Moisture	Ash	DH	PR
Substrate	21.04±0.71	2.96±0.57	66.32±1.43	7.89±0.68		
Fish Protein Hydrolysate	93.21±1.46	0.23±0.03	0.65±0.1	3.7±0.09	21.53±1.86	92.67±1.24

3.2- Physical properties of nanocapsules

The physical properties of nanocapsules carrying bioactive peptides with a combined maltodextrin-gum arabic coating are presented in Table 2. According to this table, the average size of the nanocapsules, their dispersion index and zeta potential are about 293.6 nm, 0.398 and +41.26 mV, respectively. Also, the efficiency of the nanoencapsulation process is 3.05.±89.12 percent was recorded.

Table 2- Physical properties of nanocapsules carrying bioactive peptides coated by maltodextrin- gum arabic

Physical properties	Level
Average particle size (nm)	293.6±2.83
particle dispersity index	0.398±0.06
Zeta potential (mv)	+41.26±1.53
Encapsulation efficiency (%)	89.12±3.05

3-3- Measuring the antibacterial activity of bioactive peptides and their carrier nanocapsules

3-3-1- Diameter of the growth zone

In Table 3, the antibacterial activity of free peptides and carrier nanocapsules (at 5 concentrations and two times) against Gram-positive bacteria (*Listeria monocytogenes*، *Staphylococcus aureus*، *Clostridium perfringens* and*Bacillus cereus*) from the diffusion method in agar-containing culture medium is presented as the diameter of the zone of inhibition (millimeters). According to this table, the values of the diameter of the zone of inhibition of bacteria against carrier nanocapsules are greater than those of free peptides (0.05).>pAlso, after 60 days of storing the nanoencapsulated form of peptides (carrier nanocapsules) at 4°C (refrigerator) and performing the antibacterial test again, the diameter values of the bacterial growth inhibition zone against them did not change compared to day zero and remained constant (0.05).<pHowever, by keeping the free peptides at the same temperature and time and repeating the antibacterial test, the diameters of the bacterial inhibition zone against them were significantly reduced (P < 0.05).>pWith increasing concentration of peptides and their carrier nanocapsules, the values of the diameter of the growth inhibition zone of all four Gram-positive bacteria increased significantly (P < 0.05).>p). Of course, there were two exceptions to this; the values of the diameter of the non-growth zone*Clostridium perfringens* By increasing the concentration from 120 to 150 µg/ml and also the values of the diameter of the zone of inhibition*Bacillus cereus*There was no significant change

22 -Degree of Hydrolysis

23- Protein Recovery

(0.05) with increasing concentration from 90 to 120 µg/ml.<pAccording to Table 3, bacteria*Clostridium perfringens* It was resistant to three concentrations of 30, 60 and 90 µg/ml of free peptides and their carrier nanocapsules (R); Meanwhile, this situation is for two bacteria*Staphylococcus aureus*and *Bacillus cereus*It was also recorded at concentrations of 30 and 60 µg/ml. However, such resistance was not observed for the bacteria.*Listeria monocytogenes*It was only present at a concentration of 30 µg/ml. Among the four Gram-positive bacteria studied, the overall (average) highest and lowest values of the diameter of the zone of inhibition were respectively associated with*Listeria monocytogenes*and*Clostridium perfringens* were (0.05)>p).

Table 3- Diameter of non-growth zone of Gram positive bacteria against bioactive peptides and nanocapsules carrying them on days 0 and 60 of storage at refrigerator temperature (mm)

