

PROCESSING AND PRODUCTS

The apoptotic and free radical–scavenging abilities of the protein hydrolysate obtained from chicken feather meal

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ABSTRACT This study examined the antioxidant capabilities of peptides derived from chicken feather meal (CFM) protein hydrolysates which were produced using 3 different microbial proteases (Neutrased, Alcalase, and flavourzyme) and tested at varying concentrations, namely 1, 2, and 5% by weight. The highest levels of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging activities were presented by CFM hydrolysate derived using 5 wt% Neutrased and digested for 4 h. Fractionation of this particular hydrolysate was then performed by applying 10, 5, 3, and 0.65 kDa molecular weight cutoff membranes. It was then determined that the molecular weight (MW) < 0.65 kDa fraction achieved the greatest level of free radical scavenging activity in the context of DPPH and ABTS. The MW < 0.65 kDa fraction then underwent additional fractionation using reverse-phase high-performance liquid chromatography to derive 3 main fractions designated as F₁, F₂, and F₃. All of these fractions presented a high level of activity in DPPH radical scavenging, although no significant ABTS

scavenging was observed. Quadrupole time-of-flight tandem mass spectrometry was used in determining the peptide contents of the fractions as Phe-Asp-Asp-Arg-Gly-Arg-X for F₁ (FDDRGRX, 875 Da), Val-Thr-Leu-Ala-Val-Thr-Lys-His for F₂ (VTLAVTKH, 868 Da), and Val-Ser-Glu-Ile-X-Ser-Ile-Pro-Ile-Ser for F₃ (VSEIXSIPIS, 1,055 Da). Moreover, the F₂ fraction was shown to be capable of preventing DNA damage induced by hydroxyl radicals, as indicated in tests using the plasmids pKS, pUC19, and pBR322 via the Fenton reaction. This outcome was demonstrated through *in vitro* anti-proliferative activity in human cell lines based on SW620 colon cancer, using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. The F₂ fraction at 0.5 wt.% was also shown to be capable of inducing weak early apoptosis, which could be measured by using the Fluorescein isothiocyanate Annexin V Apoptosis Detection Kit with Propidium Iodide Solution. Furthermore, an increase in caspase-3 and caspase-8 activity was observed in SW620 cells following exposure for 24 h and 48 h.

Key words: protein hydrolysate, chicken feather meal, free radical scavenging, apoptosis

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INTRODUCTION

Health care is at the forefront of people's concerns today as awareness grows of the environmental threats to health and, in particular, the underlying causes of cancer, which is a significant cause of death. GLOBOCAN provides data suggesting that for the year 2018, there

were 9.6 million deaths attributable to cancer, while 18.1 million new cases were diagnosed (Bray et al., 2018). While the latest treatment methods offer some success against cancer, especially when the condition is diagnosed early, it remains the case that the prevention of cancer is more attractive than its treatment using chemotherapy, radiotherapy, or surgery. It is also important to develop new medicines to address the problems of resistance, ineffectiveness, and harmful side effects which affect the quality of life of patients. Biotherapy is one potential source of improvement, using bioactive compounds to treat cancer. This field is attracting increased attention, and there is a growing demand for the extraction and enrichment of useful bioactive

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compounds for use in the medical field (Samarakoon and Jeon, 2012).

A number of studies have investigated bioactive peptides in terms of their physiological properties to achieve both their identification and optimization when isolated from a variety of different organisms. The proteolytic hydrolysis of food proteins allows the generation of peptides both *in vivo* and *in vitro*. Enzymatic protein hydrolysis offers one means of releasing bioactive peptides, and this technique is widely applied for the improvement of both nutritional and functional qualities of the sources of protein (Hartmann and Meisel, 2007). Peptides are understood to derive their biological properties and activities from factors such as composition, size, and amino acid structure. It is possible to use peptides in the production of nutraceuticals and functional foods which are able to prevent oxidative stress-related damage that arises in the course of various human diseases. Those amino acids that contain functional groups such as the imidazole and aromatic groups are known to be capable of interaction with free radicals. Moreover, a majority of the antioxidant peptides that are derived from foods offer low molecular weights falling in the range of 500 to 1,800 Da (Sefatiz et al., 2013).

The chicken farming sector in Thailand has witnessed significant changes in recent years, with poultry processing becoming a major industry. This leads to the increased availability of chicken feather meal (CFM) as an important by-product. Commercial poultry processing operations generate huge quantities of chicken feathers, which comprise 5–7% by weight of adult chickens. The composition of these feathers is around 90% proteins, which are both insoluble and fibrous, and contain bonds that are cross-linked with disulphide, among others. The principal constituent is insoluble keratin which has a high cysteine content (Manczinger et al., 2003). One problem is that when chicken feathers reach the environment, their effects are potentially harmful because of the high levels of amino acids they contain, including cysteine, arginine, glycine, and phenylalanine (Kumar et al., 2011). The aim of this research study was therefore to identify, characterize, and improve the antioxidative activity as well as the *in vitro* antiproliferative or cytotoxic activities of those protein hydrolysates which are derived from CFM.

MATERIALS AND METHODS

Materials

Brenntag (Mülheim, Germany) supplied the Alcalase (EC 3.4.21.62) and Flavourzyme (EC 3.4.11.1), while Novozymes (Bagsværd, Denmark) provided the Neutrase (EC 3.4.24.28). Bovine-serum albumin, 2,20-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Dulbecco's Modified Eagle Medium, L-ascorbic acid, trifluoroacetic acid, dimethyl sulfoxide, and fetal bovine serum were all obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis,

MO). Local commercial suppliers provided all other necessary chemicals that were of the greatest available purity.

CFM Hydrolysate Preparation

CFM was first obtained from Betagro Public Company (Thailand) before being dried in a hot air oven at a temperature of 60°C. It was then spun by churn and sieved through a 150- μ m mesh. In the next step, 0.5 g of CFM was mixed with 10 mL of 20-mmol phosphate buffer at a pH value of 7.2 and stirred with 150-mmol NaCl overnight at a temperature of 4°C, before hydrolysis which involved adding 1, 2.5, or 5 wt.% of either Alcalase, Flavourzyme, or Neutrase. The control used no addition. The process was then carried out in a shaker at 150 rpm and 50°C for 4 h. For Alcalase, the pH value was 8, while Flavourzyme and Neutrase were used at pH 7. To stop the reaction, the temperature was increased to around 80°C–90°C for up to 15 min. Collection of the supernatant was performed for a period of 15 min through centrifugation at $9,880 \times g$ at a temperature of 4°C for 10 min. The supernatant was collected. Protein hydrolysates were clarified by filtering through 0.45- μ m filters to remove the insoluble substrate and residual enzyme. The design of the experiment was fully randomized and performed in triplicate as proposed by Torres-Fuentes et al. (2011).

Protein Content Determination

Each of the protein hydrolysates was examined to establish the protein concentration using the Bradford procedure (Bradford, 1976) which applies bovine serum albumin for the standard. A spectrophotometer (Multiskan GO; Thermo Fisher Scientific, Waltham, MA) was then used to measure the absorbance at 595 nm (A_{595}).

