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Identification of Antioxidative Peptides from Roselle (*Hibiscus Sabdariffa* Linn) Seeds Protein Hydrolysates

Fatoumata Tounkara[1, *], Issiaka Togola[1], Mamadou Abdoulaye Konare[1], Mah Moutaga Fane[2]

[1]Laboratory of Plant, Food Biochemistry and Biotechnology, Faculty of Sciences and Techniques (FST), University of Sciences, Techniques and Technologies of Bamako (USTTB), Bamako, Mali

[2]Laboratory of Organic Chemistry and Natural Substances, Faculty of Sciences and Techniques (FST), University of Sciences, Techniques and Technologies of Bamako (USTTB), Bamako, Mali

Email address:

toucoulsup@gmail.com (F. Tounkara), togola3@gmail.com (I. Togola), konaresucces@gmail.com (M. A. Konare), clemantine_2008@yahoo.fr (M. M. Fane)

[*]Corresponding author

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Abstract: In this study Roselle seed protein isolates were digested using pepsin followed by pancreatin at different time in order to produce hydrolysate with good antioxidant. The prepared hydrolysates were as effective as antioxidants in model systems, in scavenging of free radicals. This effect was concentration-dependent and was also influenced by the hydrolysis time. Among all the hydrolysates, the 3 hours Roselle seed protein hydrolysate showed the highest antioxidant activity. Then it was separated into four fractions (I, II, III and IV) by filtration on Sephadex G-15. The antioxidant activities of the fractions were investigated using different *in vitro* methods. All fractions were effective antioxidants, with fraction III showing the highest antioxidant activity. The Reverse phase high performance liquid chromatography purification was then performed to the fraction (FIII). From the resultant, five isolated peptides from the active peak (FIII-3), were identified by liquid chromatography/ tandem mass spectrometry (LC-MS/MS), to contain Thr-Val-Glu-Asn-Leu/ Ala-Leu-Gly-Ala-Asp-Cys-Asp-Val/ Tyr-Thr-Met-Phe-Ser-Thr-Ser-Trp-Phe/ His-Asn-Asp-Pro-Glu-Phe/ Thr-Pro-Glu-Cys-Asn-Val amino acid structural sequences. The study confirmed that peptides with strong antioxidant activities from Roselle seed protein can be successfully provided through enzymatic digestion. In addition, five purified peptides are mainly responsible for the potent free radical scavenging of the fraction and the hydrolysate. Thus, they could be included as antioxidant constituents in the food industry due to their role in food quality preservation and would be expected to protect against oxidative damage in living systems. For the first time, antioxidant peptides were purified and identified from prepared Roselle seed protein hydrolysates.

Keywords: Roselle-Seeds, Protein- Hydrolysates, Identification, Antioxidative-Peptides

1. Introduction

Reactive oxygen species are the natural byproducts of oxygen metabolism in cells and are efficiently scavenged by antioxidant defense systems including antioxidant enzymes and antioxidants under normal conditions *in vivo* [1]. However, the physiological equilibrium between ROS generation and elimination will be broken when the organism is under pathological conditions (including excessive pressure, smoking, ultraviolet radiation, environmental pollution, etc.) for a long time. Excessive ROS can destroy functional molecules, which further trigger many chronic diseases in the body [2]. On the other hand, lipid oxidation in foods is generally undesirable as it may lead to changes in flavor, shape, and coloration.

Antioxidants can transform ROS into more stable forms or molecular structures to prevent the propagation of the ROS-mediated peroxidizing chain reaction by serving as a proton donor, hydrogen donor, and/or lipid peroxyl radical trap [3]. Furthermore, antioxidants can regulate *in vivo* antioxidant enzyme systems including GSH-Px, catalase (CAT), and

SOD to reduce the damage of oxidative stress [4]. In order to provide protection against serious diseases and to prevent foods from undergoing deterioration, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethoxyquin, and n-propyl gallate are widely used. Unfortunately, the long-term use and high doses of synthetic antioxidants in food are strictly limited due to their potential risks related to health [5, 6].

Therefore, there is an increasing interest in using original antioxidants with low toxicity from natural sources because they are safer and healthier [7, 8]. Bioactive peptides may act as potential physiological modulators of metabolism during the intestinal digestion of the diet.

They usually contain 3–20 amino acid residues and their activity is based on their amino acid composition and sequence [9]. The possible regulatory effects of peptides relate to nutrient uptake, immune defense [9, 10], antioxidant [11] and antihypertensive activities [12, 13]. These peptides have been isolated from various protein sources, such as hoki frame [14], rice endosperm [15], peanut [16], soybean [17] and corn gluten [18, 19].

Lastly, we reported about the antioxidant activities of Roselle seed protein hydrolysates and its derived peptide fractions [20]. From that study Roselle seed protein hydrolysate has been separated by gel filtration using Sephadex G-15, in which fraction III showed the highest antioxidant activity.