Strain	Time (day)	T	Concentrations (µg/ml)					Positive control
			30	60	90	120	150	Doxycycline
L. monocytogenes	0	F	R	6.73±0.18^{Db}	10.32±0.01^{Cc}	15.43±0.29^{Bb}	21.64±0.42^{Ab}	44.53±1.82
		N	R	8.94±0.34^{And}	14.15±1.05^{That}	18.41±1.54^{Not}	24.68±1.12^{Aa}	
	60	F	R	5.11±0.16^{Dc}	9.51±0.08^{Cd}	12.26±0.77^{Bc}	17.51±0.01^{Ad}	
		N	R	8.84±0.92^{And}	14.08±1.46^{That}	18.29±1.31^{Not}	24.51±1.83^{Aa}	
Staph. aureus	0	F	R	R	6.15±0.07^{Cf}	9.09±0.16^{Bd}	12.84±0.11^{At}	35.69±1.34
		N	R	R	7.49±0.24^{This}	12.17±0.1^{Bc}	14.24±0.26^{Of}	
	60	F	R	R	5.26±0.09^{Cg}	7.23±0.08^{Bf}	10.11±0.05^{Ah}	
		N	R	R	7.5±0.32^{This}	12.14±0.15^{Bc}	14.18±0.44^{Of}	
C. perfringens	0	F	R	R	R	6.14±0.38^{At}	6.32±0.11^{Also}	30.72±0.98
		N	R	R	R	8.3±0.42^{But}	8.38±0.23^{Eat}	
	60	F	R	R	R	5.19±0.27^{Ah}	5.26±0.21^{And}	
		N	R	R	R	8.31±0.19^{But}	8.42±0.15^{Eat}	
Bacillus cereus	0	F	R	R	9.54±0.12^{Bd}	9.46±0.1^{Bd}	15.28±0.07^{But}	39.25±1.12
		N	R	R	12.09±0.28^{Bb}	12.21±0.32^{Bc}	18.97±0.29^{And}	
	60	F	R	R	7.41±0.15^{Be}	7.19±0.34^{Bf}	12.84±0.22^{At}	
		N	R	R	12.1±0.31^{Bb}	12.2±0.22^{Bc}	19.02±0.36^{And}	

- T: Treatments, F: Free bioactive peptides, N: Nanoencapsules carrying bioactive peptides, R: Resistant
- The different lowercase and uppercase letters in each column and row indicate significant difference between the data, respectively (p<0.05).

The diameter values of the inhibition zone of gram-negative bacteria (*Escherichia coli and Salmonella Typhimurium*) against free peptides and their carrier nanocapsules (at five concentrations and two times) are shown in Table 4. As can be seen in this table, with nanoencapsulation of bioactive peptides and increasing concentration, the values of the diameter of the growth inhibition zone*Escherichia coli*and*Salmonella Typhimurium*increased significantly (0.05)>pOf course, the values of the diameter of the non-growth zone*Salmonella Typhimurium*There was no significant difference between the bioactive peptides and their carrier nanocapsules at two concentrations of 90 and 120 µg/ml (P<0.05).<pAfter 60 days of storage of the carrier nanocapsules at 4°C, their ability to create the diameter of the inhibition zone of both gram-negative bacteria did not change (consistency of the diameter of the inhibition zone) (0.05).<pHowever, this ability in free peptides decreased significantly with storage time (p<0.05).>p) which appeared as a decrease in the diameter of the zone of bacterial inhibition. According to Table 4,*Salmonella Typhimurium*at concentrations

of 30 and 60 micrograms/ml and*Escherichia coli*It was resistant to all concentrations studied except 150 µg/mL of bioactive peptides and their carrier nanocapsules.

Table 4- Diameter of non-growth zone of Gram negative bacteria against bioactive peptides and nanocapsules carrying them on days 0 and 60 of storage at refrigerator temperature (mm)

Strain	Time (day)	T	Concentrations (µg/ml)					Positive control
			30	60	90	120	150	Doxycycline
E. coli	0	F	R	R	R	R	6.85±0.02^{d}	27.36±1.64
		N	R	R	R	R	7.57±0.16^{c}	
	60	F	R	R	R	R	5.87±0.06^{and}	
		N	R	R	R	R	7.52±0.11^{c}	
S. typhimurium	0	F	R	R	6.52±0.42^{Bb}	6.54±0.18^{Bb}	9.25±0.44^{Ab}	32.47±1.28
		N	R	R	8.25±0.28^{Not}	8.23±0.31^{Not}	11.37±0.43^{A} ^{a}	
	60	F	R	R	5.55±0.36^{Bc}	5.48±0.16^{Bc}	7.56±0.12^{And}	
		N	R	R	8.21±0.22^{Not}	8.25±0.24^{Not}	11.28±0.58^{A} ^{a}	

- T: Treatments, F: Free bioactive peptides, N: Nanoencapsules carrying bioactive peptides, R: Resistant
- The different lowercase and uppercase letters in each column and row indicate significant difference between the data, respectively ($p<0.05$).