Free Radical Scavenging Assay

DPPH Radical Scavenging Activity Assay A modified version of a technique described by Saisavoey et al. (2019) was used to carry out the DPPH radical scavenging activity assay. A DPPH radical solution of 100 μ mol in methanol was then added to each of the samples in the ratio of 1:4 (v/v), amounting to 80 μ L of the sample with 320 μ L of the DPPH radical solution. This mixture was then incubated in conditions of darkness for 15 min at room temperature. Centrifugation of the solution at $12,225 \times g$ for 5 min was then performed before measurements of the absorbance were taken at 517 nm (A_{517}) using a microplate reader. The positive control was ascorbic acid (100 μ g/mL).

2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) Radical Scavenging Activity Assay The ABTS radical scavenging activity assay was performed using a modified version of the technique proposed by Saisavoey et al. (2019). In this assay, the production of ABTS cation radicals required a 1:1 (v/v) mixture of ABTS solution (7 mmol) with potassium

persulphate (2.45 mmol) placed and complete darkness for 12 h at room temperature. The ABTS cation radical solution was then diluted to reach an absorbance of 0.7 ± 0.02 at 734 nm (A_{734}). This solution was then combined in a ratio of 1:30 (v/v) with the test hydrolysate, requiring 25 μ L of the sample with 750 μ L of the ABTS cation radical solution. Incubation for 10 min in darkness followed before the measurement of A_{734} using a microplate reader. The positive control in this assay was ascorbic acid (100 μ g/mL).

Percentage Inhibition Calculations

Equation (1) was used to calculate the radical scavenging percentage:

$$\frac{[(\text{Abs control} - \text{Abs blank}) - (\text{Abs sample} - \text{Abs background})] \times 100}{(\text{Abs control} - \text{Abs blank})} \quad (1)$$

where *Abs* control represented the absorbance of the control (no sample), *Abs* blank indicated the deionized water absorbance, *Abs* sample denoted the CFM hydrolysate (or peptide sample) absorbance, and *Abs* background indicated the extent of the samples' color absorbance. The necessary CFM hydrolysate concentration for the 50% inhibition of antioxidant activity, known as the IC_{50} value, could be determined using GraphPad Prism version 6.01 for Windows (GraphPad Software Inc., San Diego, CA).

Bioactive Peptide Isolation and Enrichment

Ultrafiltration The CFM hydrolysate that offered the greatest antioxidant capability on the basis of molecular weight cutoff membranes (Pellicon XL Filter; Merck Millipore, Billerica, MA) was further fractionated via ultrafiltration to derive 5 different fractions of molecular weight (MW) ≥ 10 kDa, MW 5–10 kDa, MW 3–5 kDa, MW 0.65–3 kDa, and MW < 0.65 kDa.

Reverse-Phase High-Performance Liquid Chromatography The most active of the antioxidant protein fractions was subsequently filtered using a 0.45- μ m nylon membrane (Whatman, GE, Buckinghamshire, UK) and then underwent further separation using reverse-phase high-performance liquid chromatography (RP-HPLC; Spectra System, Thermo Fisher Scientific, San Jose, CA) with a Luna 5U C₁₈ 100A column (4.6 mm \times 250 mm, Luna 5 μ mol; Phenomenex, Torrance, CA) which had a three-phase linear gradient of 100:0% (v/v) A:B decreasing to 90:10 (v/v) A:B after 18 min, to 65:35 (v/v) A:B after 30 min, and finally to 55:45 (v/v) A:B after 40 min while maintaining a flow rate of 0.7 mL/min. The composition of mobile phase A was determined to be 0.1% (v/v) trifluoroacetic acid, while for B, it was shown to be 70% (v/v) acetonitrile in 0.05% (v/v) trifluoroacetic acid. ChromQuest Software (Thermo Fisher Scientific, San Jose, CA) was used to carry out the chromatographic analyses. The peptide

peaks were eluted by monitoring at 280 nm, allowing the subsequent determination of the amino acid sequences for the purified peptides via quadrupole time-of-flight tandem mass spectrometry (Q-TOF-MS/MS).

Electrospray-Q-TOF-MS/MS and de Novo Peptide Sequencing Electrospray (ESI)-Q-TOF-MS/MS was used to accurately identify the amino acid sequence and molecular mass for the enriched antioxidant peptide by specifically calibrating the instrument to handle peptide chains with a mass falling in the range of 50–25,000 m/z (Model Amazon SL, Bruker, Germany). *De novo* sequencing and mascot were first applied to assess the ESI-Q-TOF-MS/MS data which had been gathered, whereupon the sequences were then compared with those in the NCBI database by using BLASTp.

Protective Influence of Enriched CFM Hydrolysate on DNA Damage Induced by Hydroxyl Radicals

The Use of Transformed Escherichia coli to Prepare Plasmids The templates used to assess hydroxyl radical damage via the Fenton reaction comprised 3 plasmid types: pKS, pUC19, and pBR322. These plasmids were used to transform *Escherichia coli* before selection on ampicillin containing plates streaked on Luria-Bertani agar (2% [w/v] agar with 10 g [w/v] peptone, 10 g [w/v] yeast extract, 5 g [w/v] NaCl) with ampicillin which underwent incubation overnight at a temperature of 37°C. One colony was then chosen to be cultured in 5 mL of Luria-Bertani broth combined with ampicillin at a temperature of 37°C while undergoing agitation overnight at a speed of 250 rpm. Harvesting of the plasmid was then carried out with a Qiagen mini-prep kit used in accordance with the guidelines of the manufacturer. Elution in TE buffer (10 mmol Tris-HCl; 0.5 mmol EDTA, pH 9.0) of the plasmid DNA was performed, before storage under refrigerated conditions at -20°C before use.

Hydroxyl Radical-Induced DNA Damage Assay Among the various enriched CFM peptides, F₂ was selected for further analysis on the basis that it generated the greatest antioxidant activity. Therefore, it was further tested to assess its capabilities in limiting the DNA damage induced by hydroxyl radicals, using the Fenton reaction as explained by Sheih et al. (2009). In the next step, 4 μ L of F₂ at varying concentrations of 50, 25, 12.5, 6.25, and 3.12 μ g/mL were added to 3 μ L of the plasmid DNA, of which here were 3 types: pKS 2,961 bp, pUC19 2,686 bp, and pBR322 4,361 bp. The respective DNA concentrations were 113.5, 45.5, and 30.7 ng/ μ L, and the mixtures were allowed to stand at room temperature for 20 min. Then, 3 μ L of 2-mmol FeSO₄ was added to 3 μ L of 30% (w/v) H₂O₂ to form a mixture which underwent incubation for 30 min at a temperature

of 37°C. The DNA was then coresolved using a DNA MW marker ladder via 1% (w/v) agarose-TBE gel electrophoresis. Visualization of the resolved DNA (2,000–5,000 bp) was possible after 10 min of staining by ethidium bromide under UV transillumination.

