EVALUATION OF THE FUNCTIONAL PROPERTIES AND ANTIOXIDANT ACTIVITY OF SHRIMP RESIDUE PROTEIN HYDROLYSATES

Murtadha Adel Hanoon[1*], Dhuha Dawood Salman[2]
[1]Department of Food Science, College of Agricultural Engineering Sciences, University of Baghdad, Baghdad, Iraq.

[2]Department of Food Science, College of Agricultural Engineering Sciences, University of Baghdad, Baghdad.

*Corresponding author's email: mortada.adel2302m@coagri.uobaghdad.edu.iq

Email addresses of coauthors: dhuha.dawood@coagri.uobaghdad.edu.iq

ABSTRACT

The Amino acid, emulsifying, foaming, and antioxidant activity of protein hydrolysates prepared by enzymatic treatment of shrimp heads and shells were studied. The yield of protein hydrolysates increased with increasing hydrolysis time by the commercial's enzymes trypsin (2000U/g) and pepsin (3000U/g) were 35.5%after 2h and 34.45% after 4h respectively. The study showed a higher nutritional value for protein hydrolyzed by trypsin compared to that treated with pepsin due to the increased essential amino acids percent in the first treatment. The hydrolysate proteins showed high solubility at pH 10, especially trypsin hydrolysate, which reached 65% for the heads and 58% for the shells. The highest emulsification and foaming percentages were for the protein hydrolysates prepared from heads treated with trypsin, and were 53%, 42% respectively. The DPPH free radical scavenging assay and the reducing power assay showed antioxidant activity for all protein hydrolysates.

Key words: shrimp residue protein hydrolysates, Emulsifying capacity, Antioxidant.

INTRODUCTION

Approximately 3.8 million tons of garbage are produced globally each year (50-60 %) of the catch are processed by the shrimp industry (1), 45 to 60 percent of the overall weight is made up of the head and shell together. While some shrimp waste is added to aquaculture feed formulations and utilized as animal feed, (1), The majority of it is eventually thrown away. In addition to being unproductive, improper disposal of this waste may has a negative effects on the ecosystem. (2). Currently, the most common way of disposal processes of shrimp waste involves burying it in landfills or dumping it into the ocean, causing changes to soil, water, and marine ecosystems.

When appropriately handled to prevent oxidation and related risk factors, aquaculture or wild fish by-products are a possible source of beneficial components including protein hydrolysates and bioactive peptides that can be applied to the production of food (3). Proteolytic

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compounds are a combinate of bioactive peptides and amino acids that are created when proteins and numerous peptides are hydrolyzed. Bioactive peptides generally content of amino acids (2-20) and typically have less than (3 kilo daltons) molecular weights (4), and Because of their special structural and functional qualities, such as solubility and water-holding capacity (WHC), oil-holding capacity (OHC), emulsification, and foaming, proteins are essential for the construction and stability of food systems. They can also be crucial in the creation of novel functional food products(5).

A class of chemical molecules known as Biologically active peptides (BPs) has many health benefits, such as immune modulation, blood pressure reduction, antimicrobial, insulin mimicry, and antioxidant. Enzymatic hydrolysis, fermentation, intestinal digestion, laboratory chemical processes and other processes can produce bioactive peptides.(6) . Peptides generally content of amino acids (2-100) residues. Their biological activity depends on their natural structure and amino acid residue sequence. Peptides encoded in their original protein sequences are primarily inactive, but chemical reagents or enzymes can change them into their active state.

One crucial technique for creating functional bioactive peptides is enzymatic hydrolysis. When compared to chemical procedures, this hydrolysis encourages productivity and quality of the product, requires mild reaction conditions, and yields minimal unwanted byproducts. (7). In addition, Enzyme type, processing duration, pH, and temperature are some of the factors that affect the biological activity for resultant protein hydrolysates, which could be enhanced through this process.(6) . Various proteases such as alkalase and protease (8), neutrase (9), papain, pepsin, and trypsin (10) have commonly utilized in the synthesis of antioxidant peptides. Additionally, bacterial and fungal proteases have been applied for producing biologically active peptides. (11). The aim for this research was to prepare effective peptide hydrolysates from shrimp residue proteins and study their functional properties and antioxidant activity.