However, the structures and sequences of these responsible antioxidant peptide fractions are not known. This study is a part of our ongoing work and its aim was to purify and identify the responsible antioxidant peptides from fraction III collected from the digested Roselle seed proteins after gel filtration using Sephadex G-15. The antioxidant activities of the different the collected peptide fractions were investigated. The active peptides were further purified and sequences determined by MALDI-TOF/TOF MS/MS of the constituents responsible for the antioxidant activity.

2. Material and Methods

2.1. Material

Roselle (*Hibiscus sabdariffa L.*) seeds were obtained from the southern region of Republic of Mali. All enzymes used were food grade. Pepsin, pancreatin and DPPH were purchased from Sigma Chemical Co. (St. Louis, USA). All the other chemicals used in the experiments were analytical grade and obtained from commercial sources.

2.2. Methods

2.2.1. Extraction of Roselle Seed Protein

Protein isolates were obtained from defatted Roselle seeds flour as reported by Tounkara et al. and El-Tinay et al. [20, 21]. The defatted flour was dispersed in distilled water at flour to water ratio of 1:10 (W/V); the pH was adjusted with 1 M NaOH and stirred for 3 hours at room temperature. The extract was separated by centrifugation at 4000 rpm for 25 min. The residues were re-extracted twice as described above. The extracts were combined and the protein was precipitated. The RSP (precipitate) was washed twice with distilled water. It was then resuspended in distilled water and the pH was adjusted with 1 M NaOH prior to freeze-drying. The RSP powder was placed in Ziplock bags and stored at low temperature before subsequent uses. The protein content was determined by Kjeldahl analysis according to the AOAC method [22].

2.2.2. Preparation of Protein Hydrolysates

To produce hydrolysates from Roselle Seeds protein isolates, enzymatic digestion was performed using two enzymes (pepsin followed by pancreatin) under their optimal conditions, as reported by Tounkara el al. [20] with minor modifications. The protein samples were divided into two groups (each containing 20 g and 400 mL of water) and have been hydrolyzed in 500 mL reactor with temperature and pH control devices. Sample 1 was hydrolyzed by pepsin for 30 min followed by pancreatin for 1 h and sample 2, hydrolyzed by pepsin for 1 h followed by pancreatin for 2 h. Conditions were constantly monitored and maintained throughout the process. Upon completion of the hydrolysis, the enzymes were deactivated by heating in a boiling water bath for 10 min. The reaction mixtures were then filtered and the hydrolysates were collected. The Roselle seed protein hydrolysates (RSPH) obtained were freeze-dried and stored at -20°C for subsequent analysis. The degree of hydrolysis was determined by measuring the nitrogen content soluble in 10% trichloroacetic acid as discussed by Kim et al. [14].

2.2.3. Isolation of Antioxidative Peptides

Firstly, the active hydrolysate was fractionated by gel filtration onto Sephadex G-15. The elution peaks were monitored at 280 nm and the fractions eluted under same elution peak were pooled, lyophilized, and tested for antioxidant activities. Four fractions were collected. The most active peptide fraction from Sephadex G-15 was then purified using RP-HPLC (reverse-phase high- performance liquid chromatography) on a preparative C18 column (220 nm). The mobile phase includes eluent A, which was composed of 0.1% TFA (trifluoroacetic acid) in distilled water (v/v), and eluent B, which was composed with acetonitrile. The elution was conducted using a linear gradient of 0–50 % eluent B with a flow rate of 2 mL/min for 45 min. The absorbance of the eluted peaks was monitored at 220 nm using a UV detector. All fractions were collected lyophilized and tested for antioxidant activity assay [20].

2.2.4. DPPH Radical Scavenging Assay

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging activity of the collected peptide fractions was determined as described by Tounkara et al. and Shahidi et al. [20, 23] with minor modifications. Freeze-dried samples were dissolved in 95% ethanol at series concentrations of (0.5, 1, 1.5, 2 mg/mL for fractions after gel filtration), (0.2 mg/mL for fractions after RP-HPLC). An aliquot (0.1 mL) of the sample solution was mixed with 1.9 mL of ethanolic DPPH solution (50 μM) and the mixtures were allowed to

stand at room temperature for 30 min. The absorbance was then read at 517 nm using a spectrophotometer and the scavenging ability of DPPH by fractions was calculated as in equation (1):

$$\text{Scavenging effect (\%)} = [Abs_{control} - (Abs_{sample} - Abs_{blank}) / Abs_{control}] \times 100 \quad (1)$$

where, Abs_{sample} is the absorbance of protein hydrolysates with DPPH, $Abs_{control}$ is the absorbance of DPPH without any sample, while Abs_{blank} represents absorbance of samples without DPPH, since proteins also has absorbance at this wavelength.