Antiproliferation and Cytotoxicity Activity of Enriched CFM Hydrolysate Peptides The *in vitro* antiproliferative/cytotoxic activity of the enriched CFM peptide which had offered the greatest level of antioxidant activity—the F₂ fraction—was evaluated in terms of the effects on one nontransformed (WI-38) and 5 cancer-derived transformed human cell lines (BT474, CHAGO-K1, HEP-G2, KATO-III, and SW620). The technique used was a modified version of that proposed by Pérez et al. (2014). HEP-G2 and SW620 cells were suspended in complete medium (CM; Roswell Park Memorial Institute with 10% (v/v) fetal calf serum) before being diluted and plated at 0.5×10^4 cell/well/200 μ L in CM in 96-well plates. Meanwhile, suspensions of BT474, CHAGO-K1, KATO-III, and WI-38 cells underwent dilution and plating in CM at 1×10^4 cell/well/200 μ L in 96-well plates, controlling the ultimate OD₅₄₀ value at 1. These were then incubated at a temperature of 37°C with 5% (v/v) carbon dioxide for 24 h. The original cell culture medium was then removed, and a fresh CM was added which contained the F₂ fraction derived from the CFM hydrolysate at varying concentrations (0.5–20 μ g/mL). The same incubation procedure as before was followed, but for 72 h. Then, to each well, 10 μ L of 5 mg/mL of MTT in normal saline solution was added, before mixing and incubation for 4 h. Removal of the medium was then followed by the addition of 150 μ L per well of dimethyl sulfoxide to dissolve the insoluble purple formazan crystals before using a microplate reader spectrophotometer to take measurements of the absorbance at 540 nm (A₅₄₀). This value for A₅₄₀ was deemed to be in direct proportion to the quantity of viable cells, thereby allowing the percentage cell viability to be calculated from the following equation:

$$\text{cell survival(\%)} = \frac{\text{Abs}_{\text{sample}} \times 100}{\text{Abs}_{\text{control}}} \quad (2)$$

in which *Abs* sample represented the A₅₄₀ for the CFM fraction, while *Abs* control indicated the A₅₄₀ for the control (no sample) which was fixed at 100% cell survival. The data were then used to establish the IC₅₀ value by using the GraphPad Prism (GraphPad Software, Inc., San Diego, CA) version 6.01 for Windows. The assays were all carried out in triplicate.

Analysis of Apoptosis The cell line for which the greatest sensitivity to the F₂ fraction was reported, in the form of the lowest IC₅₀ value, then underwent further screening to assess the induction of apoptosis by F₂. First of all, the SW620 cells found in CM underwent culturing in a 25-cm² culture flask in 5 mL of CM at a density of 5×10^5 cells per flask. After an interval of 24 h, these SW620 cultures were subsequently exposed to the F₂ fraction in varying quantities of 0.25, 0.5, 1,

and 2% (v/v), representing the respective protein concentrations of 0.15, 0.3, 0.6, and 1.2 μ g/mL. Incubation followed at a temperature of 37°C with 5% (v/v) carbon dioxide for 24 h. Furthermore, other SW620 cultures received the same treatment, but using 0.5 μ g/mL of doxorubicin or simply untreated cells to serve as the respective positive and negative controls. The harvesting process of the SW620 cells involved in each treatment was completed using a scraper, followed by 5 min of centrifugation at $600 \times g$ at a temperature of 4°C, before washing in 5 mL and 0.5 mL of 20 mmol PBS buffer which contained 1% (v/v) fetal calf serum. This was followed by further centrifugation for 5 min at $600 \times g$ at 4°C before suspension in 300 μ L of Annexin V binding buffer plus 5 μ L of Fluorescein isothiocyanate conjugated Annexin V solution in the case of untreated cells or in 100 μ L in the case of treated cells. In both cases, the suspension was placed on ice in darkness for 10 min. A further 100 μ L of Annexin V binding buffer with 10 μ L of Propidium Iodide Solution was then mixed with the treated cells, before the transfer of 200 μ L of both untreated and treated cell suspensions to 96-well plates for further analysis of Fluorescein isothiocyanate-Annexin V and Propidium Iodide Solution staining through the use of a Cytomics EC500 MPL flow cytometer (BD FACSCalibur; BD Biosciences, Singapore). The resulting data were then analyzed using FlowJo (FlowJo LLC, Ashland, OR) software version 7 (Chung et al., 2010).

The SW620 cells were then exposed to the greatest concentration of the F₂ fraction (% [v/v] or protein content) capable of inducing apoptotic cells in the assay described previously, with the exception of 24 h, 48 h, and 72 h, and analyzed in the same manner.

Activity of Caspase-3 and Caspase-8

SW620 Cell Lysate Preparation Seeding of the SW620 cells was performed at 1×10^7 cells per flask, followed by the addition of the F₂ fraction at the concentration required for the IC₅₀ value in the context of the cells to be treated. Incubation at a temperature of 37°C in 5% (v/v) carbon dioxide was then carried out for periods of 24, 48, and 72 h. Both control and treated cells were subsequently harvested with a scraper before washing in 20 mmol cold PBS and centrifugation for 15 min at $600 \times g$. The resulting cell pellets subsequently underwent resuspension in $1 \times$ lysis buffer (100 μ L) before being incubated on ice for around 15–20 min. Once lysed, these cells were placed in the centrifuge for 15 min at $12,225 \times g$ at a temperature of 4°C before then placing the supernatants, or lysates, in new tubes if they were to be used immediately or in tubes for storage under refrigeration to -70°C to later carry out the analysis of the caspase activity.

Caspase-3 and Caspase-8 Activity Assay The caspase-3 and caspase-8 activity was determined through the use of colorimetric caspase-3 and caspase-8 assay kits, which allow the assessment of the hydrolysis of the acetyl-Asp-Glu-Val-Asp *p*-nitroaniline

Table 1. The IC₅₀ value of the DPPH and ABTS radical scavenging activity of the CFM hydrolysates formed from different enzymes.

Enzyme	Free radical scavenging activity (IC ₅₀) (µg/mL)*			
	Control	1 wt.%	2.5 wt.%	5 wt.%
DPPH assay				
Alcalase®	269.13 ± 10.73 ^c	68.30 ± 2.56 ^b	61.71 ± 11.05 ^b	56.76 ± 1.46 ^b
Flavourzyme®	121.43 ± 10.19 ^{c,c}	50.86 ± 4.52 ^{b,b}	31.66 ± 0.78 ^{aa,bb}	20.84 ± 0.67 ^{aa,a}
Neutrase®	96.63 ± 4.09 ^D	42.60 ± 0.48 ^C	27.63 ± 1.25 ^B	16.45 ± 0.23 ^A
ABTS assay				
Alcalase®	42.89 ± 1.87 ^b	12.92 ± 0.05 ^a	12.86 ± 0.08 ^a	9.97 ± 0.31 ^a
Flavourzyme®	64.72 ± 2.28 ^{c,c}	19.07 ± 2.19 ^{b,b}	14.88 ± 0.33 ^{aa,bb}	10.16 ± 0.19 ^{aa,a}
Neutrase®	60.18 ± 2.51 ^C	18.97 ± 1.16 ^B	13.60 ± 0.06 ^{A,B}	9.34 ± 0.08 ^A

The data are presented as the mean ± SE from triplicate results.

^{b-c, aa-cc, A-D} Values within a column or a row with different letters are significantly different ($P > 0.05$).

*Ascorbic acid was the positive control with an IC₅₀ value of 26.97 ± 0.34 and 98.44 ± 3.79 µg/mL for DPPH and ABTS, respectively.

(Ac-DEVD-*p*NA) or acetyl-Ile-Glu-Thr-Asp *p*-nitroaniline (Ac-IETD-*p*NA) peptide substrates because these result in the release of the *p*-nitroaniline (*p*NA) moiety. Monitoring of the *p*NA release was performed by following the absorbance at 405 nm (A_{405}). Both the cell lysates and the caspase-3 (5 µL) or caspase-8 (10 µL) positive control were added to 96-well plates, along with 85 µL of the respective 1 × assay buffer. Initiation of the reaction was achieved by the addition of 10 µL of caspase-3 or caspase-8 substrate to each of the wells, before mixing and incubation for up to 90 min at a temperature of 37°C. The A_{405} values were then measured and converted into *p*NA concentrations which can refer to caspase activities when interpreted using a *p*NA calibration curve. Equation 3, shown below, was used to calculate the caspase-3 and caspase-8 activities:

$$\text{Activity (mmol } p\text{NA min}^{-1}\text{mL}^{-1}) = \frac{\mu\text{mol } p\text{NA} \times d}{t \times V} \quad (3)$$

in which v denotes the sample volume (mL), d indicates the dilution factor, and t represents the time taken for the reaction in minutes.

