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ORIGINAL PAPER

Efficient purification and characterization of novel antioxidant peptides from protein hydrolysate of Malaysian fish sausage (*Keropok Lekor*) by-products

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Abstract

Biotransformation of fish by-products (FBPs) through microbial fermentation is an economical way to produce antioxidative fish protein hydrolysate. Conventional purification techniques involving column chromatography require several unit operations and are complicated. This study explored the use of an alternative bio-separation technique, an aqueous two-phase system (ATPS) in combination with ultrafiltration (UF) to purify the antioxidant peptides (APs) derived from fish sausage by-products hydrolysate. Optimised polymer–phosphate ATPS elevated peptide yield by 34.0% and the purification fold (P_F) of antioxidant activities were 3.2 for ferrous chelating and 10.3 for ABTS radical scavenging. The most effective AP purification was achieved by the ATPS–UF (10 and 3 kDa) achieving 218- and 62.6-fold purification for the ferrous chelating and ABTS radical scavenging activities, respectively. Thirteen peptides have been identified and they were characterised using amino acid analysis and an antioxidant score. The antioxidant score indicated that 61.5% of the peptides identified were APs. Abundant polar and hydrophobic amino acids in the sequences and residues were the main elements that led to the antioxidant activity. This work demonstrated the potential of combining ATPS and UF to purify APs, which is simple and effective for industrial applications.

Keywords Antioxidant peptides · Antioxidant score · Aqueous two-phase system · Fish protein hydrolysate · Ultrafiltration

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Introduction

Malaysian fish sausage (*Keropok Lekor*, KL) by-products (FBPs) can be valorised to generate fish protein hydrolysate (FPH) and bioactive peptides (BPs), which could be applied as bioactive ingredients in cosmetic and food products. The most studied bioactive compound in FPH produced by microbial fermentation is BPs that demonstrate antimicrobial and antioxidant properties [1]. The antioxidant peptides (APs) from FPH have great potential application for food, cosmetics, and nutraceutical industries because they can replace synthetic antioxidants such as butylhydroxyanisole and butylhydroxytoluene, which have carcinogenic effects [2].

Microbial fermentation of FBPs has been extensively documented to produce FPH with antioxidant properties. For instance, the APs in FPH can act as metal chelators [3], hydrogen donors to radical singlet oxygen quenchers [4, 5], or free radical terminators [6]. The molecular weight (MW) of peptides, amino acid composition, and sequence influence their antioxidant capacities [7]. Histidine and tyrosine residues, along with methionine, cysteine, tryptophan, and lysine, are the main components of APs sequence, which range in size from 3 to 16 amino acid residues and also exhibit antioxidant activity in free form [8]. However, most studies on the antioxidant activity of FPH generated from FBPs using microbial fermentation focused on the crude FPH instead of purified fractions or sequences. The hydrolysis of FBPs protein by microbial fermentation could result in hundreds of peptide fractions in a crude FPH. The separation and purification of APs will be crucial for enriching the finished product and preventing interferences with other molecules due to the relatively low yield obtained from microbial fermentation [9].

Bio-separation procedures have a direct influence on the bioactivities' efficacy and the cost of production. Two-cycle chromatography methods such as ion exchange/gel filtration chromatography and reversed-phase high-performance liquid chromatography (RP-HPLC) have been widely utilised for AP purification from FPH [10]. Also, combined techniques including ultrafiltration (UF), ion exchange/gel filtration chromatography, and RP-HPLC have been employed to purify APs [11]. However, chromatographic methods are complicated, time-consuming, producing low yield, and requiring many organic solvents (as mobile phase), which increases production costs and causing negative environmental impact [12, 13]. Therefore, it is imperative to implement alternative purification methods that are simple, effective, and environmentally friendly.

ATPS has been developed to isolate antioxidant compounds from various materials such as flavonoids from pigeon pea root [14], phenolic compounds from *Sedium dendroideum* [15], polysaccharides from *Malus hupehensis* [16], and APs from Xuanwei ham [17]. An attempt has been made to purify APs by combining the ATPS with the dialysis membrane [18, 19]. Furthermore, several studies highlighted the feasibility of combining ATPS with UF (ATPS–UF) to purify polymers *from Aloe leaves* [20], antibiotics from *Streptomyces clavuligerus* [21], bacteriocin from fish and seafood products [22], enzymes from *Cerrene unicolor* [23], and proteins from *Arthrospira platensis* [24]. Compared to the chromatography–UF and ATPS–dialysis schemes, the combination of ATPS–UF offers a more practical, affordable, and selective strategy for AP purification. On a larger scale, this will reduce complexity, and greatly increase the effectiveness of the process. However, to our knowledge, there is no report so far on the AP purification from FPH by polymer-salt ATPS–UF.

We recently developed an optimal fermentation process for producing FPH from KL FBPs with the highest protein yield and antioxidant activity using *Lactobacillus casei* LC216. The FPH was then fractionated using UF (3, 5, and 10 kDa) and characterized in terms of peptide yield, antioxidant activities, and functional properties [25]. Additionally, we have previously demonstrated that ATPS with 15% PEG2000 and 10% potassium phosphate buffer was more effective than precipitation and pH-shift techniques for purifying the APs from FPH generated from KL FBPs [26].

Thus, the objective of the study was to establish an ATPS–UF technique for the separation and purification of APs from the FPH produced from the KL FBPs by *L. casei* LC216 fermentation. Response surface methodology (RSM) was used to optimise the critical parameters in the ATPS, including temperature, sample load, and initial pH. Subsequently, peptide yields and antioxidant activities were assessed. The ATPS fraction with the highest antioxidant activity was subjected to UF to further purify the APs, which were subsequently identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Antioxidant score and amino acid analysis were used to characterise the identified peptides. The ATPS–UF scheme represents an important part of the sustainable strategy to valorise the KL FBPs to APs. The purified APs will have enormous potential for application as natural antioxidants in food products.