2.2.5. Determination of Molecular Mass and Amino Acid Sequences

The peptide fraction with the highest antioxidant activity was characterised for identification for its molecular mass and amino acid sequence. The sample was analysed by MALDI TOF–TOF mass spectrometer using a 5800 Proteomics Analyzer [Applied Biosystems at Proteomics International Pty. Ltd., Nedlands, Western Australia]. The amino acid sequence was determined by the novo sequencing method. The PEAKS Studio Version 4.5 SP2 [Bioinformatics Solutions Inc., Waterloo, ON, Canada] was used for analysis of the obtained MS/MS spectra. To align the sequence with other relevant sequences, nine amino acid sequences reported from HEWL hydrolysates were selected [24]. These sequences, along with the obtained sequence of RSPH were aligned using the CLC Main Workbench version 6.1 software (CLC bio, Aarhus, Denmark).

2.2.6. Statistical Analysis

All triplicate data sets were analysed with SPSS software (version 191 16.0, the predictive Ana-223 lytics Compagny, Chicago, USA), using one way analysis of variance (ANOVA), followed by Duncan's multiple range test.

3. Results and Discussion

3.1. The Scavenging Capacities of DPPH Radicals of Fractions

Four fractions were collected after gel separation of the 3 h RSPH [20]. The fractions were assayed for DPPH, Superoxide and hydroxyl radicals scavenging capacities. The results are presented in Figure 1 and Table 1. It was observed from the Figure 1 that Fraction III (FIII) exhibited excellent DPPH radical-scavenging activity (above 80% at 1.5 mg/mL) [20], which was higher than that of trypsin hydrolysate of the skin of both seela and ribbon fish (66 and 60% respectively at the same concentration) [25, 26].

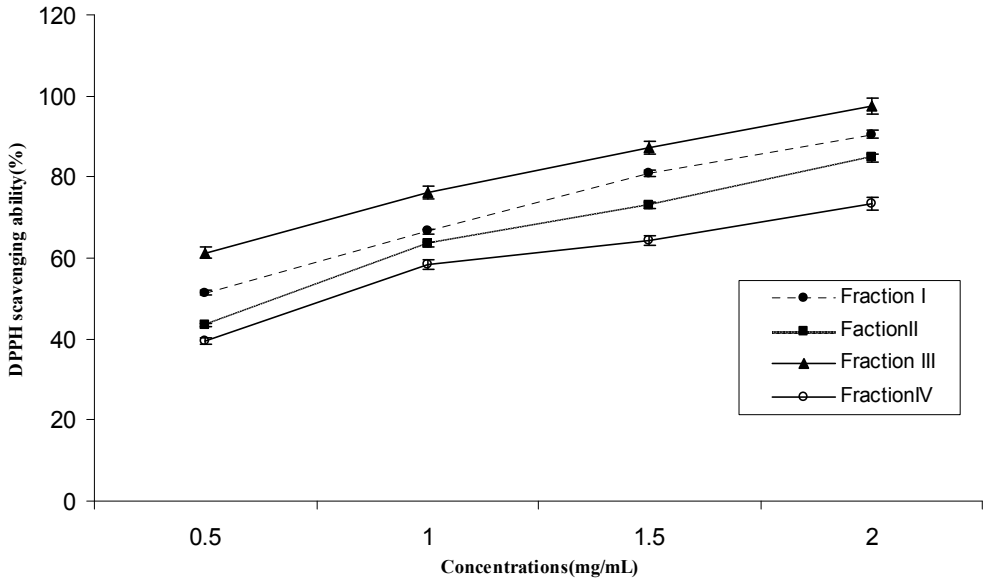


Figure 1. *The scavenging capacity of DPPH radicals (%) by various concentrations peptide fractions after gel filtration. Each value is expressed as mean ± SD of three determinations.*

3.2. Scavenging Effect on Hydroxyl Radical

The radical system used for the antioxidant activity evaluation may influence the experimental results, hence two or more radical systems are required to investigate the radical-scavenging capacities of a selected antioxidant [20, 27]. Therefore, the superoxide radical (O_2^{-}) and hydroxyl radical (HO•) scavenging capacities of the fractions after gel separation were also measured. The high reactivity of hydroxyl radicals lead to tremendous damage to the cell and its components and subsequently to the organisms as a whole [20]. Therefore, it is very important to remove hydroxyl radicals which cause detrimental effects**.** Thus, removal of hydroxyl radicals is probably one of the most effective defenses mechanism through which living body defends its self against various diseases. Table 1 shows hydroxyl and the superoxide radical-scavenging effects of the collected fractions. Among all samples, faction III exhibited the

strongest hydroxyl radical-scavenging activity (62.30%) [20]. However, fractions I, II, and IV at the same concentration (10 mg/mL) exhibited, 56.25, 46.83, and 44.98% hydroxyl radical-scavenging activity respectively, similary to the data reported by Tounkara et al. [20]. These results revealed that all tested samples possessed hydroxyl radical scavenging activity. Admittedly, the hydroxyl radical possesses the strongest chemical activity among the ROS, and easily reacts with biomolecules such as amino acids, proteins, and DNA (Je *et al*., 2005). The antioxidant activity of hydrolysates from many kinds of food proteins has been studied in recent years. Peng et al. [6] reported that whey protein hydrolysate and its peptide fractions showed antioxidant properties against hydroxyl radical similar to the results of our study.