Statistical Analysis

Every one of the triplicate outcomes is presented in the form of mean ± standard error. The statistical analysis was performed using one-way ANOVA in addition to Duncan's new multirange test applying version 15.0 of the Statistical Package for Social Sciences (SPSS) (IBM, NY). The significance level was set at $P < 0.05$.

RESULTS AND DISCUSSION

Protein Hydrolysate Free Radical Scavenging Activities Assay

DPPH and ABTS radical scavenging activity assays were used to assess the free radical scavenging capabilities of the hydrolysates which were obtained from the CFM. The results are shown in Table 1 in the form of IC₅₀ values. All of those CFM hydrolysates which were obtained via

microbial proteases demonstrated antioxidant properties, but the most effective one was that derived from 5 wt.% Neutrase treatment, which was observed to achieve the greatest level of antioxidant activity according to the DPPH and ABST assays. The IC₅₀ values were 16.45 ± 0.23 and 9.34 ± 0.08 µg/mL for the respective DPPH and ABTS assays. To draw comparisons, the hydrolysate which was obtained from 2 wt.% Neutrase treatment of bluefin leatherjacket (*Navodon septentrionalis*) was observed to have an IC₅₀ value of 9.105 µg/mL for the DPPH assay according to Chi et al. (2015a,b,c), whereas the hydrolysate from 0.1 wt.% Neutrase-treated brown seaweed (*Scytosiphon lomentaria*) showed very strong scavenging activity of DPPH radicals at 74.03% according to the study by Ahn et al. (2004).

Moreover, these findings indicated that differing antioxidant levels will result in the CFM hydrolysates from the different types and concentrations of microbial protease enzymes used. This may be the result of different hydrolyzed peptide bond patterns; for example, flavourzyme is both an endoprotease and exoprotease, while Alcalase and Neutrase are simply categorized as endoproteases (Kim et al., 2013). In this study, the CFM hydrolysate obtained from the 5 wt.% Neutrase treatment was chosen for additional study because it provided the lowest IC₅₀ value.

Enrichment of Free Radical Scavenging Peptides Derived From 5 wt.% Neutrase-Treated CFM

Fractionation of the 5 wt.% Neutrase CFM hydrolysate was carried out via ultrafiltration using 0.65, 3, 5, and 10 kDa molecular weight cutoff membranes. The findings confirmed that the fraction that did not exceed 0.65 kDa had the greatest DPPH and ABTS activity in radical scavenging, for which the respective IC₅₀ values were 1.72 ± 0.04 and 0.42 ± 0.02 µg/mL. Peptides of low MW showed greater radical scavenging ability than high MW peptides. It was reported earlier that the peptide fractions obtained from bluefin leatherjacket skin (Chi et al., 2015a), heads (Chi et al., 2015b), and blood clams (Chi et al., 2015c) demonstrated their greatest free radical scavenging activity

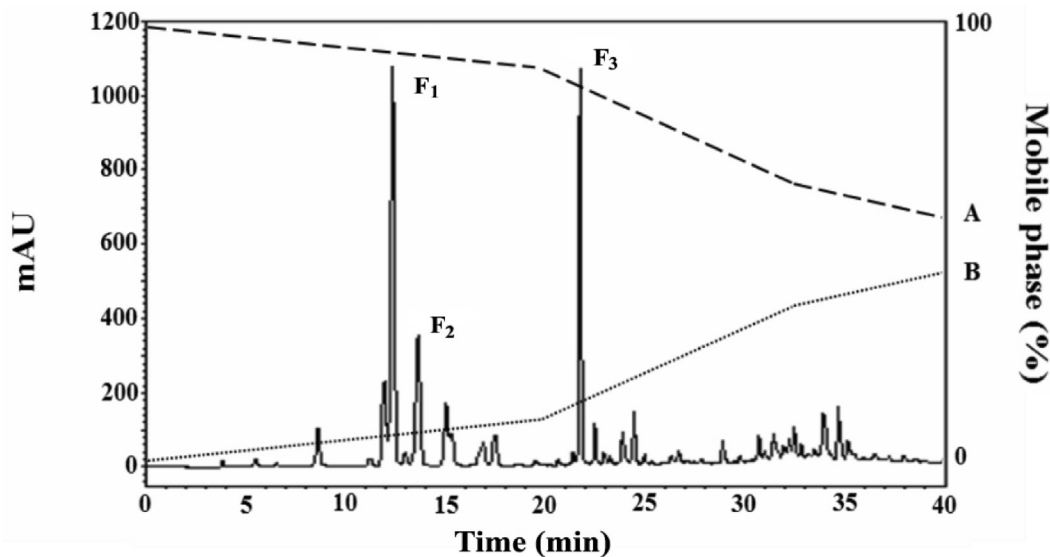


Figure 1. RP-HPLC chromatography of the molecular weight (MW) < 0.65 kDa fraction obtained from 5 wt.% Neutrase CFM hydrolysates. Abbreviation: RP-HPLC, reverse-phase high-performance liquid chromatography.

in the fractions of the lowest molecular weight (<1 kDa). These low MW peptides are able to produce this increased antioxidant activity as a result of their ability to interact more readily with the radicals, hindering the process of oxidization (Wang et al., 2012; He et al., 2013). Accordingly, it was the peptide fraction with the lowest MW (<0.65 kDa) offering the greatest antioxidant activity which was chosen to be enriched further.

Reverse-Phase High-Performance Liquid Chromatography

Purification of the MW < 0.65 kDa fraction which generated greater free radical scavenging activity was carried out by filtration using a 0.45- μ m nylon membrane followed by RP-HPLC. Three peaks were reported and collected and are designated as the F₁, F₂, and F₃ fractions, which had respective IC₅₀ values for the DPPH radical scavenging activity assay of 32.01 ± 2.17 , 22.52 ± 4.87 , and 59.66 ± 5.89 μ g/mL (Figure 1). However, these 3 fractions did not exhibit any antioxidant activity during the ABTS radical scavenging activity assay. It is important to consider that DPPH is a type of free radical capable of accepting radical electrons or hydrogen to become stable, whereas radical ABTS can be lowered when antioxidants are combined with properties of hydrogen-donating or chain-breaking. There are also, however, alternative reasons including radical stereoselectivity or extract solubility which arise in different testing systems which can explain the capacity of particular extracts to react with or quench various radical types (Zhu et al., 2008; Rival et al., 2011). Accordingly, enhanced DPPH assay activity is not necessarily indicative of a greater ability to scavenge ABTS radicals.