Materials and methods

Experiment design

Figure 1 depicts the experimental design for the separation, purification and characterisation of APs from the FPH. The FPH was fractionated using ATPS, UF, and ATPS–UF and the AP separation was evaluated in terms of peptide

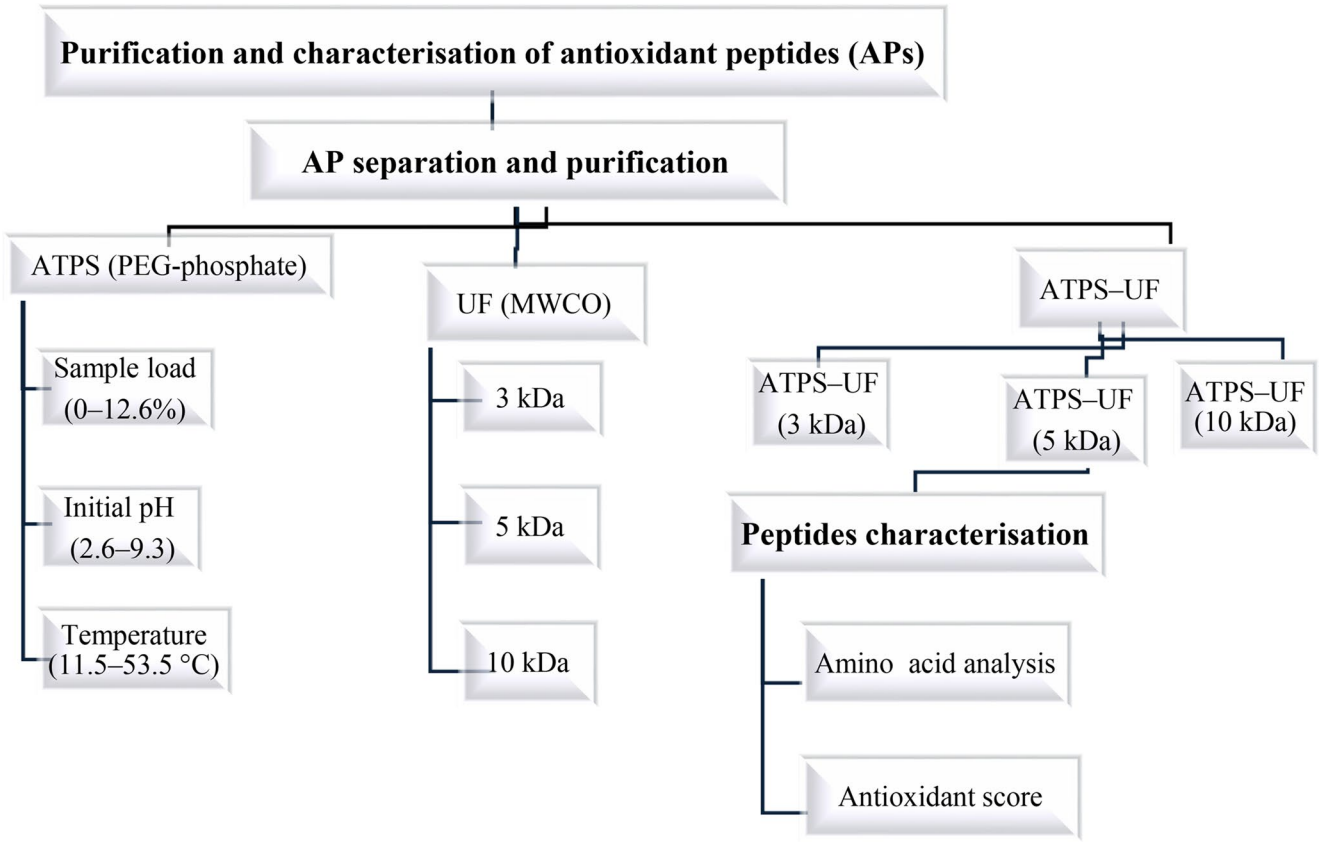


Fig. 1 Flowchart of the experimental design

hydrophobicity, MW and combination of both characteristics. The fraction with the highest antioxidant activity was subjected to LC-MS/MS analysis for peptide characterisation by the antioxidant score and amino acid analysis.

Preparation of fish protein hydrolysate (FPH)

The FPH was prepared from shortfin scad (*Decapterus macrosoma*) by-products, obtained from KL industry by *L. casei* LC216 fermentation under optimal conditions [25]. The FBPs (7.0 g) were mixed with glucose (0.5%, w/v) and distilled water (dH_2O, 35 mL) at the initial pH of 7 in 50 mL centrifuge tubes. Following that, an activated starter culture of *L. casei* LC216 containing 10^7–10^8 colonies forming units (CFU) per mL was added to the FBPs mixture to a concentration of 10% (v/v). The resulting mixture was then incubated for 24 h at 40 °C in a shaking incubator with 50 rpm of agitation (718, Hotech, Taiwan). The supernatant from the FBPs fermentation was centrifuged (Sigma 3-18KS, Sigma, Germany) at 8,000 rpm and 20 °C for 30 min to obtain clarified FPH. The FPH supernatant was freeze-dried (Martin Christ, Alpha 1–4, Germany) and stored at 4 °C before further use.

Preparation of polyethylene glycol (PEG) phase and phosphate solution

All ATPS was prepared using stock solutions of PEG (APC Pure, Europe) and potassium phosphate in dH_2O. Stock solutions of 50.0% (w/w) PEG 2000 were prepared in dH_2O for making the phase-forming components. Concentrations of PEG (15.0%, w/w) were made from the 50.0% PEG solutions. A stock solution of 40.0% (w/w) phosphate solution at pH 7 was prepared using potassium dihydrogen phosphate (KH_2PO_4, EMSURE) and dipotassium hydrogen phosphate (K_2HPO_4, EMSURE) in the volumetric ratio of 1.0:1.1. Concentrations of phosphate solutions (17.5%, w/w) were prepared from a 40.0% phosphate stock solution. The phosphate solution was adjusted with 1 N acid sulfuric (H_2SO_4) and 1 N sodium hydroxide (NaOH) to achieve the desired pH value.