3-3-2- Minimum inhibitory concentration and minimum lethal concentration

Table 5 shows the minimum inhibitory concentration and minimum lethal concentration of free and nano-encapsulated peptides against gram-positive and gram-negative bacteria. As can be seen in this table, the minimum inhibitory concentration of bioactive peptides (free and nano) for five bacterial species ranged from 50 to 500 µg/mL. This level was recorded for the minimum lethal concentration from 100 to 500 µg/mL (and more than 500 µg/mL). It was further determined that the minimum inhibitory concentration and minimum lethal concentration of the nano-encapsulated form of peptides for all studied bacteria were lower than the free peptides. Also, after 60 days of storing the nanocapsules carrying bioactive peptides at refrigerated temperature and performing the antibacterial test again, the minimum inhibitory concentration and minimum lethal concentration values for the studied bacteria did not change and remained constant. But these values in the free form of bioactive peptides (after 60 days of storage at refrigerator temperature) were not significant for all bacteria except*Bacillus cereus*and*Salmonella Typhimurium*Increase They found.Among the bacteria studied, the lowest values of minimum inhibitory concentration and minimum lethal concentration were related to*Listeria monocytogenes*As can be seen in Table 5, free peptides at day 60 and a concentration of 500 µg/mL were able to inhibit bacteria.*Clostridium perfringens*and*Escherichia coli*They were unable to eliminate these bacteria. However, the nanoencapsulated form of these peptides (carrier nanocapsules) was able to kill the aforementioned bacteria at the same concentration.

Table 5- MIC and MBC of bioactive peptides and nanocapsules carrying them against six strain of bacteria on days 0 and 60 of storage at refrigerator temperature (µg/ml)

Strain	Time (day)	Form of peptides	MIC	MBC
L. monocytogenes	0	Free	100	200
		Nanoencapsulated	50	100
	60	Free	200	400
		Nanoencapsulated	50	100
Staph. aureus	0	Free	200	400
		Nanoencapsulated	100	200
	60	Free	400	500
		Nanoencapsulated	100	200
C.perfringens	0	Free	400	500
		Nanoencapsulated	200	400
	60	Free	500	-
		Nanoencapsulated	200	500
Bacillus cereus	0	Free	200	400
		Nanoencapsulated	100	200
	60	Free	200	400
		Nanoencapsulated	100	200
E. coli	0	Free	400	500
		Nanoencapsulated	200	400
	60	Free	500	-
		Nanoencapsulated	200	500
S. typhimurium	0	Free	400	500
		Nanoencapsulated	200	400
	60	Free	400	500
		Nanoencapsulated	200	400

4-Discussion

The peptides produced in the present study and their carrier nanocapsules were able to inhibit the growth and kill bacteria that cause food spoilage. The antibacterial activity of bioactive peptides obtained from enzymatic hydrolysis of aquatic animals and their waste has been confirmed in several studies. Research resultsThey et al. (2014) showed that hydrolyzed peptides from fish muscle*Barbus callensis*Has antibacterial activity against gram-positive bacteria including*Staphylococcus aureus*، *Listeria monocytogenes*، *Bacillus subtilis*، *Enterococcus faecalis* and*Micrococcus luteus* and gram negative*Pseudomonas aeruginosa*، *Escherichia coli*، *Klebsiella pneumonia* and*Salmonella enterica* were [29].In the research of Yeganeh and Reyhani-Pool (1400), peptides obtained from enzymatic hydrolysis of shrimp waste and their carrier nanoliposomes were able to inhibit the growth of bacteria.*Staphylococcus aureus*، *Bacillus cereus* and*Escherichia coli* They showed [30]In a study that examined the intestines and viscera of a fish,[24]Using Alcalase and Flowerzyme at three times of hydrolysis of 10, 20 and 30, the antibacterial properties of the produced proteins were evaluated. It was determined that these proteins had the ability to inhibit the growth and kill bacteria.*Escherichia coli* and *Staphylococcus aureus* particle for direct object and protein hydrolyzed by alcalase was more effective in this regard in 30 minutes [31]. Of course, not all bioactive peptides (hydrolyzed proteins) with every characteristic and under every condition exhibit antibacterial activity; rather, depending on the bacterial species tested (whether it is gram-negative or gram-positive), the type of peptide source (whether

