Research Article

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# Valorisation of prawn/shrimp shell waste through the production of biologically active components for functional food purposes

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## Abstract

**BACKGROUND: The aim of the work was to develop a technology for using waste from prawn and shrimp processing as a source of active ingredients that could be used in the promotion of healthy foods. From fresh and freeze-dried prawn and shrimp shells, protein hydrolysates (carotenoproteins) were obtained using two different enzymes, Flavourzyme and Protamex.**

**RESULTS: The obtained hydrolysates were characterised in terms of protein content, degree of hydrolysis, and antioxidant and antimicrobial activity. The hydrolysate with the best antioxidant properties (FRAP value of 2933.33 μmol $L^{-1}$ TE; ORAC value of 115.58 μmol $L^{-1}$ TE) was selected and tested for its possible use as a component of functional foods. Molecular weight distribution, amino acid profile and free amino acids, the solubility of the hydrolysate in different pH ranges as well as foaming ability were determined. It was found that this hydrolysate was characterised by an amino acid profile with high nutritional value, flavour enhancement properties and excellent solubility in a wide pH range (from 97.06% to 100%). Afterward, the possibility of using carotenoproteins from prawn waste as a component of an emulsion with furcellaran and a lipid preparation of astaxanthin, taken from post-hydrolysate production waste, was investigated. The obtained complexes were stable as proved by the measurement of zeta potential ($\zeta = -23.87$ and $-22.32$ to $-27.79$ mV).**

**CONCLUSION: It is possible to produce stable complexes of the hydrolysate with furcellaran and to emulsify a lipid preparation of astaxanthin, obtained from waste following production of the hydrolysate, in them.**


**Keywords:** protein hydrolysate; antioxidant activity; food waste; furcellaran; emulsion

## INTRODUCTION

Seafood products comprise a broad spectrum of species of great significance for the food industry. These are, among others, crustaceans as part of the Decapoda order, including prawns and shrimp, which are notably valuable from a commercial point of view.[1]

The amount of waste from shrimp and prawn shellfish has dramatically increased in recent years. Thus, utilising this waste is valuable from an economic as well as an environmental point of view.[2] Waste from the processing of prawns and shrimp is rich in vital by-products, i.e. carotenoids, chitin, minerals, flavour compounds as well as proteins. In crustaceans, carotenoids are found in the form of carotenoproteins. These are stable carotenoid complexes that are attached to high-density lipoproteins. Carotenoproteins have been noted as natural colourants having bioactivity such as antioxidative properties.[3] Carotenoproteins isolated from shrimp and/or prawn by-products are also a valuable source of essential amino acids, potentially enriching food products while promoting benefits for human health. With regard to other seafood species, proteins from crustaceans are characterised by high amino acid content, including alanine, glutamic acid, arginine and glycine. They allow for an increase in palatability when compared to proteins derived from finfish. Besides, due to their favourable essential amino acid profile, the nutritional value of crustacean-based proteins is similar or even higher in comparison to red meat or soya bean. Consequently, protein hydrolysates from shrimp by-products have been applied to fortify various food product types. Efficacy concerning the extraction of proteins from crustacean by-products varies with regard to the applied processing methods. Hydrolysis is a popular strategy used in the processing of fish and shrimp waste. Its aim is to produce greatly nutrient-rich enzymatic protein hydrolysates while

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recovering bioactive molecules.[4] Enzymatic hydrolysis is a method used to improve the functional properties of proteins, because the peptides obtained usually have relatively low molecular weights, stable structures and high activities, and are easily absorbed. In recent years, many researchers have focused on antioxidant peptides extracted from various food sources because these peptides provide a natural and safe alternative to many synthetic antioxidants.[5] Many food-grade proteases are available for protein hydrolysis. The factors that affect the antioxidant activity of food protein hydrolysates include specificity of proteases used for hydrolysis, degree of hydrolysis and structural properties of resulting peptides.[6]

Carotenoids present in shrimp and prawn waste are found not only as carotenoproteins, but also in lipid fractions. Astaxanthin is a major carotenoid that can be obtained from shrimp and prawn waste. The lipid extract from crustacean cephalothorax processing by-products, having high levels of polyunsaturated fatty acids, including docosahexaenoic acid and eicosapentaenoic acid, $\alpha$-tocopherol and astaxanthin, has recently been indicated as a natural additive to food formulations.[7] Such lipid extracts can have different applications, including food dyes or functional ingredients. Consuming astaxanthin provides various pro-health benefits, such as oxidative stress and cardiovascular disease prevention.[8] Nonetheless, astaxanthin demonstrates poor water solubility and low bioavailability and it is prone to degradation via exposure to oxygen, heat as well as light. In order to eliminate those problems, emulsion-based delivery systems of astaxanthin have been developed.[9]

Emulsion-based delivery systems are a successful approach to improving water solubility and bioavailability of hydrophobic bioactive compounds.[9] Proteins (including protein hydrolysates) and polysaccharides are commonly used in the form of emulsifiers/stabilisers. Due to their amphiphilic nature, proteins demonstrate strong abilities related to the adsorption at the level of oil–water interfaces, while providing electrostatic and/or steric repulsion forces preventing the aggregation of droplets and coalescence. In contrast, polysaccharides boost viscosity of the aqueous phase, enhancing stability of emulsions via inhibiting droplet movement. Thus, they are commonly applied as stabilising/thickening agents. Interactions between proteins and polysaccharides are natural phenomena highly significant in food system stabilisation of colloidal systems, in particular, delivery systems based on emulsion processes. Combining proteins and polysaccharides in proper conditions leads to vast improvement in the stability of emulsions.[10]

Polysaccharides are in general ideal delivery agents in microcapsules because they have stable structure, are abundant in nature and are inexpensive. Polysaccharides with reactive functional groups (carboxyl (COOH)), hydroxyl (OH), amide ($NH_2$) and sulfate ($SO_4H$) groups) are promising candidates for use as carrier matrices. Furcellaran (FUR) is structurally linked with the algal polysaccharide carrageenan; nonetheless, there is a key structural dissimilarity. FUR is utilised as a gelling, and at the same time, stabilising agent in the food industry. Additionally, FUR has the ability to form complexes with a variety of proteins which enables it to be used for encapsulating applications in food and agricultural use. The interactions between proteins and polysaccharides can result in formation of completely new materials. Negatively charged FUR may act as a film-forming matrix in active films, an emulsion component and/or as a carrier material in encapsulation processes.[11] Furthermore, this safe, nontoxic, biodegradable and biocompatible material can be used in the manufacturing of films and capsules. What is more, due to its chemical structure, FUR also demonstrates mucoadhesive properties. Implementing polymers having these characteristics in the creation of new drugs allows a significant extension of contact duration between the formulation and the affected site. Concurrently, this equips the drug with a prolonged effect time and by taking substances with systemic activity into account, bioavailability may also be improved compared to that present in traditional forms.[12]

Oxidation often presents a problematic aspect for various emulsion types. A range of antioxidants are included in emulsions to improve oxidative stability. Generally, natural antioxidants are preferred over their synthetic alternatives due to the fact that the latter pose potential risks to health. Among different natural antioxidants, hydrolysed proteins have been noted as novel antioxidants because of their overall radical scavenging ability.[13]

In our previous research, fish processing waste was used to obtain FUR microcapsules with protein hydrolysates.[11] Moreover, this polysaccharide was used complexed with carp skin gelatine hydrolysates also creating a film-forming matrix for numerous active coatings.[14,15] According to our state of knowledge, there have been no other studies that have reported the formulation of a FUR–carotenoprotein stabilised emulsion system used for the encapsulation of astaxanthin oleoresins.