Purification of antioxidant peptides (APs) using aqueous two- phase system (ATPS)

The PEG-phosphate ATPS was constructed based on the compositions previously reported by Escosura and Galvez [27] at a fixed total weight of 5 g. A central composite

Table 1 Numeric and categorical factors of RSM

	Levels of factor				
Factor	-1.68	-1.0	0.0	1.0	1.68
X1 (Initial pH)	2.6	4.0	6.0	8.0	9.3
X2 (Temperature, °C)	11.5	20.0	32.5	45.0	53.5
X4 (Sample load, %)	0	2.0	6.0	10.0	12.6

design (CCD) scheme was used to optimise the separation of APs and antioxidant activity. The test consisted of 20 runs, including five-level factorial points, six axial points, and five replicates of the central points. The assessment of independent variables, including initial pH (X1), temperature (X2), and sample load (X3), was done, and each factor had five coded levels of -1.68, -1.0, 0.0, 1.0, and 1.68 (Table 1).

Ultrafiltration (UF)

The FPH and ATPS fraction with the highest antioxidant activity was subjected to centrifugal UF. The FPH and partially purified ATPS fraction (10 mg/mL) was fractionated into three fractions based on MW using 3, 5, and 10 kDa molecular weight cut-off (MWCO) filters (Merck, Amicon Ultra Centrifugal filters). The resulting fractions were analysed for peptide yield and antioxidant activities.

Peptide analysis

The method of Chen [28] was used with some slight modifications to determine the peptide yield. A 125 µL 20% (w/w) trichloroacetic acid-soluble nitrogen (TCA-SN; EMSURE) solution was added to the samples (500 µL). Following that, the mixture was centrifuged (Hettich, USA) at 100 rpm for 10 min and then kept for 30 min. The sample's peptide content was determined using the Follin method [29]. The peptide yield was calculated as the percentage of peptide concentration in the sample relative to the concentration of protein in the FBPs.

$$Peptide\ yield\ (\%) = \frac{X}{Y} \times 100\%$$

Where X represents the sample's peptide content and Y represents the sample's protein content before hydrolysis.

Investigation of antioxidant capacities

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

A fresh 0.2 mM DPPH (1000 µL) was mixed with a clear sample (200 µL) to measure the DPPH radical scavenging activity [30]. The mixture was then incubated for 30 min at 26 °C without light. The absorbance was measured using a microplate spectrophotometer at 517 nm (VersaMax Microplate Reader, SoftMax Pro, United States). The equation used to calculate the inhibition percentage is illustrated below:

$$Inhibition\ of\ DPPH\ (\%) = \left(\frac{A_{con} - A_{sam}}{A_{con}}\right) \times 100\%$$

where A_{con} represents the control's absorbance and A_{sam} represents the sample's absorbance.

2,2-azinobis-(3-ethylbenzothiazoline-6-sulfone) (ABTS) radical scavenging activity

The working solution was made by combining equal volumes of the potassium persulphate solution (2.5 mM) and the ABTS working solution (7 mM) [31]. The ABTS stock solution (1.0 to 1.3 mL) was combined with 50 mL dH_2O to produce a fresh ABTS radical solution with an absorbance of 0.70 ± 0.05 at 734 nm. A fresh 840 µL ABTS solution was mixed with 60 µL of samples. The mixture was incubated for 30 min at 26 °C without light to ascertain the ABTS radical scavenging activity. The ABTS radical scavenging activity was calculated as below:

$$ABTS\ radical\ scavenging\ activity\ (\%) = \left(\frac{A_{con} - A_{sam}}{A_{con}}\right) \times 100\%$$

where A_{con} is the absorbance of the control and A_{sam} is the absorbance of the ABTS free radical after the addition of sample solution.

Ferrous chelating activity

A solution containing 25 µL of 2 mM ferrous (II) sulphate ($FeSO_4$) was mixed with 200 µL of samples and 1,000 µL of dH_2O, for the chelation assay [32]. Subsequently, a 150 µL solution of 2 mM ferrozine was added to the mixture, stirred vigorously using a vortex, and allowed to stand at 28 °C for 10 min. The percentage of the ferrous chelation effect was calculated using the formula below:

$$Ferrous\ chelation\ effect\ (\%) = \frac{\{A_c - (A_s - A_w)\}}{A_c} \times 100\%$$

Where A_c is the absorbance of the control, A_s is the absorbance of the sample, and A_w *i*s the absorbance of the blank sample without $FeSO_4$ solution.

Antioxidant activities calculation

The DPPH and ABTS radical scavenging activities as well as ferrous chelating activity were calculated using half-maximum effective concentration (EC_{50}). To obtain the EC_{50} values, percentage inhibition of DPPH and ABTS were constructed using five different sample concentrations. Percentage inhibitions were plotted with their relative scavenging capacity on the y-axis and concentration on the x-axis.

ATPS parameters calculations

Partition coefficient (*K*) and Purification efficiency

The partition coefficient (*K*) was determined to understand the partition behaviour of the APs and other unwanted peptides in the development of the PEG-phosphate ATPS, and the *K* was calculated using the following formula [33].

$$\text{Partition coefficient (K)} = \frac{P_{tp}}{P_{bp}}$$

Where P_{tp} is the concentration of peptide (mg/mL) in TP and P_{bp} is the peptide concentration (mg/mL) in the BP.

To evaluate the purification efficiency achieved by the UF, ATPS, and ATPS–UF, the purification fold (P_F) was calculated using the formula below [34]:

$$\text{Purification fold } (P_F) = \frac{\left(\frac{1}{EC_{50,\ fraction}} \times Y_{fraction}\right)}{\left(\frac{1}{EC_{50,\ FPH}} \times Y_{FPH}\right)}$$

Whereas EC_{50} represents half-maximum effective concentration (mg/mL) and *Y* is the peptide yield (%) of the FPH fractions and FPH.

Identification of peptides

The peptides were identified using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific), connected to a Dionex system with an Easy-Spray Column Acclaim PepMap 100 C18 column (Thermo Fisher Scientific, USA; 2 µm particle size, 50 µm ⨯ 15 cm. The experiment was performed in the full MS scan range of 310–1600, followed by MS/MS scans of the 13 most intense peaks in positive ion mode. The peptide database of *Decapterus* species was loaded down from the NCBI website and the MS/MS data was searched using Proteome Discoverer software (Thermo Fisher Scientific) with De Novo GUI 1.16.1 for peptide identification purposes. The search for similar AP sequences that have already been discovered was conducted using the BIOPEP database [35].