Analysis of Peptides and de Novo Sequencing Using ESI-Q-TOF-MS/MS Analyses

Characterization of the molecular masses of the enriched fractions (F₁, F₂, and F₃) from RP-HPLC as well as determination of the peptide sequences were carried out by ESI-Q-TOF-MS/MS. For the F₂ fraction, the peptide amino acid sequences were assessed via *de novo* peptide sequencing to be Val-Thr-Leu-Ala-Val-Thr-Lys-His (VTLAVTKH, 868 Da) (Figure 2). It was not feasible, however, to assess F₁ and F₃ using the same *de novo* peptide sequencing approach, and hence, these fractions were analyzed using a database search against the SwissProt database with Mascot software, as well as through the BLASTp database of NCBI. The respective results were presented as Phe-Asp-Asp-Arg-Gly-Arg-X (FDDRGRX, 875 Da) and Val-Ser-Glu-Ile-X-Ser-Ile-Pro-Ile-Ser (VSEIXSIPIS, 1,055 Da).

Earlier reports have argued that in determining the antioxidant capabilities of peptides, composition, amino acid sequence, and molecular size play a major role (Chen et al., 2012a,b). In this study, it was found that the F₁ and F₂ fractions with respectively 7 and 8 amino acid residues showed greater DPPH radical scavenging activity than the F₃ fraction with 10 amino acid residues. Furthermore, those peptides which have 2 to 10 amino acids were observed to affect bioactivity to a greater extent than the indigenous parent proteins or large polypeptides according to Zhu et al. (2008). Moreover, it is possible for the benzene ring and indole group of aromatic amino acids containing Phe, Tyr, Trp, and His to give protons to radicals with electron deficiencies, ensuring that there is increased stability of the reactive oxygen species while the aromatic amino acids are able to support their own stability via their resonance structure (Rajapakse et al., 2005a).

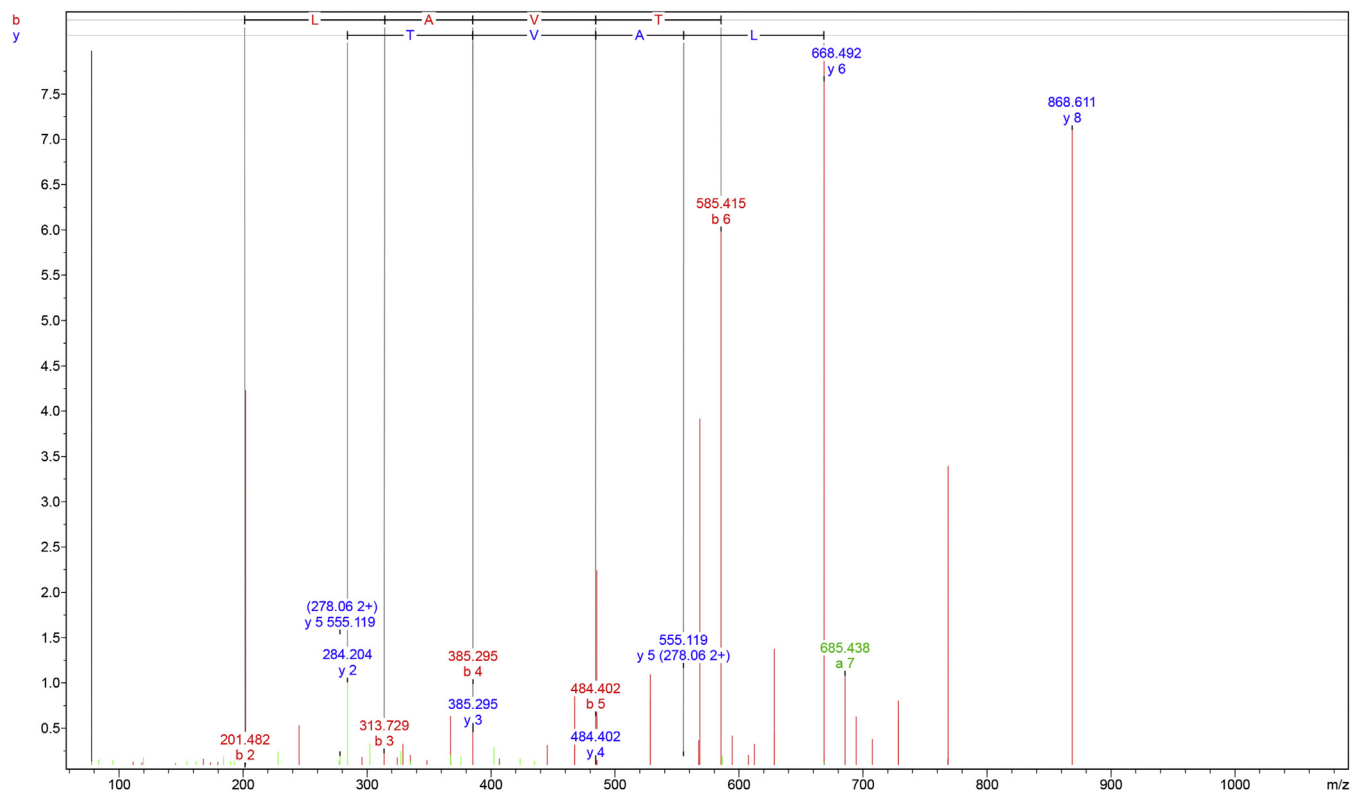


Figure 2. Mass fragmentation spectrum of subfraction F₂ (VTLAVTKH) obtained by reversed-phase high-performance liquid chromatography (Figure 1).

Moreover, antioxidant peptides comprising various different hydrophobic amino acids including nonpolar aliphatic groups containing Val, Pro, Tyr, Trp, Leu, Ile, Ala, Lys, and Met were previously observed to exhibit strong antioxidant properties (Rajapakse et al., 2005b; Elias et al., 2008; Guo et al., 2009). This is because the hydrophobic amino acid residues within the peptide sequences can lead to improved peptide solubility at the interface between water and lipids, allowing enhanced interactivity with any radical species present (Ranathunga et al., 2006). Such species would include hydrophobic radical species in addition to hydrophobic polyunsaturated fatty acids, namely omega-3 and omega-6 (Chen et al., 1998; Suetsuna and

Chen, 2002; Qian, et al., 2008a). Reports also exist to suggest that hydrogen donors that contain Gly are more active as antioxidants. Meanwhile, the SH component of Cys is known to be a radical scavenger capable of significant antioxidant action which results from any contact with radicals (Qian et al., 2008b).

One final factor that is important in determining the antioxidant activity of peptides is the positioning of the amino acids within the peptide sequence (Rajapakse et al., 2005a). For instance, when His appears at the N-terminal, this serves as an effective chelator of metal ions, while His located at the C-terminal becomes an effective scavenger of various different radicals (Chen et al., 1998). Meanwhile, tripeptides