MATERALS AND METHODS

Collecting samples and preparing specimens

Shrimp shells and heads of the Indian white shrimp (Penaeus indicus) were collected from a Baghdad local market. The samples were prepared by separating the heads and shells, washing and cleaning them to remove any remaining flesh, blood, and impurities. The waste (shells and heads) was then frozen at -18°C after packed in resalable bags.

Preparation of protein hydrolysates from shrimp residue by pepsin

The isolated protein of shrimp head and shell was prepared as described in (12) with some modifications.The protein hydrolysates of shrimp shells and heads were prepared separately according to what was mentioned in (13). 1 gram of protein isolate was mixed with 0.1 M hydrochloric acid (HCl) at a ratio of 1:20 (w/v) and incubated in a water bath at 50°C for 1 hour. The pH was then adjusted to 2 by adding 0.1 M hydrochloric acid and at a temperature of 37°C use a water bath for 10 minutes. Then the enzyme was added (3000 enzyme units per /1 gram of protein isolate).The hydrolyzed portion was withdrawn from the filtrate at different time intervals (30, 60, 90,

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120) minutes. The enzyme was inactivated by heating in a boiling water bath for 5 minutes, and the filtrate was separated by centrifugation for 15 minutes at 10,000 x g. The pH was adjusted to 7 and stored at -18°C until use.

Preparation of protein hydrolysates from shrimp residue by trypsin

the isolated protein of shrimp head and shell was prepared as described in (12) with some modifications. Enzymatic hydrolysates of isolated protein from shrimp shells and heads were prepared separately using trypsin enzyme as stated in (13). 1g of protein isolate was mixed with distilled water 1:20 (w/v) and using 0.1 M NaOH to modifed the pH to 8 .and incubated the mixture at 50°C for 1 hour in a water bath, then the mixture was placed in a water bath for 10 minutes at 37°C. The enzyme was added (2000 enzyme units per/ 1g of protein isolate). The hydrolyzed portion was withdrawn in the filtrate at different times (60, 120, 180, 240) minutes, Heating in a water bath at 95 °C for five minutes inhibited the activity of the enzyme. The process of centrifugation was carried out for 15 minutes at 10000 x g. The pH was then adjusted to 7 and the filtrate was kept at -18°C until it could be used.

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Table (1) Hydrolysis conditions for protein isolates using pepsin and trypsin.

Key	Protein isolate sample	pH	Enzyme (u/gm)	Hydrolysis time(hour)
(HP_2)	Head	2	Pepsin (3000)	2
(HT_4)	Head	8	Trypsin (2000)	4
(SP_2)	Shell	2	Pepsin (3000)	2
(ST_4)	Shell	8	Trypsin(2000)	4

Determination of Degree of Hydrolysis

The degree of protein hydrolysis of shrimp shells and heads was measured as mentioned in (14). 0.250 ml of each protein hydrolysate sample was mixed with 2 ml of sodium phosphate buffer at concentration 0.2125 M ,pH 8.2, 2 ml of 1% SDS solution, and 2 ml of 0.1% TNBS solution (2,4,6-trinitrobenzenesulfonic acid). The mixture was then incubated for 1 hour at 50°C in the absence of light . 4 ml of 0.1 M hydrochloric acid solution was then added. The tubes were incubated in a water bath at 30°C for 30 minutes. Light absorbance was measured at 340 nm using a spectrophotometer. A 55 mM leucine solution was used to prepare the standard curve, and the following formula was used to determine the degree of hydrolysis:

$$\mathbf{DH\%} = [(\boldsymbol{L_t} - \boldsymbol{L_0})/(\boldsymbol{L_{MAX}} - \boldsymbol{L_0})] \times \mathbf{100}$$

Where:

LMAX: The amount of free amino acids after acid hydrolysis using 6M hydrochloric acid (HCl) at 120°C for 24 hour.