24- *Liza ash*

it is a plant or animal peptide, from animal tissue or its waste, whether it is an animal or plant species, etc.), the concentration used [23], the characteristics of the peptides including the degree of hydrolysis [31], molecular weight [32], the composition and sequence of amino acids [33], and also the hydrolysis reaction conditions including the type of enzyme used, temperature, time, pH, enzyme to substrate ratio [31], etc., the presence or absence of this property and its level in bioactive peptides vary. A study that investigated the antibacterial properties of bioactive peptides produced from rainbow trout skin[25](with a molecular weight of less than 3 kDa) using two enzymes, alcalase and flowerzyme, were investigated (using disk diffusion and tube dilution methods), and reported that these peptides had the ability to inhibit the growth of bacteria.*Staphylococcus aureus* and*Escherichia coli*[23]. In another study, bioactive peptides produced from dogfish skin[26]Using the enzymes chymotrypsin, trypsin, and pepsin, it is able to inhibit the growth of bacteria.*Escherichia coli*،*Lactococcus lactis*and*Bacillus subtilis* Bioactive peptides produced from oysters at hydrolysis degrees of 30.12, 32.69 and 38.47% and concentrations of 0.25, 0.5, 1, 2, 3 and 4 mg/ml had no inhibitory effect on the growth of bacteria.*Pseudomonas aeruginosa*، *Staphylococcus aureus*، *Listeria monocytogenes* and*Bacillus cereus* However, by increasing the degree of hydrolysis and the concentrations used, the peptides were able to inhibit the growth of the aforementioned bacteria [35]. In the studyGhelichi et al. (2018), the antibacterial activity of proteins obtained from the hydrolysis of lyophilized common carp eggs increased with increasing hydrolysis time from 30 to 120 minutes, but these proteins were unable to inhibit the growth of*Listeria monocytogenes*، *Enterococcus faecalis*، *Pseudomonas aeruginosa*، *Klebsiella pneumonia* and *Salmonella enterica* They were not [36].

The predominant common feature of antibacterial peptides is the presence of a secondary alpha-helix structure, which can destroy the cell, possibly due to its location in the ion channel of the bacterial membrane and the change in the permeability of this structure.[23]In general and in summary, the possibilities that are raised in the context of the antibacterial mechanism of hydrolyzed proteins (bioactive peptides) are: a) The peptides bind to the bacterial cell membrane and create irreversible pores on the membrane surface, as a result of which the cytoplasm flows out of the cell and the cell is completely destroyed. b) The peptides cross the cell membrane and bind to intracellular organelles such as the ribosome and genome, disrupting the normal growth process of the cell.[37].

In this study, the nanoencapsulation process, in addition to increasing the antibacterial activity (in both methods studied) of bioactive peptides, also led to the stability of this activity during 60 days of storage at refrigerator temperature. This means that nanocapsules carrying bioactive peptides with a combined maltodextrin-gum arabic coating had a greater ability to inhibit the growth of the studied bacteria than free peptides; while the antibacterial properties of nanocapsules, unlike free peptides, did not decrease during the storage period (the values of the diameter of the bacterial growth inhibition zone remained constant, as well asMIC andMBCNanocapsules after 60 days of storage at refrigerator temperature). The increase in antibacterial activity of bioactive peptides by performing nanoencapsulation can be related to the increase in contact surface. Also, the stability of antibacterial activity of nanocapsules (during 60 days of storage at refrigerator temperature) can probably be explained by the fact that the combined coating used (maltodextrin-gum arabic) was able to protect the core structure (bioactive peptides) against environmental conditions such as refrigerator temperature, humidity, etc. and maintain its properties. In the study of Reyhani-Pool and Yeganeh (2023), the pigment astaxanthin extracted

25 -*Oncorhynchus mykiss*

26 -*Squalus acanthias*

from microalgae*Haemococcus pluvialis*[27]Nanoencapsulated using a combined maltodextrin-sodium caseinate coating (and freeze-drying method), increased antibacterial activity and stability of this activity (during 60 days of storage at refrigerator temperature) were reported in astaxanthin.[4]In another study, it was proven that nanoencapsulation of the pigment phycocyanin using maltodextrin and sodium caseinate coatings (and freeze-drying method) can enhance the antibacterial activity of the pigment and help to stabilize this property (during 60 days of storage at refrigerated temperature).[26]The findings of the two aforementioned studies are consistent with the results of the present study. In contrast, in the study by Yeganeh and Reyhani-Pool (1400), in which bioactive peptides obtained from enzymatic hydrolysis of shrimp waste were encapsulated in nanoliposomes, it was determined that the growth inhibitory activity of*Staphylococcus aureus*، *Bacillus cereus* and*Escherichia coli* There is no significant difference in free bioactive peptides and carrier nanoliposomes.[30].