The first objective of the work reported here was to develop a technology for obtaining protein hydrolysates with high antioxidant activity from prawn and shrimp processing waste, which could be used as an emulsion component and, at the same time, being a source of antioxidants in functional foods. During the extraction of these carotenoproteins, residues were formed from which a lipid fraction containing astaxanthin was then extracted. Therefore, the next aim was to investigate the possibility of producing an emulsion composed of FUR, protein hydrolysates and lipid fraction containing astaxanthin, which could be a component of functional food. Such an approach would make it possible to use waste from shrimp processing as a source of many active ingredients that could be used in promoting healthy foods.

## MATERIALS AND METHODS

### Materials

The shells of prawns (*Parapenaeus longirostris*) and shrimp (*Nephrops norvegicus*) were obtained from local processor Centaurus d.o.o. This was waste from the deshelling step in the production of prawn and shrimp vacuum-packed frozen meat. Prior to use, the shrimp and prawn shells were thoroughly washed with distilled water and then ground. After that, the shells were stored at a temperature of −20 °C, awaiting further analysis. FUR (type 7000) was purchased at Est-Agar AS (Karla village, Estonia). The FUR chemical composition ($M_w$ of $2.951 \times 10^5$) was: carbohydrates, 79.61%; protein, 1.18%; fat, 0.24%. TWEEN 80 was procured at Chempur (Piekary Śląskie, Poland).

The Novozymes company (Bagsværd, Denmark) donated food-grade enzymes, i.e. Flavourzyme® (1000 LAPU $g^{-1}$) and Protamex® (1.5 AU-A $g^{-1}$). All chemical reagents were obtained from Merck Chemicals Ltd (Nottingham, UK) and were applied as received and not subjected to any further purification processes.

### Methods

*Preparation of protein hydrolysate from shrimp and prawn shells*

The carotenoprotein was prepared by enzymatic hydrolysis according to the method proposed by Dayakar *et al.*,[3] with some modifications.



  

The prawn or shrimp shells were ground in a grinder (model 980, Moulinex, France). One section of the raw material prepared in this manner was freeze-dried (FreeZone 2.5, Labconco, Kansas City, MO, USA) while the second was used without drying. The samples were mixed with distilled water at a ratio of 1:10. Using an MR (Heidolph, Germany) magnetic stirrer, the suspension was stirred at 350 rpm for a period of 15 min. Based on the data available in the literature, two food-grade enzyme preparations were chosen based on their potential to produce protein hydrolysates demonstrating antioxidant activity[16,17]: Flavourzyme® and Protamex® (Novozymes, Bagsværd, Denmark). The conditions of hydrolysis for the studied enzymes were established according to the available literature sources: Flavourzyme – pH 6.0, 50 °C; Protamex – pH 7.0, 50 °C. The suspension created from the samples was heated to the determined temperature while the pH level was adjusted with 1 mol $L^{-1}$ HCl. Addition of the enzyme preparations totalled 2% for raw material content. Hydrolysis was performed for 120 min. Termination of the reaction was carried out by maintaining the hydrolysates at a temperature of 95 °C for a duration of 15 min, followed by filtration via a muslin cloth. The residue was reused for the astaxanthin extraction. The achieved supernatant was referred to as carotenoprotein. The liquid fraction was further freeze-dried. Hydrolysis was repeated three times.

### *Degree of hydrolysis and protein content in obtained carotenoproteins*

Protein content of obtained protein hydrolysates was determined via the Kjeldahl method, and crude protein was calculated using a 6.25 conversion factor.

The degree of hydrolysis was established based on the Taylor[18] method, as described in detail by Karami *et al.*[19]

### *Determination of biological activity of obtained carotenoproteins*

*Antimicrobial activity: minimal inhibitory concentration (MIC).* Shrimp and prawn hydrolysates were dissolved in distilled water at a concentration of 50 mg $mL^{-1}$. For the determination of MIC by the microdilution method, the prepared shrimp/prawn hydrolysates were diluted with Mueller–Hinton broth at a 1:1 ratio (to a concentration of 25 mg $mL^{-1}$) and 100 µL of the prepared mixture was added to the first wells of a 96-ell microtiter plate. Following a standard two-fold dilution procedure, a concentration range of 12.5–0.2 mg $mL^{-1}$ was tested. A working inoculum ($1 \times 10^5$ CFU $mL^{-1}$) of Gram-positive pathogen bacterium *Listeria monocytogenes* ATCC 7644 was prepared based on a growth curve in the log phase. The bacterial concentration of working inoculum was additionally tested according to the method proposed by Koch (the standard dilution procedure). An amount of 50 µL of the bacterial inoculum was added to each well, and the plate was then shaken at 600 rpm for 1 min on a microtiter plate shaker. In addition to the samples on the plate, bacterial inoculum was tested as a positive control (50 µL of inoculum and 50 µL of broth media), shrimp/prawn hydrolysates as a negative control (50 µL of broth media and 50 µL shrimp/prawn hydrolysates) and broth media as a blank (100 µL of broth media). After incubation at 37 °C for 24 h, 20 µL of the bacterial metabolic activity indicator 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT; 2 mg $mL^{-1}$) was added to the wells. Plates were shaken for 1 min before incubation at 37 °C for 1 h. MIC values represent the lowest concentration at which the hydrolysates inhibit bacterial growth and were determined visually in wells where no reduction of INT to red formazan was observed.[20]

*Antioxidant activity.* The method described by Benzie and Strain[21] and modified by Čagalj *et al.*[20] was used for ferric-reducing/antioxidant power (FRAP). Briefly, 300 µL of FRAP reagent solution was added to the microplate wells, and the absorbance at 592 nm was then measured. The change in absorbance was measured 4 min after adding 10 µL of the sample to the microplate wells. The results obtained for standard Trolox solutions were compared to the measured absorbances. Micromoles of Trolox equivalents per litre of extract (µmol $L^{-1}$ TE) were used to express the FRAP results.

Prior to analyses of oxygen radical absorbance capacity (ORAC), 100-fold dilution of the extracts was done. The ORAC method used was described by Burčul *et al.*[22] Briefly, microplates were thermostatically controlled at 37 °C for 30 min after addition of 25 µL of the sample (or puffer for blank and Trolox for standard) and 150 µL of fluorescein. At excitation and emission wavelengths of 485 and 520 nm, measurements were taken every minute for 80 min after the addition of 25 µL of 2,2′-azobis(2-amidinopropane) dihydrochloride. ORAC results were expressed in µmol $L^{-1}$ TE.

### *Characterisation of protein hydrolysates (PDF) with the highest biological activity*

*Electrophoresis (SDS–PAGE).* Via different enzymes, sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) of hydrolysate digestion was performed as described by Laemmli.[23] This was done by applying Mini-PROTEAN (Bio-Rad, USA) precast gels. The implemented markers were Precision Plus Protein™ Unstained Protein Standards (95–6.5 kDa; Bio-Rad, USA). Separation was carried out at 15%, which allowed one to split the acrylamide gels. Following this step, staining took place according to the standard Coomassie procedure (Bio-Rad, USA).

*Free amino acids and sum of amino acids.* The exact methodology for this procedure has been described in our previous paper.[24]

*Solubility of protein at different pH levels.* The examined samples were distributed in distilled water, the concentration being 1%. After that, the solution pH level was altered from 2 to 10 with 0.1 N HCl or 0.1 N NaOH. The samples were shaken for a 30 min period at room temperature and then centrifuged (10 000 × *g*, 30 min, 25 °C). Protein content in supernatant was established via the method proposed by Kjeldahl. Protein solubility was given as the percentage ratio between content of water-soluble protein and total protein.