L0: The amino acid content at the time zero

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Lt: Amino acid content at the times studied

Amino acids content

Amino acids were characterized according to (15) for shrimp shell and head protein hydrolysates using a High Performance Liquid Chromatography (HPLC) Solvent Delivery System 2100 manufactured by SYKAM, Germany. 0.2 g of samples were digested in 12 ml of 6N hydrochloric acid (HCl) with 0.1% phenol at 110°C for 24 hours. Then, they were cooled in an iced water bath to room temperature, and the samples were filtered using Whatman No. 0.8 filter paper, and the filtrate was washed with distilled water. After that, they were concentrated using a rotary evaporator at 50°C. Washing and evaporation were repeated twice, the pH was adjusted to 2.2 using 1M NaOH, then was added (0.02M) HCl 3.5 ml, the solution was injected into the HPLC and detected at 338 nm under the following separation conditions: sample volume 5 µL, temperature 40°C, addition of O-Phthaldialdehyde (OPA) reagent, which was prepared by mixing 25 ml of 100 mmol Proax solution with 2.5 ml of 20% SDS solution and 0.04 g of O-Phthaldialdehyde were dissolved in methanol 1 ml with ß-mercaptoethanol 100 µL and distilled water was added to bring the amount up to 50 ml , and For comparison, standard amino acids were utilized.

Solubility of protein hydrolysates at different pH

The solubility of the samples was determined according to the method reported by (16, 17) with several adjustments. 100 mg of protein hydrolysates were in 20 ml of distilled water, and 0.5 M sodium hydroxide (NaOH) or 0.5 M hydrochloric acid (HCl) were added until the pH reaches to 2, 4, 6, 7, 8, 10, and 12. After 30 minutes of stirring at room temperature using a magnetic stirrer, the samples were centrifuged at 10,000 x g for 20 minutes. The Bradford method was used to measure the amount in the filtrate, and the percentage solubility was estimated according to the following equation:

$$PSI(\%) = \frac{P_1}{P_0} \times 100$$

Where:

PSI: solubility index.

P1: content in the filtrate.

P0: content in the original sample.

Emulsifying Properties

The method indicated by (18, 19) was adopted with several adjustments for measuring the emulsifying property (EA) and emulsion stability (ES). 5 ml of protein hydrolysates (10 mg/ml) were mixed with 5 ml of oil and homogenized using a homogenizer at 10,000 x g for 1 minute. Centrifugation was performed for 5 minutes at 2,000 x g. The mixture (10 mg/ml) was prepared at different pH from 2 to 10. The volume of the emulsified layer and the total contents of the tube were measured. The emulsifying property (EA) was calculated as follows:

$$EA(\%) = \frac{H_1}{H_0} \times 100$$

Where:

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EA (%): emulsifier activity.

H1: volume of the emulsified layer in the tube.

H0: total volume.

The stability of the emulsions (ES) was determined at temperatures (80)°C for 30 minutes and PH 10 before centrifugation at 2000 x g for 5 minutes. The volume of the emulsified layer was measured using a graduated cylinder. The stability of the emulsion was calculated as follows:

$$ES(\%) = \frac{H_{1After}}{H_{0Befor}} \times 100$$

Where:

H1 After: Volume of the emulsion layer in the tube after heating.

H0 Before: Total volume before heating.

Foaming Properties

Foam capacity and foam stability were calculated according to (20). 0.25 g of protein hydrolysates were mixed with 25 ml of distilled water. Homogenized at 7000 x g for 2 minutes, then placed in a 100 ml graduated cylinder. The foam volume before and after homogenization was recorded, and the foam capacity was estimated according to the following equation:

$$FC(\%) = \frac{V_1 - V_0}{V_0} \times 100$$

Where:

FC(%): Foam capacity.

V1: Total volume after homogenization.

V0: Total volume before homogenization.

For foam stability, the foam volume was measured after being left for periods of (5, 15, 30) minutes, and then the following equation was applied:

$$FS(\%) = \frac{F_t}{F_0} \times 100$$

Where:

FS(%): Foam stability.

F0: Foam volume at time zero.

Ft: Foam volume at a specific time.

Antioxidant Activity

Determination of Free Radical Scavenging Activity (DPPH)

The free radical scavenging activity of protein hydrolysates was determined using the method previously used (21) with several adjustments. 1 ml of 0.1 mM DPPH solution dissolved in 95% ethanol mixed with 1 ml of sample (2 mg/ml). The mixture was left away from light at room temperature for 30 minutes and centrifuged at 10,000 x g for 5 minutes. The absorbance of the samples was measured at a wavelength of 517 nm. Butylated hydroxyl toluene (BHT) was used as a comparative sample at a concentration of 0.1 mg/ml.