Table 2 Scale calculates antioxidant scores to peptides according to the presence and position of specific amino acids in the amino acid sequence

Peptide characteristics	Point
Trp within the amino acid sequence	5.0
Tyr within the amino acid sequence	2.0
Short sequence length (2 to 10 residues)	2.0
His, Lys, Pro, Phe, Val, or Iso within the amino acid sequence	1.0
Tyr, Trp, Val, or Lys residues at N-terminus	0.5
Trp, Tyr, or Met residues at the C-terminus	0.5

Trp tryptophan, *Tyr* tyrosine, *His* histidine, *Lys* lysine, *Pro* proline, *Phe* phenylalanine, *Val* valine, *Iso* isoleucine, *Met* methionine

Antioxidant score

The antioxidant scores of the identified peptides were calculated using the scoring scheme described in Table 2 [36]. Based on the presence and position of specific amino acid residues in the peptide sequences, the antioxidant scores of the peptides were used to assess their activity.

Statistical analysis

All the data were obtained from triplicate experiments and the data were used to calculate mean values and standard deviation (SD). One-way analysis of variance (ANOVA) was used with Tukey's test at a significance level of $p<0.05$ using Minitab Statistical Software (version 14).

Results and discussion

Antioxidant peptide (AP) purification by aqueous two-phase system (ATPS)

According to our previous studies, the top phase (TP) comprised 15.0% PEG 2000 and 17.5% phosphate ATPS had the highest partition coefficient (*K*) value, peptide yield, and antioxidant activities. To improve AP purification in the PEG-phosphate system, initial pH, temperature, and sample loading can be manipulated. The assessment of independent variables, such as initial pH (X1), temperature (X2), and sample load (X3), and their relationship with peptide yield and antioxidant activities, demands standardisation via RSM. The design of experiments and dependent variable values are presented in Table 3.

The experimental settings of initial pH (2.6–9.3), temperature (11.5–53.5 °C), and sample load (2.0–12%, w/w) were selected based on previous reports [18, 37, 38]. Five replicates of the central point values yielded between 18.6 and 31.1% for peptide yield and 0.30–0.38 mg/mL for ABTS EC_{50}. The peptide yield obtained for all 20 runs ranged from

Table 3 CCD matrix and responses of dependent variables for peptide yield and antioxidant activity

	Coded variable			Response	
	Variables			Peptide yield (%)	ABTS EC_{50} (mg/mL)
No.	X1 Initial pH	X2 Temperature (°C)	X3 Sample load (%)	Experimental	Experimental
1.	4.0	20.0	10	10.2	0.33
2.	6.0	53.5	6.0	14.5	0.34
3.	6.0	32.5	6.0	18.6	0.38
4.	6.0	32.5	12.6	45.7	0.03
5	6.0	32.5	6.0	26.9	0.31
6.	4.0	45.0	2.0	9.6	0.37
7.	6.0	32.5	6.0	18.1	0.34
8.	2.6	32.5	6.0	11.1	0.33
9.	6.0	32.5	6.0	26.8	0.32
10.	6.0	11.5	6.0	20.9	0.34
11.	6.0	32.5	6.0	24.8	0.30
12.	9.3	32.5	6.0	30.4	0.12
13.	8.0	20.0	10.0	38.6	0.17
14.	8.0	45.0	2.0	9.4	0.24
15.	4.0	45.0	10.0	9.9	0.37
16.	4.0	20.0	2.0	9.8	0.35
17.	6.0	32.5	6.0	31.1	0.30
18.	6.0	32.5	2.0	11.6	0.36
19.	8.0	20.0	2.0	16.0	0.38
20.	8.0	45.0	10.0	44.5	0.02

9.4% to 45.7%, and the maximum value was found in design points 4 (45.7%) and 20 (44.5%). The range of ABTS's antioxidant activity, determined by EC_{50}, was 0.02–0.38 mg/mL in all runs. Furthermore, the maximal ABTS radical scavenging activity was also reached at design points 4 (0.03 mg/mL) and 20 (0.02 mg/mL), coinciding with the design points that gave the highest peptide yield.

Analysis of variance (ANOVA)

The ANOVA was performed for the evaluation of the three independent variables and the interaction effects of variables on different responses. The high F-value for peptide yield (10.42) and ABTS EC_{50} (59.35) indicate that the models in the current studies are significant. A p-value of <0.05 indicates that the model terms are significant, which explains why the probability that any observed difference between groups is just a coincidence (only 0.1%, and 0.0% for peptide yield and ABTS radical scavenging, respectively). The insignificant p-value ($p>0.05$) for the lack of fit (peptide yield: 0.219; ABTS EC_{50}: 0.359) suggests that the model fits the experiment. The coefficient of determination (R^2) of the model was 90.3% for peptide yield and 98.2% for ABTS radical scavenging activities ($R^2 \geq 80.0$ to be considered well-fitting).

Fig. 2 Contour plots and surface plots of the initial pH, and sample load on (**a**): peptide yield, and (**b**): ABTS EC_{50} and temperature and initial pH on: (**c**) peptide yield and (**d**): ABTS EC_{50}

Analysis of peptide yield and ABTS radical scavenging activity

Regression analysis on the model produced the following best explanatory model equation.