*Foam-forming ability.* Foam formation ability was measured by placing a sample (0.5 g) in 50 mL of distilled water, which was then left for swelling (duration: 15 min). The solution was dissolved at a temperature of 60 °C. The foam was created through homogenising the mixture at 10 000 rpm for a period of 5 min (Unidrive X1000, CAT Scientific, Paso Robles, CA, USA). The homogenised solution was further poured into a 250 mL measuring cylinder. Foam-forming ability was calculated as the volume ratio of foam *versus* liquid, while foam stability was estimated as the ratio of the initial foam volume to its volume after 30 min.



  

*Assessing potential use of carotenoid protein as antioxidant component and wall material in emulsion with oleoresin*

*Producing astaxanthin-rich fraction (ARF) and quantification of carotenoids.* The solid residue after obtaining carotenoproteins was freeze-dried and used as a raw material for the production of ARF. The ARF extraction was carried out in the same way as done by Dmytrów *et al.*[25] The quantification of carotenoids was performed according to the method described by Gómez-Estaca *et al.*[26] The content of astaxanthin in ARF was, on average, 37.56 ± 2.30%.

*Production of emulsion with FUR, PDF and ARF. Preparing initial solutions to obtain emulsions*: FUR and PDF solutions (1000 ppm; ionic strength 0.005 mol $L^{-1}$) were prepared and stirred for 24 h at 90 °C (for FUR) and 40 °C (PDF). The solution of the lipid fraction with ARF was prepared by mixing 0.05 g of ARF in 5 mL of sunflower oil. The solution prepared in this way was stirred for 48 h at 50 °C until complete dissolution.

*Preparing oil-in-water FUR–PDP emulsions enriched with ARF*: FUR–PDF complexes were prepared in three ratios: 2:1; 1:1 and 1:2. Then, 50 µL of ARF solution and 50 µL of TWEEN 80 were added to the prepared solutions and left on a magnetic stirrer for 24 h at 40 °C. To determine the stability of the obtained emulsions, the zeta potential was tested on the first and fifth days of refrigerated storage.

*Zeta potential of obtained emulsion.* The zeta potential of the emulsion was measured at 25 °C via dynamic light scattering (Zetasizer Ultra Red, Malvern Instruments Ltd, Worcestershire, UK).

### Statistical analysis

Statistical analysis was performed using Statistica 13.0 software (Tibco, Palo Alto, USA). To determine the differences between hydrolysates, a three-way analysis of variance (ANOVA) was performed with type of material (shrimp/prawn), drying (freeze-dried/wet) and enzyme (Flavourzyme/Protamex) used during the hydrolysis process. Tukey *post hoc* test was performed do determine the differences between individual groups. To determine the significance of each of the individual factors, additional 31-way ANOVAs were performed. To select the hydrolysate with the highest antioxidant potential the results of FRAP and ORAC analyses were standardised using equal weights for both FRAP and ORAC. The standardised results were summed and the one-way ANOVA with Tukey *post hoc* test was performed to distinguish hydrolysate with the highest antioxidant potential. The confidence interval used for all statistical analysis was 95% ($P < 0.05$).

## RESULTS AND DISCUSSION

### Screening of best material for production of carotenoprotein with high antioxidant activity

*Degree of hydrolysis and protein content in obtained carotenoproteins*

The physicochemical properties of protein hydrolysates are dependent on the protein substrate, enzyme specificity for the applied conditions of proteolysis and hydrolysis. Additionally, it has been shown that the protein antioxidant activity can be increased via enzymatic hydrolysis, and selected peptides or fractions have stronger antioxidant potential than others.[27] Enzymatic hydrolysates have recently been researched and used as nutraceutical resources. Many researchers are particularly interested in the enzymatic hydrolysates made from by-products obtained from processing fish and crustaceans.[28] In the present study, wet and freeze-dried shells of shrimp and prawns were hydrolysed for 2 h by two specific enzymes: Flavourzyme and Protamex. The degree of hydrolysis values for the hydrolysates are given in Table 1.

The cleavage of peptide bonds via protease causes the decomposition of protein tertiary structure and the reduction of protein molecular weight.[29] In addition, this reaction elevates concentrations of free amino and carboxyl groups as well as their functional characteristics.[28] All obtained hydrolysates were characterised by a high degree of hydrolysis, but among them, the PWF and PDF samples showed the highest degree of hydrolysis value (47.01% and 46.81%, respectively). Similarly to our research, a degree of hydrolysis level higher than 40% was achieved from the protein hydrolysate that was extracted from Pacific white shrimp (*Litopenaeus vannamei*) waste via the papain enzyme.[3] Contrary to the above, degrees of hydrolysis from protein hydrolysates which were isolated from *Acetes chinensis* and *Acetes indicus*, respectively, employing the Alcalase enzyme, were observed to be approximately 26.32% and 29.57%.[30,31] As stated by Dayakar *et al.*,[3] degree of hydrolysis varies according to season, species, gender, size and conditions of hydrolysis. The degree of hydrolysis

**Table 1.** Degree of hydrolysis (DH), protein content and antioxidant activity of protein hydrolysates from different raw materials of shrimp and prawn shells obtained using different enzymes

| Sample | Type of material | Type of enzyme | DH (%) | Protein (%) | FRAP (µmol $L^{-1}$ TE) | ORAC (µmol $L^{-1}$ TE) |
|---|---|---|---|---|---|---|
| SWP | Shrimp – wet | Protamex | $30.56^{a} \pm 0.31$ | $55.23^{c} \pm 0.37$ | $1212.82^{cd} \pm 57.74$ | $120.43^{abc} \pm 0.60$ |
| SWF | Shrimp – wet | Flavourzyme | $44.01^{d} \pm 0.41$ | $51.10^{b} \pm 0.93$ | $1094.87^{c} \pm 73.92$ | $128.34^{bc} \pm 1.35$ |
| SDP | Shrimp – freeze-dried | Protamex | $45.55^{de} \pm 0.45$ | $60.47^{d} \pm 0.30$ | $1264.10^{d} \pm 31.09$ | $121.05^{abc} \pm 1.01$ |
| SDF | Shrimp – freeze-dried | Flavourzyme | $39.13^{c} \pm 0.34$ | $60.55^{d} \pm 0.44$ | $807.69^{b} \pm 7.69$ | $118.24^{ab} \pm 3.42$ |
| PWP | Prawns – wet | Protamex | $30.32^{a} \pm 0.24$ | $58.95^{d} \pm 0.56$ | $800.00^{b} \pm 7.69$ | $131.05^{c} \pm 2.02$ |
| PWF | Prawns – wet | Flavourzyme | $47.01^{e} \pm 0.42$ | $53.98^{bc} \pm 1.21$ | $630.77^{a} \pm 42.83$ | $123.04^{abc} \pm 1.21$ |
| PDP | Prawns – freeze-dried | Protamex | $35.84^{b} \pm 0.41$ | $57.56^{cd} \pm 0.60$ | $1200.00^{cd} \pm 13.32$ | $112.46^{a} \pm 3.50$ |
| PDF | Prawns – freeze-dried | Flavourzyme | $46.81^{e} \pm 0.29$ | $40.72^{a} \pm 0.10$ | $2933.33^{e} \pm 101.57$ | $115.58^{a} \pm 9.82$ |

Different superscripts in the same column indicate significant differences between mean values ($P < 0.05$). Results are expressed as mean ± standard deviation.
SWP, wet shrimp shells hydrolysed with Protamex; SWF, wet shrimp shells hydrolysed with Flavourzyme; SDP, dry shrimp shells hydrolysed with Protamex; SDF, dry shrimp shells hydrolysed with Flavourzyme; PWP, wet prawn shells hydrolysed with Protamex; PWF, wet prawn shells hydrolysed with Flavourzyme; PDP, dry prawn shells hydrolysed with Protamex; PDF, dry prawn shells hydrolysed with Flavourzyme.