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The following formula was used to determine the samples' percentage of free radical scavenging capacity.:

RSI % = Control - sample / Control ×100

Where:

Sample: measurement of the sample's optical absorbance at a wavelength of 517 nm.

Control: measurement of the blank optical absorbance at a wavelength of 517 nm (2 ml of DPPH solution + 1 ml of distilled water).

Estimation of reducing power:

The method described by (22) was followed with some modifications to estimate the reducing power of proteins hydrolysates. 1 ml of the sample was taken at a concentration of (20 mg/ml) and mixed with 2.5 ml of sodium phosphate buffer at a concentration of 0.2 and a pH of 6.6. Then, 2.5 ml of a 1% potassium ferricyanide solution was added. The mixture was mixed and incubated at 50°C for 20 minutes. Then, 1 ml of a 10% trichloroacetic acid (TCA) solution was added, followed by centrifugation at 10,000 x g for 10 minutes. 2.5 ml of the filtrate was withdrawn, and 2.5 ml of distilled water was added, followed by 0.3 ml of a 1% ferric chloride (FeCl3) solution. The optical absorbance of the samples was measured at a wavelength of 700 nm.

Butylated hydroxyl toluene (BHT) at a concentration of 0.3 mg/ml was used as a comparison sample, and the same workflow was performed. The higher the optical absorbance value, the greater the reducing power.

RESULTS

Degree of protein hydrolysis:

The figure(1,2) shows significant differences at the level of ≤ 0.05 in the degree of pepsin enzymatic hydrolysis of the protein isolate from (head and shell) shrimp with increasing reaction time, ranging from 22.08% - 36.6% and 17.47% - 34.45% respectively. While the degree of trypsin enzymatic hydrolysis was 23.86% - 36.71% and 21.26% - 35.50% respectively. It was also found that the degree of hydrolysis increases with increasing time. However, no significant difference was observed between the degree of head and shell hydrolysates, Except for pepsin hydrolysis at 30 minutes. Shrimp head hydrolysates showed higher degrees of hydrolysis compared to shrimp shell hydrolysates when using pepsin and trypsin enzymes. This is attributed to differences in protein structure and the nature of the dominant amino acids. These results indicate a higher percentage of the amino acids targeted by pepsin (tyrosine, leucine and phenylalanine) and trypsin (arginine and lysine) in head proteins compared to shell proteins.
(23, 24).

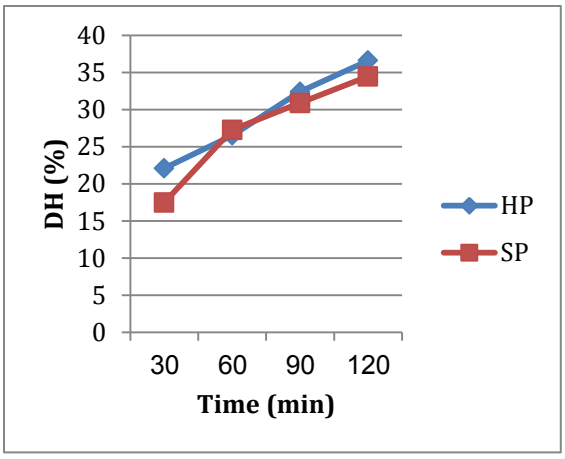
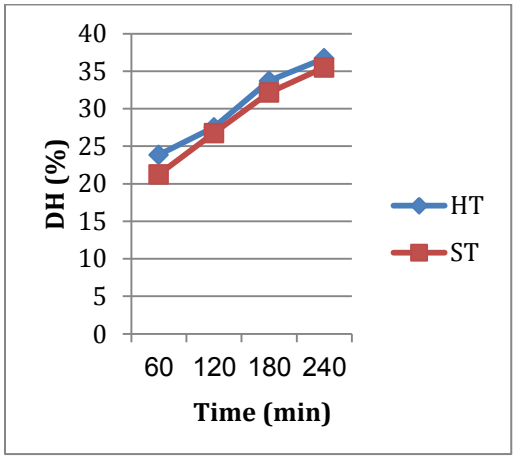