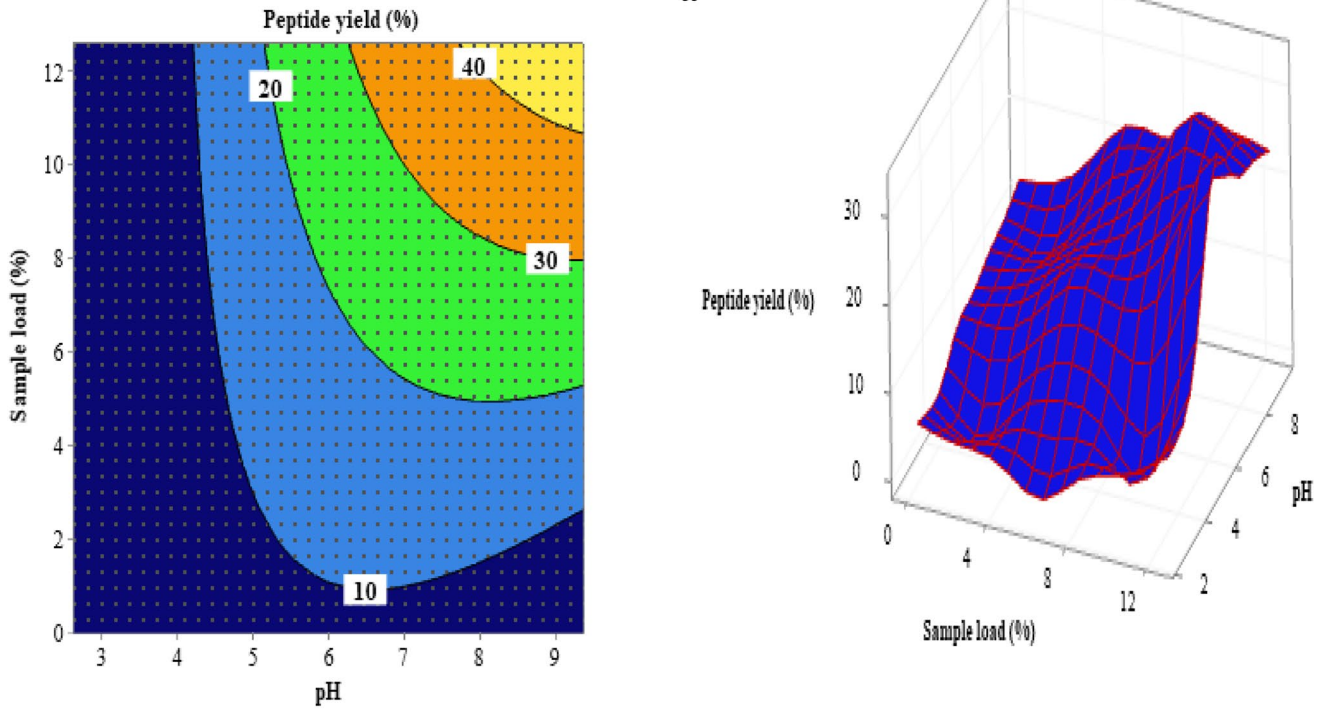
$$Peptide\ yield\ (\%) = -36.6 + 10.55X1 + 0.973X2 - 63.4X3 \\ -0.846X1 * X1 - 0.01758X2 * X2 + 1.6X3 * X3 - 0.0025X1 * X2 \\ +13.03X1 * X3 + 13.03X1 * X3 + 0.455X2 * X3$$

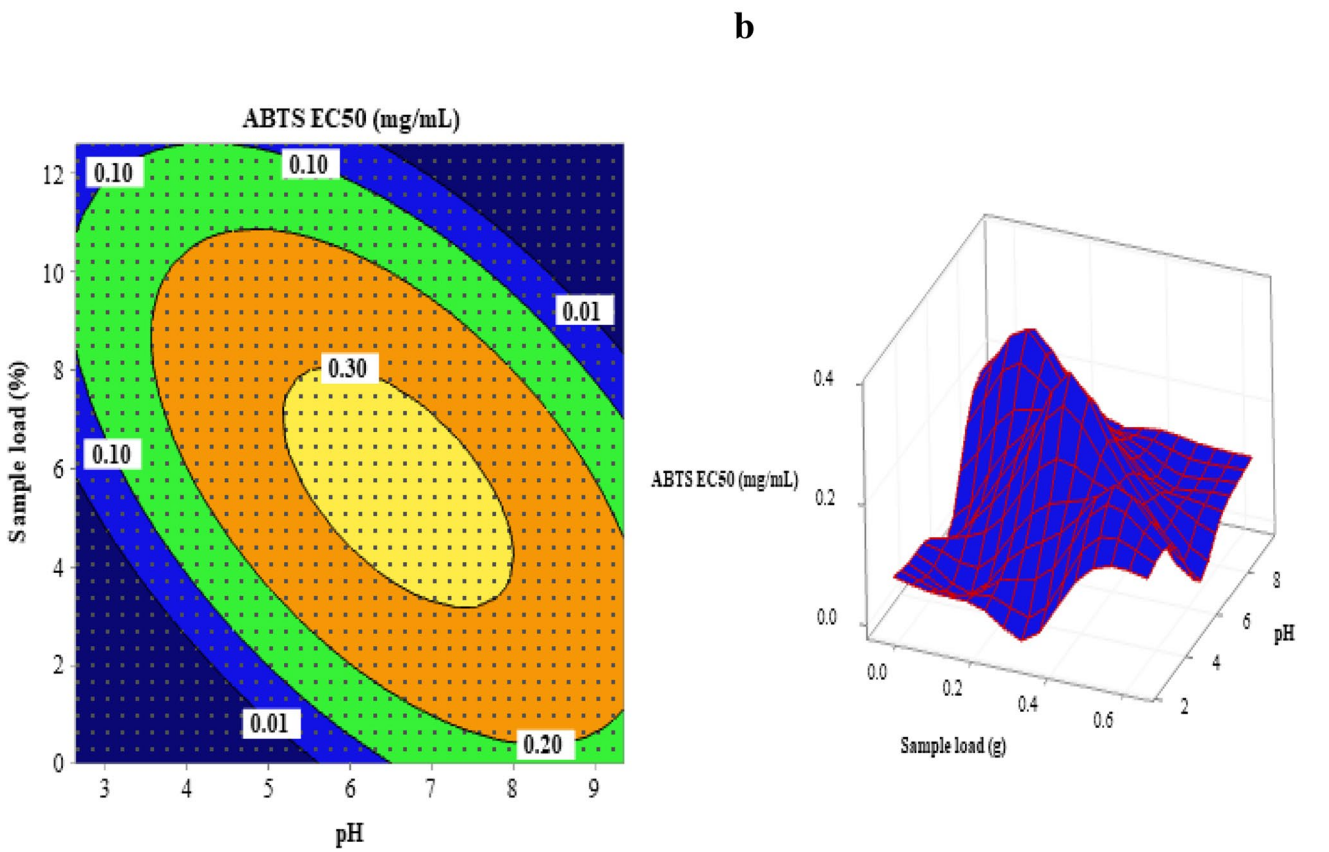
$$ABTS\ EC_{50}(mg/mL) = -1.207 + 0.3492X1 - 0.00107X2 + 2.653X3 \\ -0.01945X1 * X1 + 0.000044X2 * X2 - 2.075X3 * X3 \\ -0.000604X1 * X2 - 0.2279X1 * X3 + 0.00434X2 * X3$$