  

was not affected by the choice of the raw material but freeze-drying the samples resulted in a higher degree of hydrolysis value.

As presented in Table 1, all obtained hydrolysates had high protein content (40.72–60.55%) and could be essential protein sources. The final protein concentration was not affected by the drying method, but in terms of material used, the use of shrimp resulted in generally higher protein content. Furthermore, hydrolysis with Flavourzyme resulted in lower overall protein content.

The elevated protein content resulted from protein solubilisation during hydrolysis, insoluble undigested non-protein substance removal and partial lipid removal following hydrolysis.[27] Based on the content of protein, the carotenoprotein extracted with Protamex and Flovourzyme could be applied as an inexpensive protein source used for human supplementation or in diets comprising cultured salmonid species.

*Biological activity of obtained carotenoproteins*

According to data found in the literature on the subject, crustacean shells can be a source of biologically active peptides with bactericidal properties.[32] Therefore, the antimicrobial activity of the obtained hydrolysates was tested. No growth inhibition of *L. monocytogenes* was observed by any of the tested hydrolysates indicating lack of antimicrobial properties. Djellouli *et al.*[33] also noted a lack of antimicrobial properties among hydrolysates obtained from crustacean shells. However, after incubation of the hydrolysates at 100 °C in the presence of glucosamine, those authors found a significant increase in antimicrobial activity of the hydrolysates against Gram-negative and Gram-positive bacteria. According to those authors, the incubation of glucosamine with protein hydrolysates from crustacean disposals could yield molecules with promising applications of interest to various industries. Therefore, further studies should be conducted to assess the antimicrobial activity of Maillard reaction products, where the obtained carotenoproteins from shrimp and prawns would be used as a substrate.

Prawn and shrimp carotenoproteins contain both carotenoids and hydrolysed proteins. The carotenoid and protein hydrolysates with peptides are well known for their antioxidant properties. The peptides found in the cephalothorax extract are assumed to be greatly responsible for the antioxidant activity of these preparations.[34] The nature of the parent protein, pre-hydrolysis treatment as well as the specificity of the enzymes applied in the reaction foster the biological activity of the antioxidant peptides released in the process.[35]

Protein hydrolysate antioxidant activity does not depend on only one mechanism for the reason that proteins comprise numerous amino acids having various antioxidant properties. Some antioxidant components are considered more effective radical scavengers or lipid peroxidation inhibitors, while others exhibit reducing effects.[36] To confirm the antioxidant capacity and investigate the antioxidant reaction type of the achieved carotenoproteins, two antioxidant assays were performed: ORAC and FRAP. The obtained results of the utilised antioxidant tests demonstrated that all of the studied hydrolysates had high antioxidant activity, with significant influence of the type of material and enzyme used (Table 1).

The hydrolysate from freeze-dried prawns obtained using Flavourzyme (PDF) had the highest ability to reduce iron ions (2933.33 μmol $L^{-1}$ TE), while much lower values were obtained for hydrolysates prepared using Protamex and wet materials (PWP) (800.00 μmol $L^{-1}$ TE). In the case of shrimp hydrolysates, higher iron ion reduction values were obtained for samples treated with the Protamex enzyme (1212.82 and 1264.10 μmol $L^{-1}$ TE) compared with those obtained with Flovourzyme (1094.87 and 807.69 μmol $L^{-1}$ TE). As suggested by Pattanaik *et al.*,[37] carotenoproteins that were extracted from shrimp processing waste play a key role in shifting electrons to free radicals, inhibiting or retarding their growth. FRAP is generally used to assess the ability of a substance to convert the TPTZ–Fe(III) complex to a TPTZ–Fe (II) complex. Sowmya *et al.*[34] stated that carotenoproteins from shrimp (*Penaeus monodon*) heads may reduce the $Fe^{3+}$/ferric cyanide complex to a ferrous form. As a consequence, when carotenoprotein can be found in appropriate concentrations, the stage of propagation can be inhibited.

The value for ORAC was not influenced by the raw material selection. These values for the hydrolysates prepared using different conditions were within the range of 112.46 to 131.05 μmol TE. The highest value for ORAC was found in PWP samples (131.05 μmol TE), while the lowest value was recorded for the PDP sample (112.46 μmol TE). Nevertheless, there were no visible differences in ORAC values between the hydrolysates created from shrimp. ORAC is an assay method that determines antioxidant activity of a compound/extract through the scavenging peroxyl radicals. On the other hand, the ORAC assay is restricted to measuring hydrophilic chain-breaking antioxidant capacity but only against peroxyl radicals.[38]

For both assay types (ORAC as well as FRAP), the antioxidant activity maximal and minimal values have not been established in the same runs. This was to be expected as ORAC and FRAP assays, as previously described, involve different reaction mechanisms.

In their research, Dayakar *et al.*[3] noted that carotenoproteins obtained via enzymatic hydrolysis of *P. vannamei* heads and shell waste have wide application potential in the food industry because of their strong antioxidant ability. Shrimp processing waste is a rich source of bioactive substances, specifically, free amino acids, sugar reductors, polysaccharides as well as phenolic compounds, playing a significant part in antioxidative properties. Also, Pattanaik *et al.*[37] suggest that carotenoprotein can be efficiently extracted through enzymatic hydrolysis using papain enzyme from shell waste of *Parapeneopsis stylifera*, and can serve as an effective antioxidant as well as being a rich source of essential amino acids and carotenoids.

To choose the hydrolysate with highest antioxidant potential we have standardised and summed up the results of both antioxidant assays. This allowed choosing the hydrolysate with significantly highest antioxidant properties, which was the preparation obtained from freeze-dried prawn shells with Flavourzyme (PDF). The preparation was further tested, allowing evaluation of its potential application as components of emulsions and functional food products.

## Characterisation of carotenoprotein with highest antioxidant activity as a component of emulsions and functional food products

*Amino acid profile and free amino acid content*

A food's nutritional value is dependent on the type and quantity of amino acids accessible for body functions. Table 2 presents the total amino acid and free amino acid profiles of the PDF hydrolysate. The total content of amino acids in the hydrolysate was 65.74 g (100 g of dry weight)$^{-1}$ and the sum of free amino acids was 5.94 g (100 g)$^{-1}$, which confirms the high degree of hydrolysis.