Figure (1) the degree of hydrolysis over time, (HP) hydrolyzed head protein using pepsin, (SP)hydrolyzed shell protein using pepsin



(2) the degree of hydrolysis over time,(HT) hydrolyzed head protein using trypsin, (ST)hydrolyzed shell protein using trypsin **Figure**

Amino acid content

Table (2) showed the amino acid content in protein hydrolysat by pepsin and trypsin the result showed differences between the samples. The head hydrolysates by pepsin and trypsin (HP_2 and HT_4) showed higher polar amino acid content compared to the shell hydrolysates (SP_2 and ST_4,) were 7873.234 ppm and 7783.508 ppm for the heads 4457.966 and ppm , 4514.958 ppm for the shells, respectively. For non-polar amino acids, the values were 1846.684 ppm and 1815.157 ppm for the heads compared to 891.771 ppm and 933.561 ppm for the shell , while essential amino acids were 4174.193 ppm and 4109.957 ppm for the heads and 2580.513ppm , 2633.198 ppm for the shells, respectively. This result reflecting The head proteins have higher concentrations of all three types of amino acids.The results indicate a higher content of amino acids in the hydrolyzed proteins from the head compared to the shell, whether treated with trypsin or pepsin. This may be due to the greater number of sites broken down by these enzymes Therefore, the shrimp heads can be utilized to produce amino acid-rich protein hydrolysates, especially essential ones, as well as functional amino acids Table (2,3).

Table (2) Amino acid content in protein hydrolysat from head and shell shrimp

Amino Acid	HT_4(ppm)	HP_2(ppm)	ST_4(ppm)	SP_2(ppm)
Aspartic Acid N	11.125	12.561	11.950	10.219
Glutamic Acid N	353.399	364.517	304.753	300.812
Serine N	2099.531	2110.497	518.698	511.507
Histidine E	1145.075	1160.611	1008.040	998.611
Glysine N	844.150	856.203	635.822	628.789
Arginine S	486.706	498.603	201.632	197.300
Alanine N	160.567	161.145	119.705	113.161
Tyrosine S	536.965	541.084	143.058	138.624
Cystine S	996.265	1001.115	879.703	868.812
Valine E	258.605	267.681	187.618	181.919

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Methionine E	538.420	543.350	215.640	203.076
Phenylalanine E	239.666	248.252	160.770	152.515
Isoleucine E	290.807	292.894	118.770	111.806
Leucine E	327.092	333.362	131.058	129.294
Lysine E	1310.292	1328.043	811.302	803.292
Total	9598,665	9719.918	5448.519	5349.735

HT :head trypsin, HP: head pepsin, ST :shell trypsin, SP: shell pepsin

Table (3) Concentration of the total polar, non-polar, and essential amino acids in protein hydrolysates.

Sample	Polar A.A(ppm)	non-polar A.A(ppm)	EssentialA.A(ppm)
HT_4	**7783.508**	**1815.157**	**4109.957**
HP_2	**7873.234**	**1846.684**	**4174.193**
ST_4	**4514.958**	**933.561**	**2633.198**
SP_2	**4457.966**	**891.771**	**2580.513**

Antioxidant Activity

DPPH Free Radical Scavenging Activity

The results of the DPPH free radical scavenging assay showed a significant difference ($p \leq 0.05$) between all hydrolysates and the standard (BHT). The inhibition percentage was 32.4324% for the pepsin hydrolysate of the heads (HP2) and 33.0115% for the shells (SP2), while it was 29.1505% for the trypsin hydrolysate of the heads (HT4) and 30.3088% for the shells (ST4). however, the BHT exhibited the highest antioxidant activity it was 43.3525% Figure (3). The results were consistent with what was reached by (25) who prepared five protein hydrolysates derived from Decapterus maruadsi fish using different enzymes. The DPPH test showed a rise whose degree varied according to the enzyme utilized, where it was pepsin (32.63%) and trypsin (39.37%). These results indicate that shrimp hydrolysates, whether from the heads or shells, possess a notable ability to scavenge free radicals, although less than that of synthetic BHT. The antioxidant activity in the hydrolysates is attributed to the presence of small peptides resulting from the enzymatic hydrolysis process, which contain amino acids effective in hydrogen donation, such as tyrosine (Tyr), histidine (His), and leucine (Leu). These amino acids act to neutralize the DPPH· free radical through electron and hydrogen transfer reactions (26).