The results of the pioneering research showed that the sensitivity of gram-positive bacteria compared to gram-negative bacteria (the study subject) toBioactive peptides and especially their carrier nanocapsules are more. This higher sensitivity is probably related to the absence of a lipopolysaccharide layer in the cell wall of gram-positive bacteria. The lipopolysaccharide layer in the cell wall of gram-negative bacteria can significantly prevent the entry of bioactive peptides into the cell and neutralize their destructive effects. [23]. The greater sensitivity of Gram-positive bacteria to natural antibacterial compounds than Gram-negative bacteria has also been demonstrated in other studies [4, 26, 38].

In the present study, *Escherichia coli*It was only sensitive to a concentration of 150 µg/ml of bioactive peptides and their carrier nanocapsules. Other bacteria were also resistant to some concentrations in the agar diffusion and tube dilution methods. This resistance of bacteria to bioactive peptides and their carrier nanocapsules has various reasons that are generally divided into two categories; the first category is related to the characteristics of bacteria such as the presence of genes resistant to antibacterial compounds in the cell, the presence of a lipopolysaccharide layer in the wall of gram-negative bacteria, and cellular resistance to harsh environmental conditions. [34]. The second category is related to the physicochemical properties of bioactive peptides and their carrier nanocapsules, which can include things such as the insensitivity of bacteria to the concentrations used for bioactive peptides and their carrier nanocapsules (meaning that with increasing the concentration of peptides and nanocapsules, bacterial growth is inhibited, which was also recorded and confirmed in the present study), the non-optimal molecular weight [32] and the degree of hydrolysis [31], the absence or deficiency of some amino acids with antimicrobial properties in the peptide chain, the unsuitability of the physical properties of the carrier nanocapsules (average particle size, particle size distribution index, zeta potential, and microencapsulation efficiency) for exerting antibacterial properties, etc.

5- Conclusion

Bioactive peptides produced from lanternfish using the commercial microbial enzyme Protamax, with a degree of hydrolysis of 21.53±1.86% and a molecular weight of less than 3 kilodaltons, have the ability to inhibit the growth and proliferation of gram-positive and gram-negative bacteria that cause food spoilage. Also, nanoencapsulation of these peptides using freeze-drying and a combined maltodextrin-gum arabic coating not only increases their antibacterial activity, but also significantly contributes to the stability of this property during their storage at refrigerated

27- *Haematococcus pluvialis*

temperature. These peptides and carrier nanocapsules are more powerful in combating the growth and proliferation of gram-positive bacteria (probably due to the absence of a lipopolysaccharide layer in the wall). For this reason, their use in foods where there is a possibility of the presence of gram-negative bacteria,It should be carefully and expertly evaluated. Part of this evaluation is related to increasing the concentration of bioactive peptides and their carrier nanocapsules in food formulations. Finally, in order to use bioactive peptide-based preservatives in food formulations, their nanoencapsulated form with a double-layer coating is proposed.

6-Thank you and appreciation

This research was conducted with financial support from Sari University of Agricultural Sciences and Natural Resources under contract number 03-1404-03, for which we would like to express our gratitude.

Financing

The author declares that he has not received any funding.

Authors' Contributions

All activities were carried out by the author.

Competitive interests

The author declares that he has no financial conflicts of interest or competing interests in this study.

7-Resources

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مجله علوم و صنایع غذایی ایران

سایت مجله: www.fsct.modares.ac.ir

مقاله علمی_پژوهشی

اثر حامل ترکیبی مالتودکسترین-صمغ عربی و روش خشک‌کردن انجمادی بر فعالیت ضد باکتریایی پپتیدهای زیست‌فعال فانوس-ماهی (*Benthosema pterotum*) علیه باکتری‌های عامل فساد مواد غذایی

سهیل ریحانی پول[1*]، سکینه یگانه[2]، فرزانه شاکریان[3]، فائزه ترک پهنابی[4]، آرش رخشانی[3]

۱- دانش‌آموخته دکتری تخصصی، گروه فرآوری محصولات شیلاتی، دانشکده شیلات و محیط زیست، دانشگاه علوم کشاورزی و منابع طبیعی گرگان، گرگان، ایران

۲- استاد، گروه شیلات، دانشکده علوم دامی و شیلات، دانشگاه علوم کشاورزی و منابع طبیعی ساری، ساری، ایران