The essential amino acids noted in the hydrolysate from the prawn by-products comprise 48.10% of all amino acids. These



  

**Table 2.** Amino acid profile and free amino acid content in prawn shell protein hydrolysates

| | Amino acid profile (%) | Free amino acids (mg (100 g)$^{-1}$) |
|---|---|---|
| Aspartic acid | 8.66 ± 0.11 | 42.36 ± 0.43 |
| Serine | 4.08 ± 0.03 | 411.07 ± 4.15 |
| Glutamic acid | 14.29 ± 0.24 | 78.64 ± 0.79 |
| Glycine | 8.91 ± 0.25 | 557.39 ± 5.63 |
| Histidine | 2.45 ± 0.34 | 433.58 ± 4.38 |
| Arginine | 11.09 ± 0.17 | 1291.81 ± 13.05 |
| Threonine | 3.56 ± 0.22 | 363.19 ± 3.67 |
| Alanine | 7.13 ± 0.20 | 421.56 ± 4.26 |
| Proline | 5.10 ± 0.07 | 324.43 ± 3.28 |
| Cysteine | 0.55 ± 0.05 | 37.37 ± 0.38 |
| Tyrosine | 3.61 ± 0.14 | 553.88 ± 5.59 |
| Valine | 5.27 ± 0.06 | 333.04 ± 3.36 |
| Methionine | 1.88 ± 0.15 | 268.04 ± 2.71 |
| Lysine | 7.87 ± 0.12 | 11.51 ± 0.12 |
| Isoleucine | 4.33 ± 0.04 | 16.71 ± 0.17 |
| Leucine | 7.21 ± 0.29 | 576.84 ± 5.83 |
| Phenylalanine | 4.51 ± 0.06 | 218.59 ± 2.21 |
| Sum of amino acids (g (100 g of hydrolysates)$^{-1}$) | — | 65.74 ± 0.60 |
| Sum of free amino acids (g (100 g of hydrolysates)$^{-1}$) | — | 5.94 ± 0.60 |

Results are expressed as mean ± standard deviation.

values surpass the 40% reference value proposed by FAO/WHO[39] for infants. The hydrolysate demonstrated a notably high content of flavour enhancers: glutamic acid (14.29% of total amino acids), aspartic acid (8.66% of total amino acids), glycine (8.91% of total amino acids) and alanine (7.13% of total amino acids), all of which are compounds which influence the umami taste making the product a potential flavour enhancer.[40]

The hydrolysate contains also high levels of arginine and lysine (11.09% and 7.87% respectively) both of which are considered as essential or semi-essential amino acids, exhibiting various metabolic functions in the human body.

Food-derived peptides may exhibit antioxidant activity through selected amino acids which act as metal-chelating and hydrogen-/electron-donating agents. This occurs via interactions with free radicals, thus terminating the radical chain reaction or averting their formation.[41] The determination of free amino acid formation can well mirror the process of hydrolysis.[42] In the hydrolysate free amino acid profile, the arginine and leucine levels were noted as the highest (1291.81 and 576.84 mg (100 g)$^{-1}$, respectively). In general free amino acids are not efficient antioxidants. However, extensive proteolysis in protein hydrolysates may cause a decrease in antioxidant activity. Nonetheless, the antioxidative result of protein hydrolysates is not exclusively determined by separate amino acids but their combination with peptides.[43] In accordance with the research by de Queiroz *et al.*,[44] significant action is expressed in the functional properties of food-related proteins by the presence of hydrophobic amino acids, e.g. methionine, leucine and alanine. Hydrophobic amino acids that can be found in protein hydrolysates demonstrate potentially exceptional antioxidant properties and can be incorporated in other food products as supplements.

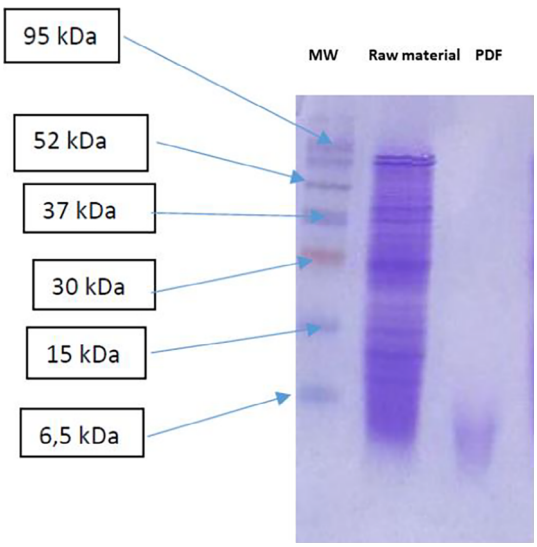


**Figure 1.** SDS–PAGE protein profile of raw material and prawn shell protein hydrolysates.

*Electrophoresis (SDS–PAGE) of prawn shell protein hydrolysates*

Figure 1 shows the SDS–PAGE patterns of the PDF samples. The Flavourzyme enzyme preparation was efficient in degrading the largest proteins of the substrate mixture to low-molecular-weight peptides and free amino acids, as shown by the lack of high protein bands with high molecular weight (more than 6.5 kDa). In contrast, the raw material had various protein bands with high molecular weight.

As noted by de Queiroz *et al.*,[44] protein hydrolysates have various applications depending on the formed peptide size and amino acid sequence. Hydrolysates having a low hydrolysis degree show possible technologically functional characteristics, i.e. antioxidant activity, better solubility and, *inter alia*, foaming ability. Those having a high degree of hydrolysis are typically used as nutritional supplements, in particular in medical diets. In the current study, we chose the hydrolysate demonstrating the highest antioxidant activity. It could be seen in electropherograms that the hydrolysate obtained with the Flavourzyme enzyme contained many low-molecular-weight peptides less than 6.5 kDa. Therefore, it can be assumed that this hydrolysate will exhibit high antioxidant activity. From previous studies,[45] low-molecular-weight peptides which were rich in aromatic and hydrophobic amino acids in chains, expressed very high antioxidant potentials. Similarly, Nwachukwu and Aluko[46] reported the considerable hydroxyl radical-scavenging ability of soybean protein hydrolysates and peptide fractions having a higher hydrolysis degree (lower molecular weight).

The study of Gamage *et al.*[47] aimed to characterise extracted proteases of *Aspergillus flavus*, *Aspergillus niger* and *Penicillium* sp. to be utilised as potential sources to produce shrimp waste protein hydrolysates with degraded antigenic proteins. The shrimp waste samples treated with proteases had a great reduction in protein bands compared to the untreated sample. There was a much greater concentration of smaller peptides because hydrolysis of shrimp waste protein is a process to degrade the long protein chains in shrimp head and shell into small peptides and amino acids. According to literature data[48] the major allergens identified from shrimp were tropomyosin (33 kDa), myosin light chain (20 kDa) and argine kinase (40 kDa). Therefore, it is

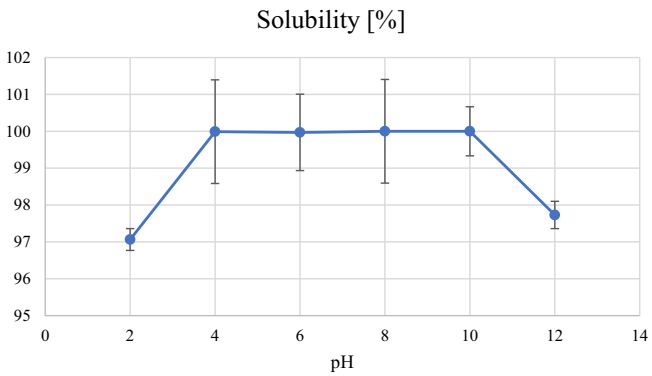


**Figure 2.** Solubility of prawn shell protein hydrolysate at different pH values.

very important to degrade the potential antigenic protein in shrimp waste before further processing. Enzymatic hydrolysis of carotenoproteins can be a way to reduce allergenicity.