Reducing power

The results of the reducing power assay showed a significant difference ($p \leq 0.05$) between all samples and the standard (BHT)

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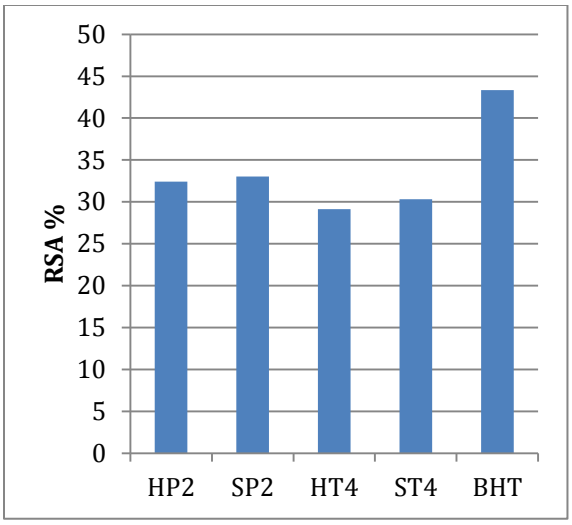
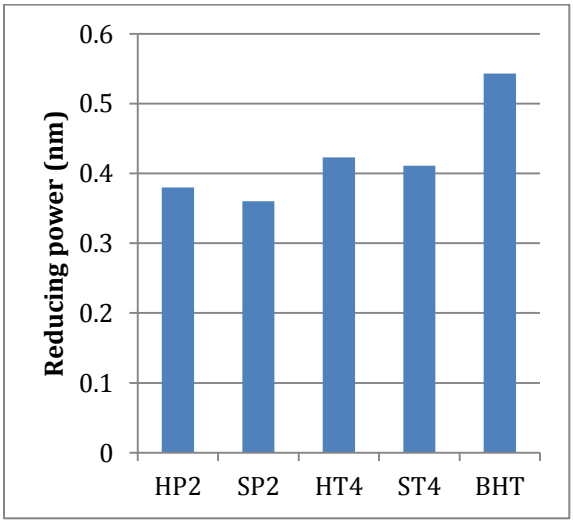
Figure (4). Absorbance values at 700 nm were 0.38 nm for pepsin hydrolysates of heads (HP2) and 0.36 nm for shells (SP2), while they were slightly higher in trypsin hydrolysates of heads (HT4) at 0.423 nm and shells (ST4) at 0.411 nm. The standard compound BHT recorded the highest value at 0.543 nm. (25) Description of the preparation Comprising five hydrolyzed proteins obtained from Decapterus maruadsi fish using different enzymes, and it was found that all five hydrolysates had (0.229, 0.234 , 0.260, 0.309, and 0.33) reducing power for pepsin, neutrase, papain ,alcalase, and trypsin hydrolysates, respectively.These results indicate that all hydrolysates possess good reducing power, but it is lower than that of synthetic BHT, reflecting their relatively limited ability to donate electrons and convert iron ions from Fe^{3+} to Fe^{2+} . Reducing power is an indicator of the ability of compounds or peptides to serve as donors of electrons, Consequently, this contributes to avoid oxidation and neutralize free radicals..The superior reducing power of trypsin hydrolysates (HT4 and ST4) compared to pepsin hydrolysates (HP2 and SP2) is attributable to the differences in the composition of the resulting peptide; trypsin produces peptides rich in basic amino acids such as lysine (Lys) and arginine (Arg), which are known for their high ability to interact with metal ions (27). However, the hydrolysates from the heads (HT4 and HP2) outperformed those from the shells (ST4 and SP2), suggesting that the proteins extracted from the heads possess structures rich in active amino acids and active sites capable of electron transfer. These findings support those of previous studies, as (28) indicated that shrimp hydrolysates rich in amino acids (Met, His, Trp and Tyr) exhibit higher reducing capacity due to their activity in electron transfer reactions.