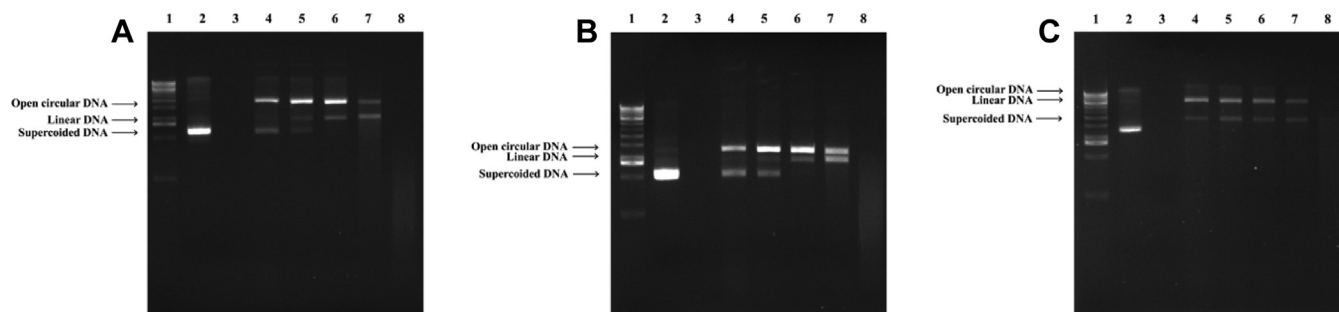


Figure 3. Representative patterns of agarose gel electrophoresis of the (A) pKS, (B) pUC19, and (C) pBR322 plasmids after hydroxyl radical treatment via the Fenton reaction in the presence of the F₂ fraction at various concentrations. Lane 1: marker 1 kbp; lane 2: plasmid DNA (pKS is 2,961 bp, 113.5 ng/μL; pUC19 is 2,686 bp, 45.5 ng/μL; and pBR322 is 4,361 bp, 30.7 ng/μL); lane 3: plasmid DNA with FeSO₄ and H₂O₂ treatment (as DNA damage control); lanes 4–8: plasmid DNA with FeSO₄ and H₂O₂ treatment in the presence of the F₂ fraction at 50, 25, 12.5, 6.25, and 3.12 μg/mL, respectively.

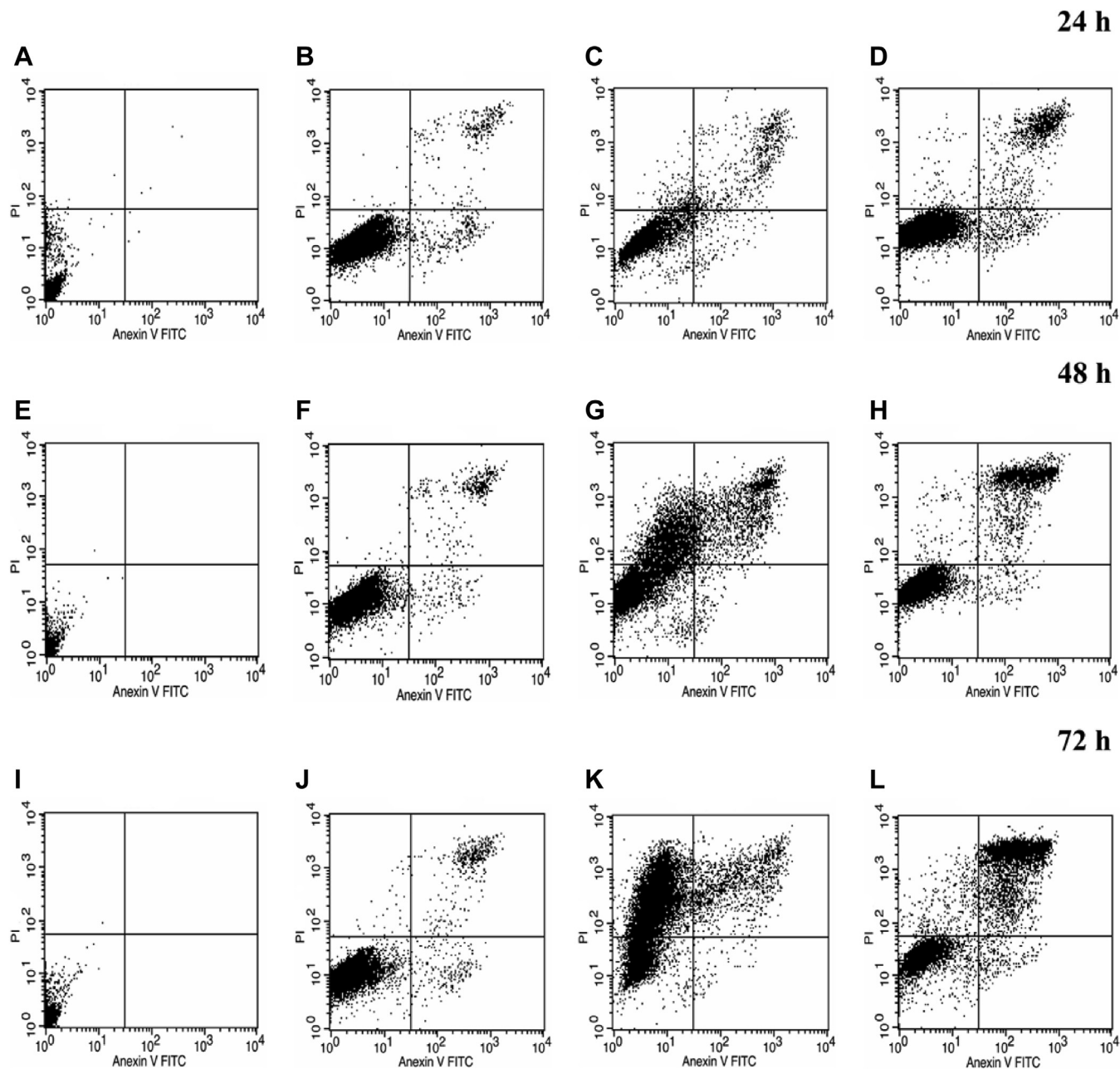


Figure 4. Flow cytometry analysis of SW620 cells cultured for 24 h (A–D), 48 h (E–H), and 72 h (I–L) in CM with (A, E, and I) nothing (blank control) and (B–D, F–H, and J–L) cells labeled with Fluorescein isothiocyanate-annexin V and Propidium Iodide Solution after culture with (B, F, and J) nothing (negative control), (C, G, and K) the F₂ fraction with IC₅₀ concentration, and (D, H, and L) 0.5 µg/mL of doxorubicin (positive control). Quadrants: Bottom left, the normal or live cells; top left, the necrotic cells; bottom right, the early apoptotic cells; top right, the late apoptotic cells.

that have Trp or Tyr at the C-terminal act as powerful radical scavengers (Saito et al., 2003). Therefore, the F₁ (FDDRGRX) fraction observed to achieve DPPH radical scavenging activity with an IC₅₀ value of 32.01 ± 2.17 µg/mL might attribute this to the existence within the sequence of Phe, which is known to be an aromatic amino acid. For F₂ and F₃, (VTLAVTKH and VSEIXSIPIS, respectively), the F₂ amino acid sequences included hydrophobic amino acids such as Val and Pro, which would explain the antioxidant properties. Also, in the F₂ fraction was His, which might further explain the high level of antioxidant activity.

Protective Influence of the F₂ Fraction on DNA Damage Induced by Hydroxyl Radicals

The substrates used in testing to determine the protective influence on DNA of the F₂ fraction against hydroxyl radical-induced damage were 3 different plasmids: pKS, pUC19, and pBR322. Plasmids were induced to create DNA strand breakages under the Fenton reaction which can take 3 forms: supercoiled, linear, and open circular DNA. The F₂ fraction was used in varying concentrations of 50, 25, 12.5, 6.25, and 3.12 µg/mL to prevent DNA damage induced by hydroxyl radicals.