A PEG-phosphate ATPS RSM optimisation observed that identical conditions and factors generated the highest peptide yield and ABTS EC_{50}. The initial pH and sample load have a considerable impact on the peptide yield and the ABTS EC_{50} of TP PEG-phosphate ATPS by the single-factor and two-way interactions. The findings indicated that the ideal pH range to obtain the highest peptide yield and ABTS radical scavenging activity with the lowest EC_{50} value was between 7.0 and 9.0 (Fig. 2a). Peptides can be separated into distinct phases based on the hydrophobic and hydrophilic forces in the ATPS, which can be influenced by the system pH. The initial pH of the ATPS influences the protein molecules' surface charge and ionisation, which are important for the partitioning behaviour [39].

Moreover, the peptide yield and ABTS EC_{50} in the TP increased as the sample load was increased. The highest peptide yield and ABTS radical scavenging activity was obtained using a 10.0–12.0% sample load (Fig. 2a, b). A higher sample load would be beneficial so that the ATPS could handle larger feed volumes. It was discovered that when the FPH concentration exceeded 12%, a negative impact on the TP peptide recovery was observed due to peptide precipitation at the interphase, which in turn, decreased the peptide yield and ABTS radical scavenging activity. Selvakumar [40] reported that the total protein partition coefficient gradually increased from 1.2 to 1.45, as yeast loading was increased from 5 to 20% (w/w). The difference in optimal sample load between this study and other studies may be because of the difference in physicochemical characteristics of each protein such as molecular size, charge, and hydrophobicity.

The optimum temperature range yielding the highest peptide yield was found to be 20–40 °C, while the ABTS EC_{50} was unaffected by the temperature of ATPS (Fig. 2c, d). Similar results for the partition coefficient in TP PEG-salt ATPS from fish proteins were reported by Nagaraja [37], indicating a decrease in protein yield at higher temperatures (> 40 °C). Temperature affects the


a
Peptide yield (%)
20
40
30
10
Sample load (%)
pH
Peptide yield (%)
Sample load (%)
pH


b
ABTS EC50 (mg/mL)
0.10
0.10
0.01
0.30
0.10
0.01
0.20
Sample load (%)
pH
ABTS EC50 (mg/mL)
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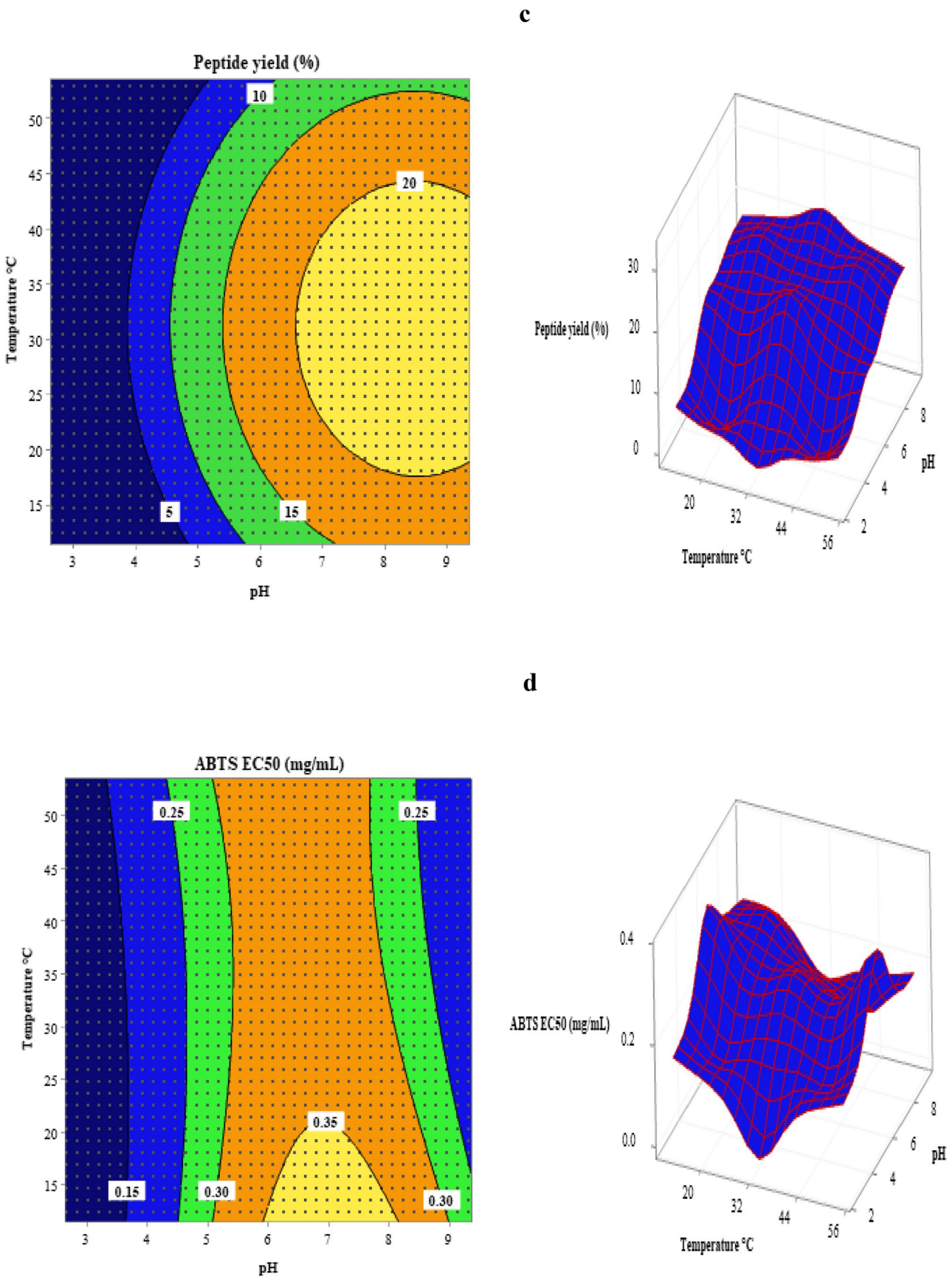

Fig. 2 (continued)

protein partitioning in the ATPS through changes in physicochemical characteristics such as interfacial tension, density, and viscosity [41]. Following the optimisation, the ABTS radical scavenging activity and peptide yield were successfully enhanced by 85.3% (EC_{50} decreased from 0.13 to 0.02 mg/mL) and 34.0% (33.2% to 44.5%), respectively.