) Free radical scavenging of **3Figure (** protein hydrolysates from shrimp residues



) Reducing power of protein **4Figure (** hydrolysates from shrimp residues

Functional properties of protein hydrolysates

Solubility

The solubility index (SPI) of the shrimp hydrolysates showed significant differences between the samples at the most pH values ($P<0.05$), except at pH 6 where no

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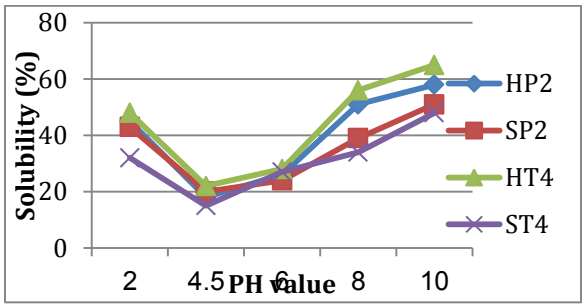
significant differences were observed. The highest solubility values was at pH 10 for all hydrolysates, . The sample HT4 (trypsin hydrolysate of heads) showing the highest value (65%), followed by HP2 (58%), SP2 (51%), and ST4 (48%). The pH 4.5 Showed the lowest solubility for all samples, were close to the isoelectric point, Figure (5) . The nature and arrangement of amino acids in the protein chain (protein fundamental structure), where an amino acid may be hydrophilic or hydrophobic, charged, or uncharged also affect solubility When a protein has a large number of polar amino acids on its surface, it dissolves well in polar liquids. Animal proteins commonly have a U-shaped solubility curve because they are almost insoluble at pH 4–5 and most soluble at low and high pH values. (29).



) Solubility ratio at different pHs **5Figure (** for protein hydrolysates

Emulsifying Properties

The emulsifying activity (EA) results of protein hydrolysates from shrimp heads and shells using pepsin and trypsin enzymes showed significant differences at most pH values (P<0.05), except at pH 4.5 where no significant difference was observed between the treatments.Generally, the emulsifying activity increased with increasing pH, and the highest values were recorded at pH 10, reaching (53%) for the trypsin hydrolysate from heads (HT4) and (51%) for the trypsin hydrolysate from shells (ST4), followed by the pepsin hydrolysates (HP2 and SP2) with values of (48%) and (42%), respectively. The lowest values were observed at pH 4.5 for all hydrolysates.

In contrast, the emulsion stability (ES) results showed significant differences among all treatments (P<0.05), with the trypsin hydrolysates recording higher values (42% for HT4 and 41% for ST4) compared to the pepsin hydrolysates (38% for HP2 and 33% for SP2). Figure (6,7) , (30) explained that the protein hydrolyzes (Acetes indicus) prepared by enzymatic hydrolysis using alcalase enzyme showed emulsifying properties that varied according to the protein hydrolysates' amount , with a 20 mg/ml of concentration showing the maximum emulsion stability (23%) and emulsifying capacity (26%).

that varied with the concentration of the protein hydrolysates, with the highest emulsifying capacity (26.67%) and emulsion stability (23.33%) being found at a concentration of 20 mg/ml**.** (31) He pointed out that protein properties, such as solubility, surface charge, isoelectric point, and surface hydrophobicity, play a major role in its emulsifying ability. A protein's emulsifying ability It is additionally affected by several of variables, including aqueous phase viscosity, processing conditions, temperature, pH, and ionic strength.