۳- دانشجوی تحصیلات تکمیلی، گروه شیلات، دانشکده علوم دامی و شیلات، دانشگاه علوم کشاورزی و منابع طبیعی ساری، ساری، ایران

۴- کارشناس آزمایشگاه، پژوهشکده اکولوژی دریای خزر، موسسه تحقیقات علوم شیلاتی کشور، سازمان تحقیقات، آموزش و ترویج کشاورزی، ساری، ایران

اطلاعات مقاله

تاریخ های مقاله :

تاریخ دریافت: ۱۴۰۴/۰۵/۲۸

تاریخ داوری: ۱۴۰۴/۱۲/۰۸

تاریخ پذیرش: ۱۴۰۳/۰۳/۰۵

کلمات کلیدی:

فانوس‌ماهی،

پپتیدهای زیست‌فعال،

نانوریزپوشانی،

صمغ عربی،

لیستریا مونوسیتوژنز،

MIC

DOI: 10.48311/fsct.2026.84087.0

* مسئول مکاتبات:

soheylreyhani@gmail.com

چکیده

طی دو دهه اخیر تحقیقات پیرامون استفاده از نگهدارنده‌های طبیعی در مواد غذایی بجای ترکیبات شیمیایی به دلیل ایمن‌بودن مورد توجه محققین قرار گرفته است. پپتیدهای زیست‌فعال یکی از ترکیبات طبیعی هستند که پتانسیل بالایی برای جلوگیری از فساد (اکسیدایتو و میکروبی) مواد غذایی دارند. هدف تحقیق حاضر نانوریزپوشانی پپتیدهای زیست‌فعال تولیدشده از فانوس‌ماهی (*Benthosema pterotum*) (با وزن مولکولی کمتر از ۳ کیلودالتون) با استفاده از روش خشک‌کردن انجمادی و پوشش ترکیبی مالتودکسترین-صمغ عربی و سپس ارزیابی فعالیت ضد باکتریایی نانوکپسول‌های حامل و پپتیدهای آزاد علیه باکتری‌های عامل فساد مواد غذایی از جمله *لیستریا مونوسیتوژنز*، *استافیلوکوکوس اورئوس*، *کلستریدیوم پرفرنژنس*، *باسیلوس سرئوس*، *اشریشیاکلی* و *سالمونلا تیفی‌موریوم* بود. مطابق نتایج، نانوریزپوشانی پپتیدهای زیست‌فعال، علاوه بر اینکه موجب افزایش فعالیت ضد باکتریایی آن‌ها شد (افزایش مقادیر قطر هاله عدم رشد باکتری‌ها و کاهش MIC و MBC)، بلکه موجب پایداری این خصوصیت طی ۶۰ روز نگهداری نانوکپسول‌های حامل در دمای ۴ درجه سانتی‌گراد گردید ($p<0/05$). افزایش غلظت پپتیدهای زیست‌فعال و نانوکپسول‌های حامل آن‌ها (از ۳۰ به ۱۵۰ میکروگرم بر میلی-لیتر) غالبا با افزایش قطر هاله عدم رشد باکتری‌ها همراه بود ($p<0/05$). در بین باکتری‌ها، بیشترین مقادیر قطر هاله عدم رشد و کمترین میزان MIC و MBC برای *لیستریا مونوسیتوژنز* ثبت شد. همچنین حساسیت باکتری‌های گرم مثبت در برابر پپتیدهای زیست‌فعال و نانوکپسول‌های حامل، بیشتر از باکتری‌های گرم منفی گزارش گردید. پپتیدهای آزاد در روز ۶۰ و غلظت ۵۰۰ میکروگرم بر میلی‌لیتر قادر به مهار باکتری‌های *کلستریدیوم پرفرنژنس* و *اشریشیاکلی* بودند اما نتوانستند این باکتری‌ها را حذف کنند. این در حالیست که فرم نانوریزپوشانی‌شده این پپتیدها (نانوکپسول‌های حامل) در همین غلظت قادر به کشندگی باکتری مذکور بودند. براساس نتایج، نانوریزپوشانی پپتیدهای زیست‌فعال با استفاده از روش خشک‌کردن انجمادی و پوشش ترکیبی مالتودکسترین-صمغ عربی موجب افزایش و پایداری فعالیت ضد باکتریایی و در نتیجه کارائی بیشتر آن‌ها در فرمولاسیون مواد غذایی می‌گردد.