*Functional properties of prawn shell protein hydrolysates*

Solubility is among the most significant functional properties of proteins as well as protein hydrolysates. A great number of other functional properties, e.g. emulsification and foaming, are influenced by solubility. Protein solubility is affected by pH and there is a demand in the food industry for high solubility levels across a broad range of pH values.[27]

Figure 2 shows the nitrogen solubility changes for different pH values of PFD hydrolysate. It can be seen that this hydrolysate has very good solubility in a wide range of pH. In acidic conditions (pH 2–4), the nitrogen solubility was almost complete, reaching 97.06%. Above a pH of 4.0, nitrogen solubility increased and remained at 100% until reaching a pH of 10. A somewhat lower solubility level was achieved at pH 12 (97.73%). The pH level has an effect on the charge of the weakly acidic and basic side chain groups. In general, hydrolysates exhibit lower solubility at their isoelectric points. Solubility variations may be ascribed to peptide net charge, increasing as the pH shifts away from the pI, and surface hydrophobicity, further promoting aggregation through hydrophobic interactions.[49] Basically, protein hydrolysate is soluble over a wide pH range, showing low influence of pH, whereas native proteins with tertiary and quaternary structure are affected considerably by pH.[50] Consequently, enzymatic hydrolysis may be applied as a valuable alternative for improving the solubility of animal proteins. In accordance with the study carried out by Egerton *et al*.,[51] a high protein hydrolysate solubility could cause a pleasant appearance and smooth feel in the mouth of any given food or beverage. The exceptional solubility of prawn by-product hydrolysate suggests that it could have potential application in formulated food systems.

The acquired hydrolysate does not demonstrate any foaming properties. This may be because of its high hydrolysis degree (48.81%). In accordance with Gharbi and Labbafi,[52] excessive hydrolysis could have a negative effect on foaming properties due to the reduction in surface hydrophobicity. Chen and Chi[53] noted that foaming properties of egg white protein hydrolysates experienced a decrease along with an increase in hydrolysis degree. In tandem with the increase in hydrolysis degree, the peptides' net charge also underwent an increase. Therefore, electrostatic barriers to adsorption may rise, which may potentially cause a reduction in peptide absorption and foaming ability. Granting that extensive hydrolysis may induce an increase in diffusion rate (due to reduced particle size and the enhanced solubility), it reduces hydrolysate adsorption to the interface (caused by reduced surface hydrophobicity and greater net charge).[52]

## Assessing the potential use of prawn shell protein hydrolysates as emulsion components

All emulsions can be considered as thermodynamically unstable and tend to break with time. This results in two separated liquid phases. Food emulsions can become unstable because of various physicochemical mechanisms, including flocculation, coalescence, gravitational creaming, sedimentation and Ostwald ripening. As these are thermodynamically unstable systems, information regarding emulsions' kinetic stability is significant for the development of products with suitable properties that would last for an appropriate duration.[54]

Properties concerning electrostatic complexes formed between proteins and polysaccharides are dependent on the mass ratio of protein to polysaccharide in a solution, which further relies on factors such as biopolymer flexibility, conformation and linear charge

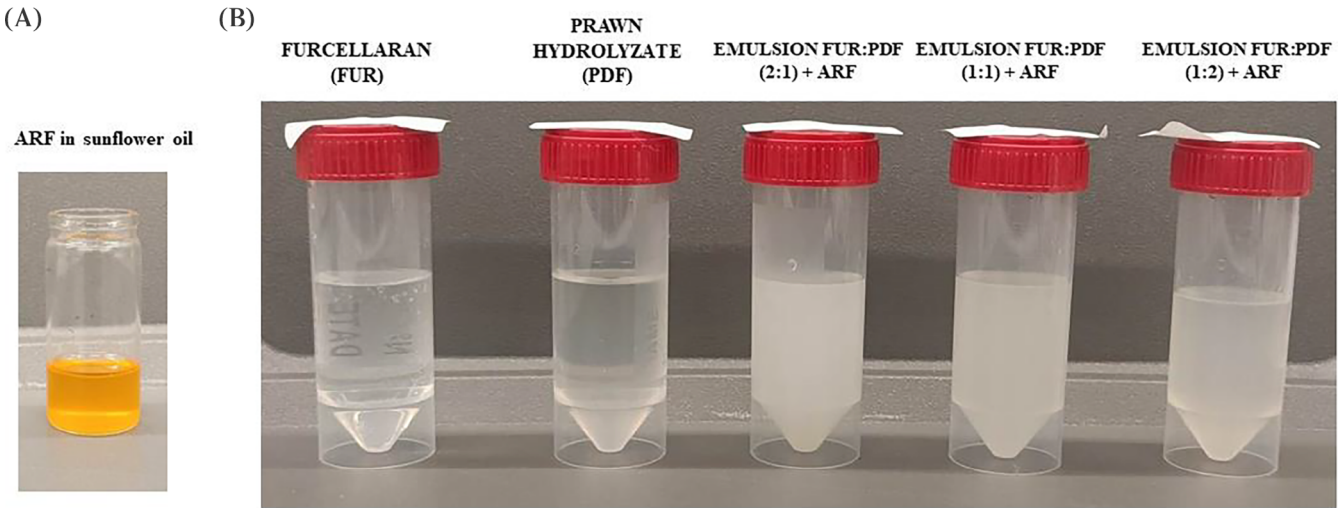


**Figure 3.** Appearance of (a) ARF in sunflower oil; (b) FUR and PDF solutions as well as their emulsions in different ratios of components with ARF.

**Table 3.** Zeta potential values of obtained FUR–PDF complexes and their oil-in-water emulsions enriched with ARF (1st and 5th days of storage)

| | FUR:PDF (1:1 ratio) | FUR:PDF (2:1 ratio) | FUR:PDF (1:2 ratio) |
|---|---|---|---|
| FUR–PDF complex | $-23.87^{b} \pm 1.43$ | $-27.79^{a} \pm 1.37$ | $-22.32^{b} \pm 0.74$ |
| FUR–PDF + ARF emulsion | $-25.52^{b} \pm 0.63$ | $-23.01^{b} \pm 1.68$ | $-22.66^{b} \pm 1.24$ |
| FUR–PDF + ARF emulsion (after 5 days of storage) | $-18.80^{c} \pm 0.54$ | $-15.33^{c} \pm 2.3$ | $-17.68^{c} \pm 2.44$ |

Different superscripts in the same column indicate significant differences between mean values ($P < 0.05$). Results are expressed as mean ± standard deviation.

density flexibility.[55] As a consequence, we designed an emulsion of an astaxanthin lipid preparation with various FUR and PDF ratios (Fig. 3).

A chief gauge of colloidal dispersion stability is the electric potential found in the interfacial layer located at the particle interface. Higher zeta potential suggests higher electrostatic repulsion and separation distance between droplets causing a decrease in flocculation as well as aggregation. In cases where the zeta potential value is within the range of −10 and +10 mV, the system is virtually assumed as neutral. But when the zeta potential reaches a level above −30 and +30 mV, it is considered strongly anionic or cationic respectively.[56]

Thus, to provide an indirect quantitative evaluation of emulsification quality, we measured the zeta potential in complexes of FUR and PDF (at different polysaccharide-to-protein hydrolysate ratios) and, in emulsions, these complexes with ARF (Table 3).