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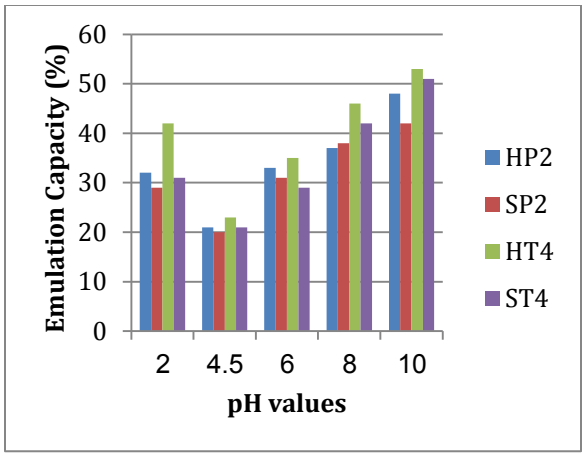
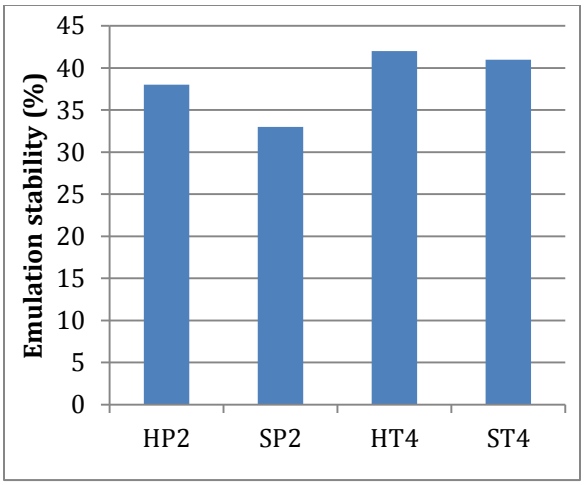


) Emulsifying capacity ratio at **6Figure (** different pH levels for protein hydrolysates.

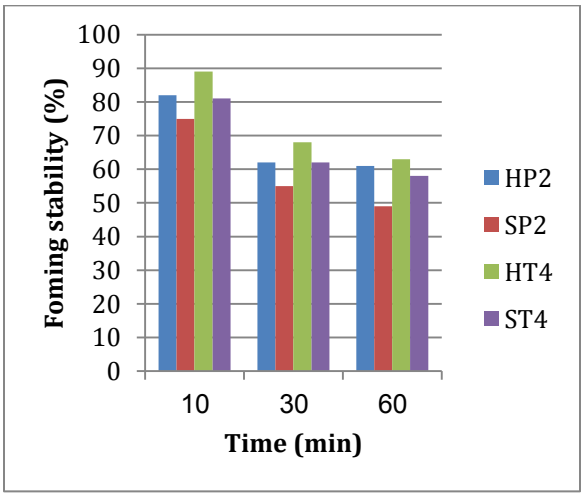
Foaming Properties

The foaming capacity and stability of the all protein hydrolysates results for the shrimp hydrolysates showed significant differences at (P<0.05), Figure (8) shows the Thehighest foaming capacity was for HP2 and SP2, reaching 172% and 181%, respectively. The lowest percentage was for HT4 and ST4, reaching 72% and 86%. As for foam stability, The amount of foam was seen to drop over time.. The HT4 sample was the best from all sample after 10 minutes, and the lowest foam level after 60 minutes was at SP2 (figure 9).(32) showed that the extraction methods significant effect on the the foam properties and stability.



) Emulsification stability **7Figure (** ratio of protein hydrolysates

(33) showed that the carp protein isolate's enzymatic hydrolysates had an 87.4% foam capacity and a 28.4-minute foam stability.



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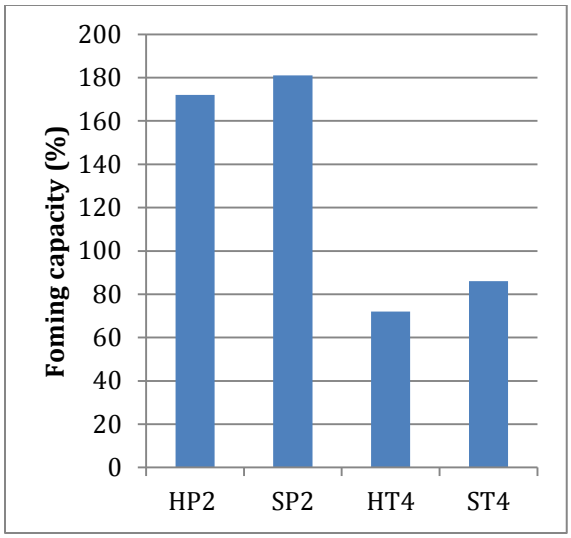

Figure (8) Foaming ability of proteolytic hydrolysates

Figure (9) Foam stability to time ratio of protein hydrolysates

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