Purification of antioxidant peptides (APs) using combination of aqueous two-phase system (ATPS) and ultrafiltration (UF)

The FPH was subjected to UF, ATPS, and ATPS–UF to evaluate the separation and purification of APs relative to the peptide molecular weight (MW), and hydrophobicity and combination of these factors. The FPH ATPS fractions that provided the highest antioxidant activity were further fractionated based on the MW (10, 5, and 3 kDa). Table 4 displays the peptide yield, antioxidant activities, and purification fold (P_F) achieved by the UF, ATPS, and ATPS–UF.

The 10 kDa UF fraction (0.2 mg/mL) exhibited the most effective AP separation and enrichment, with a 3.8-fold purification for the ABTS radical scavenging activity. The ferrous chelating activity was not affected by the UF. Picot [42] discovered that an UF operation did not substantially change the antioxidant activity of a FPH product because of the possibility of peptide breakdown throughout the process and the potential for a synergistic effect of peptides with varying MWs.

Table 4 Peptide yields, antioxidant activities and P_F achieved by the UF, ATPS, and ATPS–UF

Step	Peptide yield (%)	ABTS EC_{50} (mg/mL)	P_F	Ferrous chelating EC_{50} (mg/mL)	P_F
FPH	83.7 ± 4.5^{a}	0.8 ± 0.04^{a}	1.0	5.0 ± 0.9^{b}	1.0
Scheme 1 UF (kDa)					
10	79.3 ± 6.3^{ab}	0.2 ± 0.01^{a}	3.8	5.4 ± 1.1^{b}	0.9
5	62.2 ± 2.8^{b}	0.3 ± 0.04^{a}	2.0	13.1 ± 1.9^{a}	0.3
3	61.2 ± 1.9^{b}	0.3 ± 0.03^{a}	2.0	19.4 ± 3.3^{a}	0.2
Scheme 2 ATPS	43.1 ± 6.1^{c}	0.04 ± 0.01^{b}	10.3	0.8 ± 0.1^{c}	3.2
Scheme 3 ATPS-UF					
10	36.4 ± 3.6^{c}	0.03 ± 0.00^{b}	11.6	0.01 ± 0.0^{c}	218.0
5	30.4 ± 3.0^{c}	0.09 ± 0.00^{b}	3.2	0.8 ± 0.2^{c}	2.3
3	26.2 ± 1.9^{c}	0.004 ± 0.00^{c}	62.6	1.0 ± 0.3^{c}	1.6
Vit. C	-	0.01 ± 0.00			
EDTA	-			0.02 ± 0.00	

Each value is expressed as mean ± SD. Mean values in a column that does not share superscripted letters are significantly different from others at $p < 0.05$ by one-way ANOVA. Vitamin C and EDTA serve as positive controls

In ATPS, the APs partitioned to the TP while most of the impurities were partitioned to the bottom phase. As a result, the APs become more concentrated and purified in the PEG-rich phase. The ATPS fraction exhibited AP separation and enrichment, with 10.3- and 3.2-fold purification for the ABTS radical scavenging activity and ferrous chelating capacity, respectively.

In the present study, a UF was applied to the ATPS fraction to purify APs and improve the P_F for antioxidant activities. The most effective AP purification was achieved by the combination of ATPS–UF (3 and 10 kDa), achieving 62.6- and 218-fold purification for the ABTS radical scavenging and ferrous chelating activities, respectively. For an initial purification, the ATPS might be effective in removing several contaminants. Applying ATPS before UF significantly reduced the bulk volume of the sample that the UF must process by half [43]. Centrifugal UF has been employed to remove PEG after ATPS operation and to improve the purity of the targeted compounds, including polysaccharides, C-phycocyanin, and B-phycoerythrin [20, 44, 45]. Patil and Raghavarao [46] employed UF after ATPS to concentrate phycocyanin, remove PEG, and raise the purity level (from 1.2 to 4.1, based on the absorbance of the phycocyanin to that of protein).

The different MW distribution of peptide in each UF and ATPS–UF fraction from FPH was responsible for the different effects on the ferrous chelating and ABTS radical scavenging capacities. The ATPS–UF (3 kDa) fraction had a high ABTS radical scavenging activity (0.004 mg/mL), however, it had a low ferrous chelating capacity (1.0 mg/mL). Fractions with lower MW peptides (5.0–48.0 kDa) would consist of more active and shorter peptides, which may act as electron donors and react with free radicals to change them into more stable compounds and disrupt chain reactions [47]. Tkaczewska [48] discovered that the carp (*Cyprinus carpio*) skin hydrolysate's ability to chelate ferrous ions is not influenced by peptide MW but by the presence of specific peptide structures like Glu, Asp, His, and Lys residues. The APs from ATPS–UF (3 kDa) exhibited strong antioxidant activity, which could be due to the enrichment of APs based on their hydrophobicity in the PEG-rich phase ATPS and low-MW peptides with UF.

The purified APs from ATPS–UF (3 kDa) had a higher ABTS radical scavenging activity (ABTS EC_{50}: 0.004 mg/mL) compared to that purified from Loma fermented fish (ABTS EC_{50}: 0.64 mg/mL) by a three-step conventional scheme (UF, size exclusion chromatography, and RP-HPLC) [49]. Furthermore, the purification levels of ABTS radical scavenging activity (P_F= 62.6) and ferrous chelating capacity (P_F= 218.0) achieved in this study were excellent compared to that attained for antioxidant purification (P_F= 6.5) from FPH produced from *Scorpaena notata* by *Penicillium digitatum* using UF, gel filtration chromatography, and RP-HPLC [34].