The mixed solutions become more negatively charged as the amount of FUR was increased (from $\zeta = -23.87$ and −22.32 to −27.79 mV), which could indicate that FUR and protein hydrolysates formed stable molecular complexes. The intense negative charge on the surface could have been caused by the occurrence of anionic FUR.[57] After adding ARF to the complex, the zeta potential did not change significantly in either group. In an earlier paper, a shrimp (*L. vanamei*) lipid extract was encapsulated using a partially purified soy phosphatidylcholine having lower electronegative zeta potential (−42 mV).[58] After 5 days of storing the emulsion, a decrease in zeta potential was noted. Therefore, it may be concluded that the emulsions in the tested system are not stable during storage. Thus, further research should be conducted in an attempt to increase the stability of these emulsions or ARF encapsulation as well as the hydrolysate with the addition of another polysaccharide.

## CONCLUSIONS

Shells comprising waste from the processing of prawns and shrimp can be an excellent substrate for the production of protein hydrolysates with a high protein content and good antioxidant power. The hydrolysate from freeze-dried prawn shells, obtained during enzymatic hydrolysis using Flavourzyme, is characterised by very good amino acid profile and excellent solubility in a wide pH range. Therefore, it has the potential to be used as an ingredient in functional foods such as protein-fortified snacks and beverages to enhance their nutritional content and bioactive properties. Furthermore, the obtained protein hydrolysates could be included in aquaculture feeds to enhance the growth and health of farmed aquatic species. In addition, it is possible to produce stable complexes of this hydrolysate with FUR and to emulsify a lipid preparation of astaxanthin, obtained from waste following production of the hydrolysate, in them. However, further research is essential to increase the storage stability of these emulsions.

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## DATA AVAILABILITY STATEMENT

Research data are not shared.

## REFERENCES

1 Ortea I, Cañas B, Calo-Mata P, Barros-Velázquez J and Gallardo JM, Identification of commercial prawn and shrimp species of food interest by native isoelectric focusing. *Food Chem* **121**:569–574 (2010).
2 Wang C-H, Doan CT, Nguyen VB, Nguyen AD and Wang S-L, Reclamation of fishery processing waste: a mini-review. *Molecules* **24**:2234 (2019).
3 Dayakar B, Xavier KM, Ngasotter S, Layana P, Balange AK, Priyadarshini B *et al.*, Characterization of spray-dried carotenoprotein powder from Pacific white shrimp (*Litopenaeus vannamei*) shells and head waste extracted using papain: antioxidant, spectroscopic, and microstructural properties. *LWT.* **159**:113188 (2022).
4 De Aguiar Saldanha Pinheiro AC, Martí-Quijal FJ, Barba FJ, Tappi S and Rocculi P, Innovative non-thermal technologies for recovery and valorization of value-added products from crustacean processing by-products – an opportunity for a circular economy approach. *Foods* **10**:2030 (2021).
5 Liu Y, Wan S, Liu J, Zou Y and Liao S, Antioxidant activity and stability study of peptides from enzymatically hydrolyzed male silkmoth. *J Food Process Preserv* **41**:e13081 (2017).
6 Rao PS, Bajaj R and Mann B, Impact of sequential enzymatic hydrolysis on antioxidant activity and peptide profile of casein hydrolysate. *J Food Sci Technol* **57**:4562–4575 (2020).
7 Šimat V, Rathod NB, Čagalj M, Hamed I and Generalić MI, Astaxanthin from crustaceans and their byproducts: a bioactive metabolite candidate for therapeutic application. *Mar Drugs* **20**:206 (2022).
8 Montero P, Calvo M, Gómez-Guillén M and Gómez-Estaca J, Microcapsules containing astaxanthin from shrimp waste as potential food coloring and functional ingredient: characterization, stability, and bioaccessibility. *LWT* **70**:229–236 (2016).
9 Liu X, McClements DJ, Cao Y and Xiao H, Chemical and physical stability of astaxanthin-enriched emulsion-based delivery systems. *Food Biophys* **11**:302–310 (2016).
10 Boonlao N, Shrestha S, Sadiq MB and Anal AK, Influence of whey protein-xanthan gum stabilized emulsion on stability and in vitro digestibility of encapsulated astaxanthin. *J Food Eng* **272**:109859 (2020).
11 Tkaczewska J, Jamróz E, Piątkowska E, Borczak B, Kapusta-Duch J and Morawska M, Furcellaran-coated microcapsules as carriers of *Cyprinus carpio* skin-derived antioxidant hydrolysate: an in vitro and in vivo study. *Nutrients* **11**:2502 (2019).
12 Andrews GP, Laverty TP and Jones DS, Mucoadhesive polymeric platforms for controlled drug delivery. *Eur J Pharm Biopharm* **71**:505–518 (2009).
13 Cheng Y, Xiong YL and Chen J, Antioxidant and emulsifying properties of potato protein hydrolysate in soybean oil-in-water emulsions. *Food Chem* **120**:101–108 (2010).
14 Jamróz E, Tkaczewska J, Juszczak L, Zimowska M, Kawecka A, Krzyściak P *et al.*, The influence of lingonberry extract on the properties of novel, double-layered biopolymer films based on furcellaran, CMC and a gelatin hydrolysate. *Food Hydrocoll* **124**:107334 (2022).



  