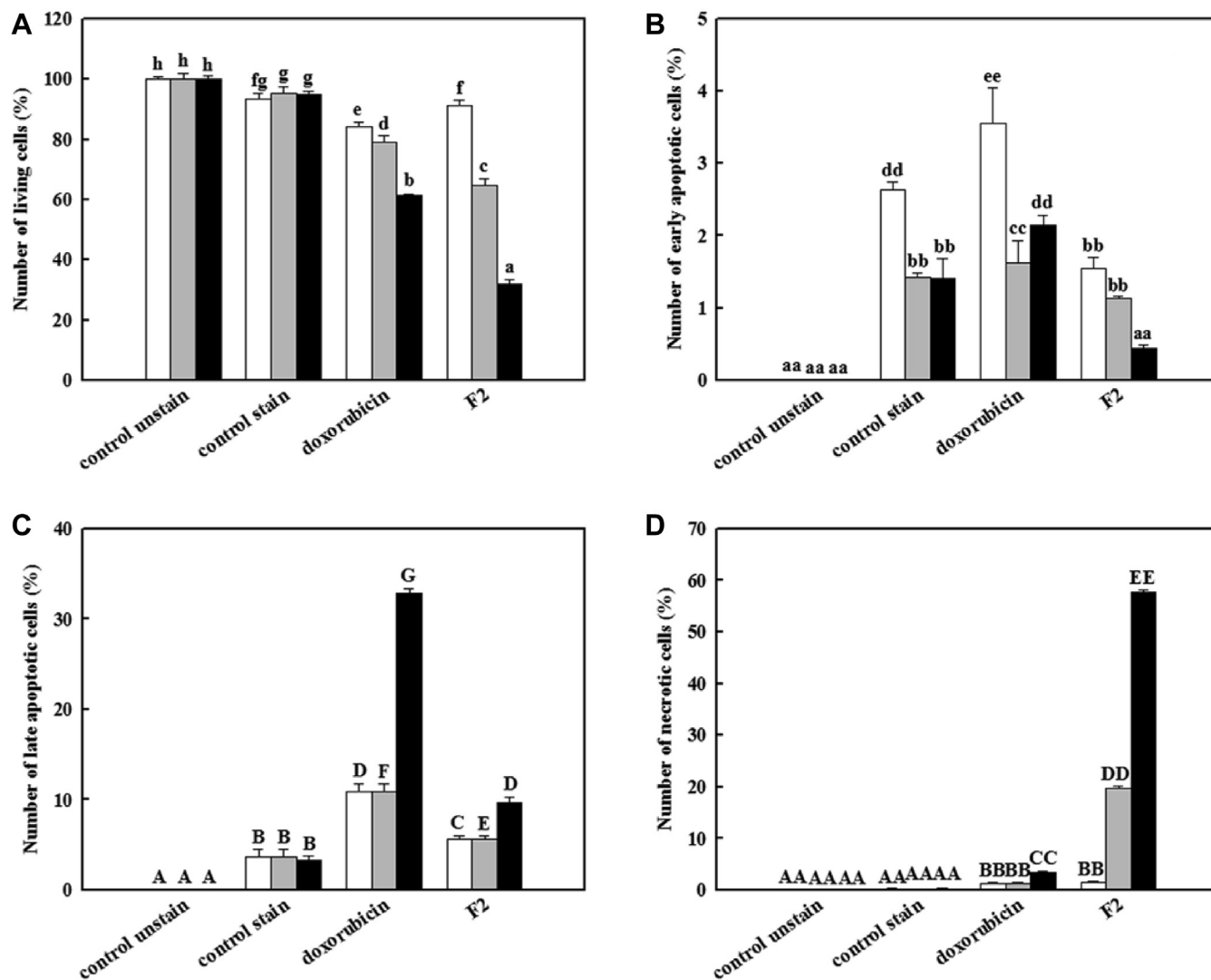


Figure 5. Proportion (%) of SW620 cells that were (A) normal live cells, (B) early apoptotic cells, (C) late apoptotic cells, and (D) necrotic cells after treatment for 24 h, 48 h, or 72 h with nothing (control stain), doxorubicin at 0.5 $\mu\text{g}/\text{mL}$, or the F₂ fraction at the IC₅₀ concentration. Data are shown as mean \pm 1 standard error of mean, and the results are obtained in triplicate. Means indicated using a different letter show a significant difference.

The findings showed that supercoiled DNA could be wholly converted to become linear or open circular DNA during the Fenton reaction in the absence of F₂, but when F₂ was present, a smaller proportion of supercoiled DNA underwent this conversion at 50 and 25 $\mu\text{g}/\text{mL}$ when testing the pKS and pUC19 plasmids, although this was not observed in the case of pBR322, as shown in Figure 3. The F₂ concentrations which were examined were all capable of reducing the potential for DNA damage induced by hydroxyl radicals to varying degrees, with the exception of the 3 $\mu\text{g}/\text{mL}$ concentration, which was too low to achieve any protection in any of the 3 plasmids.

Sheih et al. (2009) observed that it is possible for supercoiled DNA to undergo complete conversion to different forms when exposed to peptides. It was also reported by Lee et al. (2012) that purified peptides are able to prevent DNA damage induced by hydroxyl radicals depending on the dose involved, from 0.125 to 0.1 mg/mL. In this study, the findings indicate that greater F₂

concentrations, such as 50, 25, 12.5, and 6.25 $\mu\text{g}/\text{mL}$, from 5 wt.% Neutrased CFM were more effective in preventing DNA damage under the Fenton reaction. The antioxidant qualities of the F₂ fraction and ability to prevent DNA damage might be applicable in the context of an antiproliferative assay. Accordingly, it can be stated that 5 wt.% Neutrased CFM hydrolysate can create peptides capable of restricting the oxidative damage caused to DNA.

Antiproliferative Activities of the F₂ Fraction From 5 wt.% Neutrased CFM Hydrolysates

One way to carry out initial screening for potential activity against cancer or for apoptotic cell death patterns is to use the antiproliferative activity assay. This research study made use of WI-38 to serve as the human normal fibroblast cell line, while BT474, Chago-K1, HepG2, KATO-III, and SW620 served as cancer-derived cell

Table 2. Caspase-3 and caspase-8 activity levels in SW620 cells exposed to the F₂ with the concentration corresponding to IC₅₀, or doxorubicin at 0.5 μg/mL for 24, 48, and 72 h.

Enzyme/treatment	Caspase activity (pmole pNA/min/mL) after:		
	24 h	48 h	72 h
Caspase-3			
Untreated (control)	$2.59 \times 10^{-5} \pm 0.17 \times 10^{-5a}$		
F ₂ fraction	$2.73 \times 10^{-5} \pm 0.05 \times 10^{-5a}$	$5.33 \times 10^{-5} \pm 0.38 \times 10^{-5b}$	$3.24 \times 10^{-5} \pm 0.03 \times 10^{-5a}$
Doxorubicin (0.5 μg/mL)	$8.18 \times 10^{-5} \pm 0.26 \times 10^{-5c}$	$8.90 \times 10^{-5} \pm 0.67 \times 10^{-5c}$	$9.12 \times 10^{-5} \pm 0.32 \times 10^{-5c}$
Caspase-8			
Untreated (control)	$1.70 \times 10^{-5} \pm 0.30 \times 10^{-5 a,a}$		
F ₂ fraction	$2.58 \times 10^{-5} \pm 0.30 \times 10^{-5 a,a,b,b}$	$5.18 \times 10^{-5} \pm 0.57 \times 10^{-5 c,c}$	$3.38 \times 10^{-5} \pm 0.06 \times 10^{-5 b,b}$
Doxorubicin (0.5 μg/mL)	$5.38 \times 10^{-5} \pm 0.18 \times 10^{-5 c,c}$	$7.15 \times 10^{-5} \pm 0.26 \times 10^{-5 d,d}$	$11.05 \times 10^{-5} \pm 0.74 \times 10^{-5 e,e}$

The data are presented as the mean ±SE from triplicate results. Values within a column or a row with different letters are significantly different ($P > 0.05$)

lines from humans. These cells were all exposed to varying concentrations of the F₂ fraction before incubation for 72 h. MTT assay was then performed to determine the quantity of viable cells in each case. The antiproliferative activity of the F₂ fraction was shown to be much stronger in the context of SW620 cells, achieving an IC₅₀ value of 26.37 ± 2.87 μg/mL, than is the case with the BT474, Chago-K1, Hep-G2, and KATO-III cell lines, which demonstrated respective IC₅₀ values of 33.39 ± 0.43 , 32.47 ± 0.48 , 32.52 ± 0.78 , and 32.53 ± 2.44 μg/mL.