UF was applied to enrich APs in the ATPS workflow. Although UF slightly reduces peptide yield and adds processing steps, it remains more cost-effective and scalable than column chromatography due to lower equipment and solvent requirements. Thus, UF can be considered an optional step to enhance specific activity, balancing functional benefits against operational costs, and moderating previous claims of simplicity and industrial feasibility. Although the peptide yield was lower, the antioxidant peptide enrichment is very effective as indicated by the value of PF. Furthermore, the ABTS EC_{50} value (0.004 mg/mL) of the ATPS–UF (3 kDa) fraction was much lower than that of positive control (vitamin C, EC_{50}: 0.01 mg/mL). Hence, the ATPS–UF (3 kDa) fraction was subjected to further investigation for AP identification.

Identification and characterisation of antioxidant peptides (APs)

Thirteen prominent peaks were selected for the sequence analysis through a database and computation (Table 5). These peptides were all considered to be novel as they were not found in the BIOPEP database. The sequence included in the ATPS–UF (3 kDa) fraction is composed of 13–18 amino acids. The obtained sequence, comprising 13–18 amino acids, was found to be consistent with the findings from the literature, i.e., BPs normally consist of 2–20 amino acids [7, 50].

A scale was developed to give a score on the APs based on the presence and position of specific amino acids within the sequence and residues. Peptide sequences scoring above 5.0 could represent APs [51]. Based on the antioxidant score, the presence of APs was detected, which included 61.5% of the identified peptides in the ATPS–UF (3 kDa) fraction. Eight sequences were identified as APs and the highest antioxidant score was achieved by ASKKPVLLGMKPYK (9), DGQPWVMSPACMKR (9), and RHLYNPQQPAPDF (7) sequences.

Table 5 Identified peptides based on liquid chromatography-tandem mass spectrometry and antioxidant score

Amino acid sequence	m/z	Charge	Antioxidant score
HKSGEEAEKCCVLK	520.92	3	5
GMLLALSMQFYDR	520.92	3	6
NLPVLVLMSVPLHK	520.65	3	6
MLKRAPPASPVGSSR	518.62	3	6
ASKKPVLLGMKPYK	520.65	3	9
GCHRNLDGLHGEMQNR	612.95	3	2
ARPGRCCNAAYTASGNPK	612.98	3	5
EEHRCCGVDSKLPTHK	613.63	3	6
VRELGPNMEMAGPEHR	613.63	3	4
ELNCSQGPPSSDSKR	802.67	2	3
DGQPWVMSPACMKR	803.67	2	9
RHLYNPQQPAPDFK	820.42	2	7
MAPGLPGATGVHVMSGR	819.42	2	5

The 19 amino acids comprising the sequences' content were in the correct order: Pro > Gly> Leu > Lys > Ser = Arg > Ala > Met =Val > His = Glu > Cys =Asn > Gln > Asp > Tyr = Thr > Phe > Trp. Notably, 52.3% and 47.7% of the identified APs contained hydrophobic amino acids (HA) and polar amino acids (PA), respectively. In these sequences, Pro (P), Gly (G), and Leu (L) were the most prevalent HA, and Lys (K), Ser (S), and Arg (R) were the most prominent PA. The isolated peptide sequences from the FPH of tuna by-products, including HA and PA, have been found to have a strong radical scavenging action [52]. This study demonstrated that a smaller MW peptide and the presence of HA and PA amino acids within the sequences and residues were the key factors contributing to the antioxidant activity of the ATPS–UF fraction (3 kDa).

Conclusion

A systematic approach was implemented to purify the APs from FPH produced by *L. casei* LC216 fermentation. The optimal conditions for the PEG-phosphate system have been identified to be 15.0% (w/w) PEG 2000 and 17.5% (w/w) phosphate at 45 °C, pH 8, with 10% (w/w) sample load. This led to a 34.0% increase in peptide yield, and 10.3- and 3.2-fold purification for the ABTS radical scavenging activity and the ferrous chelating capacity, respectively. The combination of ATPS and UF (3 kDa and 10 kDa) raised the P_F to 62.6 and 218.0 for ABTS radical scavenging and ferrous chelating activities, respectively, by enriching the APs based on hydrophobicity and MW. The presence of HA and PA amino acids in the AP sequences and residues, as well as lower MW APs were found to be the primary contributors to the antioxidant activities. Thus, the ATPS–UF combination has been proven to be effective for AP purification from the KL FPH and is expected to be a viable proposition for the large-scale purification of the APs. This study showed that *L. casei* LC216 fermentation released APs with demonstrated in-vitro antioxidant activity, further research is needed to validate these results through in-vivo and clinical studies. This would provide more comprehensive evidence of FPH's efficacy and safety for use in functional food applications.

Glossary

ABTS	2,2-azinobis-3-ethylbenzothiazoline-6-sulfone.
ANOVA	Analysis of Variance.
APs	Antioxidant peptides.
ATPS	Aqueous two-phase system.
BPs	Bioactive peptides.

BP	Bottom phase.
CCD	Central composite design.
dH_2O	Distilled water.
DPPH	2,2-diphenyl-1-picrylhydrazyl.
EC_{50}	Effective concentration at 50% inhibition.
EDTA	Ethylenediaminetetraacetic acid.
FBPs	Fish by-products.
$FeSO_4$	Ferrous (II) sulphate.
FPH	Fish protein hydrolysate.
KL	*Keropok Lekor*.
LC-MS/MS	Liquid chromatography-tandem mass spectrometry.
MW	Molecular weight.
MWCO	Molecular weight cut-off.
OFAT	One-factor-at-a-time.
PEG	Polyethylene glycol.
P_F	Purification fold.
RP-HPLC	Reverse phase-high performance liquid chromatography.
RSM	Response surface methodology.
UF	Ultrafiltration.

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Author contributions All authors contributed to the study concept and experimental design. Material preparation, data collection, and analysis were performed by NYR, FWFW, and SRI. The first draft of the manuscript was written by NYR and FWFW. The final manuscript was edited, reviewed, and approved by all authors.

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Declarations

Conflict of interest There are no relevant financial or nonfinancial interests to disclose for the authors.

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