15 Tkaczewska J, Kulawik P, Jamróz E, Guzik P, Zając M, Szymkowiak A *et al*., One-and double-layered furcellaran/carp skin gelatin hydrolysate film system with antioxidant peptide as an innovative packaging for perishable foods products. *Food Chem* **351**:129347 (2021).
16 Cumby N, Zhong Y, Naczk M and Shahidi F, Antioxidant activity and water-holding capacity of canola protein hydrolysates. *Food Chem* **109**:144–148 (2008).
17 Tkaczewska J, Jamróz E, Kulawik P, Morawska M and Szczurowska K, Evaluation of the potential use of carp (*Cyprinus carpio*) skin gelatine hydrolysate as an antioxidant component. *Food Funct* **10**:1038–1048 (2019).
18 Taylor W, Formol titration: an evaluation of its various modifications. *Analyst* **82**:488–498 (1957).
19 Karami Z, Peighambardoust SH, Hesari J, Akbari-Adergani B and Andreu D, Antioxidant, anticancer and ACE-inhibitory activities of bioactive peptides from wheat germ protein hydrolysates. *Food Biosci* **32**:100450 (2019).
20 Čagalj M, Skroza D, Razola-Díaz MC, Verardo V, Bassi D, Frleta R *et al*., Variations in the composition, antioxidant and antimicrobial activities of *Cystoseira compressa* during seasonal growth. *Mar Drugs* **20**: 64 (2022).
21 Benzie IF and Strain JJ, The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': the FRAP assay. *Anal Biochem* **239**: 70–76 (1996).
22 Burčul F, Generalić Mekinić I, Radan M, Rollin P and Blažević I, Isothiocyanates: cholinesterase inhibiting, antioxidant, and anti-inflammatory activity. *J Enzyme Inhib Med Chem* **33**:577–582 (2018).
23 Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685 (1970).
24 Tkaczewska J, Zając M, Jamróz E and Derbew H, Utilising waste from soybean processing as raw materials for the production of preparations with antioxidant properties, serving as natural food preservatives: a pilot study. *LWT* **160**:113282 (2022).
25 Dmytrów I, Szymczak M, Szkolnicka K and Kamiński P, Development of functional acid curd cheese (Tvarog) with antioxidant activity containing astaxanthin from shrimp shells preliminary experiment. *Foods* **10**:895 (2021).
26 Gómez-Estaca J, Calvo MM, Sánchez-Faure A, Montero P and Gómez-Guillén MC, Development, properties, and stability of antioxidant shrimp muscle protein films incorporating carotenoid-containing extracts from food by-products. *LWT – Food Sci Technol* **64**:189–196 (2015).
27 Sila A, Sayari N, Balti R, Martinez-Alvarez O, Nedjar-Arroume N, Moncef N *et al*., Biochemical and antioxidant properties of peptidic fraction of carotenoproteins generated from shrimp by-products by enzymatic hydrolysis. *Food Chem* **148**:445–452 (2014).
28 Kim S-B, Yoon NY, Shim K-B and Lim C-W, Antioxidant and angiotensin I-converting enzyme inhibitory activities of northern shrimp (*Pandalus borealis*) by-products hydrolysate by enzymatic hydrolysis. *Fish Aquatic Sci* **19**:29 (2016).
29 Adler-Nissen J, *Enzymic Hydrolysis of Food Proteins*. Elsevier Applied Science Publishers, Essex, UK (1986).
30 Cao W, Zhang C, Hong P and Ji H, Optimising the free radical scavenging activity of shrimp protein hydrolysate produced with alcalase using response surface methodology. *Int J Food Sci Technol* **44**: 1602–1608 (2009).
31 Dhanabalan V, Xavier M, Murthy LN, Asha KK, Balange AK and Nayak BB, Evaluation of physicochemical and functional properties of spray-dried protein hydrolysate from non-penaeid shrimp (*Acetes indicus*). *J Sci Food Agric* **100**:50–58 (2020).
32 Amparyup P, Donpudsa S and Tassanakajon A, Shrimp single WAP domain (SWD)-containing protein exhibits proteinase inhibitory and antimicrobial activities. *Dev Comp Immunol* **32**:1497–1509 (2008).
33 Djellouli M, López-Caballero ME, Arancibia MY, Karam N and Martínez-Alvarez O, Antioxidant and antimicrobial enhancement by reaction of protein hydrolysates derived from shrimp by-products with glucosamine. *Waste Biomass Valorization* **11**:2491–2505 (2020).
34 Sowmya R, Rathinaraj K and Sachindra N, An autolytic process for recovery of antioxidant activity rich carotenoprotein from shrimp heads. *Marine Biotechnol* **13**:918–927 (2011).
35 Pimentel FB, Alves RC, Harnedy PA, FitzGerald RJ and Oliveira MBP, Macroalgal-derived protein hydrolysates and bioactive peptides: enzymatic release and potential health enhancing properties. *Trends Food Sci Technol* **93**:106–124 (2019).
36 Ketnawa S, Benjakul S, Martínez-Alvarez O and Rawdkuen S, Fish skin gelatin hydrolysates produced by visceral peptidase and bovine trypsin: bioactivity and stability. *Food Chem* **215**:383–390 (2017).
37 Pattanaik SS, Sawant PB, Xavier KM, Dube K, Srivastava PP, Dhanabalan V *et al*., Characterization of carotenoprotein from different shrimp shell waste for possible use as supplementary nutritive feed ingredient in animal diets. *Aquaculture* **515**:734594 (2020).
38 Sinthusamran S, Benjakul S, Kijroongrojana K, Prodpran T and Kishimura H, Protein hydrolysates from Pacific white shrimp cephalothorax manufactured with different processes: compositions, characteristics and antioxidative activity. *Waste Biomass Valorization* **11**:1657–1670 (2020).
39 FAO/WHO J, *Protein quality evaluation*. Food and Agriculture Organization of United Nations, Rome, p. 1 (1991).
40 Wang W, Zhou X and Liu Y, Characterization and evaluation of umami taste: a review. *TrAC Trends Anal Chem* **127**:115876 (2020).
41 Matemu A, Nakamura S and Katayama S, Health benefits of antioxidative peptides derived from legume proteins with a high amino acid score. *Antioxidants* **10**:316 (2021).
42 Yang R, Wang J, Liu Z, Pei X, Han X and Li Y, Antioxidant effect of a marine oligopeptide preparation from chum salmon (*Oncorhynchus keta*) by enzymatic hydrolysis in radiation injured mice. *Mar Drugs* **9**: 2304–2315 (2011).
43 Tkaczewska J, Peptides and protein hydrolysates as food preservatives and bioactive components of edible films and coatings: a review. *Trends Food Sci Technol* **106**:298–311 (2020).
44 de Queiroz ALM, Bezerra TKA, de Freitas PS, da Silva MEC, de Almeida Gadelha CA, Gadelha TS *et al*., Functional protein hydrolysate from goat by-products: optimization and characterization studies. *Food Biosci* **20**:19–27 (2017).
45 Sonklin C, Laohakunjit N and Kerdchoechuen O, Assessment of antioxidant properties of membrane ultrafiltration peptides from mungbean meal protein hydrolysates. *PeerJ* **6**:e5337 (2018).
46 Nwachukwu ID and Aluko RE, Structural and functional properties of food protein-derived antioxidant peptides. *J Food Biochem* **43**: e12761 (2019).
47 Gamage M, Liyanage N, Pathirana I, Ediriweera S and Prabodha L, Evaluation of the potential of fungal acid proteases of *Aspergillus flavus*, *Aspergillus niger* and *Penicillium* sp. to produce shrimp-waste protein hydrolysates with degraded antigenic proteins. *J. Dry Zone Agric* **8**: 21–37 (2022).
48 Veličković TĆ and Gavrović-Jankulović M, *Food Allergens: Biochemistry and Molecular Nutrition*. Springer, London, UK (2014).
49 Sorgentini DA and Wagner JR, Comparative study of foaming properties of whey and isolate soybean proteins. *Food Res Int* **35**:721–729 (2002).
50 Dey SS and Dora KC, Antioxidative activity of protein hydrolysate produced by alcalase hydrolysis from shrimp waste (*Penaeus monodon* and *Penaeus indicus*). *J Food Sci Technol* **51**:449–457 (2014).
51 Egerton S, Culloty S, Whooley J, Stanton C and Ross RP, Characterization of protein hydrolysates from blue whiting (*Micromesistius poutassou*) and their application in beverage fortification. *Food Chem* **245**:698–706 (2018).
52 Gharbi N and Labbafi M, Influence of treatment-induced modification of egg white proteins on foaming properties. *Food Hydrocoll* **90**:72–81 (2019).
53 Chen C and Chi YJ, Antioxidant, ACE inhibitory activities and functional properties of egg white protein hydrolysate. *J Food Biochem* **36**:383–394 (2012).
54 Albano KM, Cavallieri ÂLF and Nicoletti VR, Electrostatic interaction between proteins and polysaccharides: physicochemical aspects and applications in emulsion stabilization. *Food Rev Intl* **35**:54–89 (2019).
55 Jones OG and McClements DJ, Recent progress in biopolymer nanoparticle and microparticle formation by heat-treating electrostatic protein–polysaccharide complexes. *Adv Colloid Interface Sci* **167**:49–62 (2011).
56 Kasprzak MM, Berski W, Krystyjan M, Jamróz E, Florczuk A, Tkaczewska J *et al*., Effects of fibre addition and processing on the stability, rheology and in vitro gastric digestion of whey protein-xanthan gum stabilised emulsions with high oil phase. *LWT* **178**:114465 (2023).
57 Jamróz E, Janik M, Juszczak L, Kruk T, Kulawik P, Szuwarzyński M *et al*., Composite biopolymer films based on a polyelectrolyte complex of furcellaran and chitosan. *Carbohydr Polym* **274**:118627 (2021).
58 Marín D, Alemán A, Montero P and Gómez-Guillén M, Encapsulation of food waste compounds in soy phosphatidylcholine liposomes: effect of freeze-drying, storage stability and functional aptitude. *J Food Eng* **223**:132–143 (2018).




  