It was previously reported that antioxidant peptides have the ability to inhibit the proliferation of cancer cells in addition to inducing cell apoptosis (Hsu et al., 2011; Chi et al., 2015c). In this research study, however, there was no significant effect reported for the F₂ fraction in terms of cytotoxicity toward the human normal fibroblast cell line (WI-38).

SW620 Cell Apoptosis Induced by the F₂ Fraction of the CFM Hydrolysate

To assess the long-term potential for cancer treatment drugs, the F₂ fraction from the 5 wt.% Neutrased CFM hydrolysate underwent investigation of its capacity for the induction of apoptosis. In particular, colon cancer (SW620) was an area of interest, and therefore, the most sensitive cell line of SW620 was used in testing. The cells were first of all exposed to the IC₅₀ concentration and were then harvested after 24 h, 48 h, or 72 h before being left to stand for 24, 48, or 72 h after harvesting before the analysis for cell apoptosis. Figures 4 and 5 present the results for the F₂ fraction at 24 h for early apoptosis, late apoptosis, and necrosis as 1.55 ± 0.15 , 5.68 ± 0.33 , and 1.51 ± 0.20 , respectively, with the data representing the percentage of the cell population. At 48 h, similar results were obtained as 1.13 ± 0.03 , 14.3 ± 0.09 , and 19.84 ± 0.33 , respectively. At 72 h, the figures were 0.44 ± 0.05 and 9.69 ± 0.06 , respectively, indicating a decline in both early and late apoptosis, although necrotic cells had risen in terms of their percentage to reach 57.83 ± 0.26 . The results presented here, however, were significantly lower than those in the case when using doxorubicin.

It is apparent that low levels of apoptosis were reported at 24 h and 48 h for SW620 cells exposed to the F₂ fraction, and therefore, these times might be most appropriate for cancer treatments. Moreover, it was reported by Chen et al. (2012a,b) that 200-μmol Baicalin, which is a flavone derivative that can be isolated and sublimated from the dry roots of *Scutellaria*, has been linked with antiproliferative activity in the context of SW620 cells, while leading to an increased proportion of apoptotic cells. Meanwhile, Pérez et al. (2014) reported that *Ilex laurina* and *Ilex paraguariensis* have leaves that can restrict the viability of SW620 cells. The respective IC₅₀ values were found to be 115 and 133.4 μg/mL, and the test results indicated early apoptotic cell proportions of 25 and 30% when exposing SW620 cells for 48 h. In addition, Ji et al. (2011) described the use of a triterpene-enriched extract from *Ganoderma lucidum* (GLAI) when inducing apoptosis in SW620 cells. GLAI is a triterpene-enriched extract derived from the fruit of *Ganoderma lucidum*.

Caspase-3 and Caspase-8 Activities

The concentration of the F₂ fraction matching IC₅₀ was chosen to assess its capacity for the induction of caspase-3 or caspase-8 activities in the cell lysates of those SW620 cells which had been exposed to F₂ for periods of 24 h, 48 h, or 72 h. Caspase-3 is a vital caspase as part of the proapoptotic cascade and has a critical role to play in different types of apoptosis (Wu et al., 2014). Meanwhile, caspase-8 serves as a common factor which stimulates the process of apoptosis (Nagata, 1997). Therefore, when these caspases increase their activity, this can be seen as an indication of apoptosis and hence might be considered an alternative assay. The activity of caspase-3 in the case of SW620 cells exposed to the F₂ fraction for periods of 24, 48, and 72 h was shown to be at its lowest after the 24-h period ($2.73 \times 10^{-5} \pm 0.05 \times 10^{-5}$ pmole pNA/min/mL) while the highest values came after 48 h ($5.33 \times 10^{-5} \pm 0.38 \times 10^{-5}$ pmole pNA/min/mL). At 72 h, a decline had begun to take effect, as shown in Table 2.

In the case of caspase-8 and SW620 cells, the F₂ fraction was similarly at its most effective state after 48 h ($5.18 \times 10^{-5} \pm 0.57 \times 10^{-5}$ pmole pNA/min/mL) as

can be seen in Table 2. When the activities of caspase-3 and caspase-8 are combined, it can be seen that the F₂ fraction resulted in maximized activity after 48 h (171.2 pmole pNA/min/mL) than was the case after just 24 h (59.9 pmole pNA/min/mL) or 72 h (23.8 pmole pNA/min/mL). Maximum caspase-3 and caspase-8 induction levels at 48 h when exposed to the F₂ fraction matched the exposure time required for maximal apoptosis when observed via flow cytometry, suggesting links between the process of apoptosis and the induction of the activities of caspase-3 and caspase-8. Increasing the proportion of apoptotic cells should, therefore, increase the activity of caspase-3 and caspase-8. Earlier studies have shown that in SW620 cells, caspase-3 and caspase-8 show increased activity after exposure to 200 µmol of Baicalin for 48 h, leading to apoptosis (Chen et al., 2012a,b). Moreover, GLAI was also reported to induce the activity of caspase-3 in SW620 cells after exposure to 10, 25, and 50 µg/mL for 24 h (Ji et al., 2011).

CONCLUSIONS

This research demonstrated that 5 wt.% Neutrase hydrolysate which was derived from CFM was capable of generating peptides offering the greatest levels of antioxidant activity. The assessment of the activity levels for the various membrane-separated ultrafiltration fractions indicated that low MW peptides (MW < 0.65 kDa) played an essential part in antioxidant activity, with strong performance recorded in DPPH and ABTS assays for free radical scavenging. Additional fractionation via RP-HPLC created the 3 principal fractions, F₁, F₂, and F₃, among which F₂ was shown to offer the highest activity level in the DPPH assay, although the ABTS assay brought about no activity from any of the fractions, indicating strong antioxidant activity in the ABTS assay from at least one of the minor fractions from RP-HPLC. Furthermore, F₂ was also shown to inhibit DNA damage caused by hydroxyl radical activity when the concentration of F₂ was increased beyond 3 µg/mL. There were no cytotoxic effects reported for the F₂ fraction in the context of untransformed WI-38, but such effects were reported for all the 5 transformed cancer cell lines which were tested. The greatest antiproliferative activity effect was recorded for the SW620 cell line representing colon cancer. This resulted partly from the weak apoptosis induction with stronger caspase-3 and caspase-8 activity induction when SW620 cells were exposed to the F₂ fraction at 0.5% for 48 h. The results presented here indicate that the peptides derived from CFM via Neutrase enzymatic hydrolysis might usefully serve as natural antioxidants capable of enhancing the functional and antioxidant properties in foods and nutraceuticals.

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