3.3. Scavenging Effect on Superoxide Radical

Superoxide radicals are generated by a number of biological reactions. Although they do not directly initiate lipid oxidation, superoxide radical anions are potential precursors of highly reactive species such as hydroxyl radicals and hydrogen peroxide [20, 28]. Not only superoxide anion radicals but also their derivatives are cell damaging, which can cause damage to the DNA and membrane of cells. Therefore it is of great importance to scavenge superoxide anion radicals. Pyrogallic acid can automatically oxidise under alkaline conditions to produce superoxide radicals directly, the constant rate of this autoxidation reaction being dependent on the pyrogallic acid concentration [20, 29]. Table 1 shows the superoxide radical-scavenging effects of the fractions. Among all samples, faction III possessed the highest superoxide radical-scavenging activity (70.87%). However, fraction I, fraction II, and fraction IV at the same concentration (10 mg/mL) exhibited 25.43, 38.69 and 32.44% superoxide radical-scavenging ability respectively [20]. All tested samples possessed superoxide radical scavenging activity. Based on the results described above, all the collected fractions, especially fraction III have good free radical-scavenging activities and could be a potential source of natural antioxidants. Between all tested samples, the Fraction FIII showed excellent DPPH (Figure 1), superoxide and hydroxyl radical scavenging ability (Table 1). For this reason, it was then selected for reverse phase chromatography analysis (RP-HPLC) analysis.

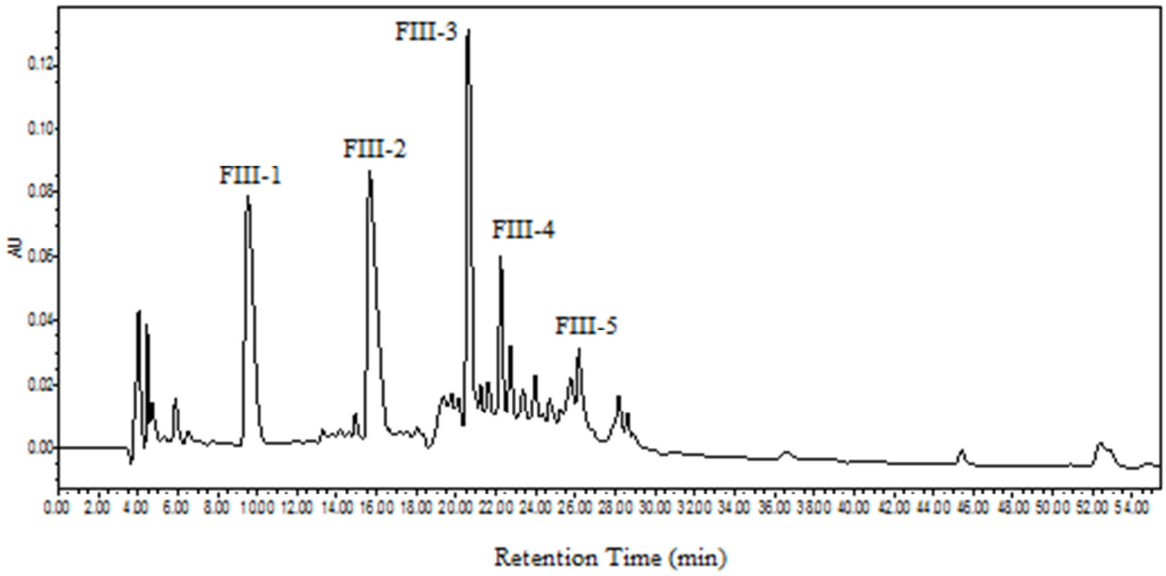
Table 1. *The Hydroxyl and Superoxide radical scavenging effects (%) by concentration (10mg/mL) of samples (source: Tounkara et al. [20]).*

Test sample	Superoxide	Hydroxyl
Fraction I	25.43± 0.75^{a}	56.25± 0.97^{a}
Fraction II	38.69± 0.33^{b}	46.83± 1.02^{b}
Fraction III	70.87± 0.78^{c}	62.30± 0.69^{c}
Fraction IV	32.44± 0.57^{b}	44.98± 1.05^{d}

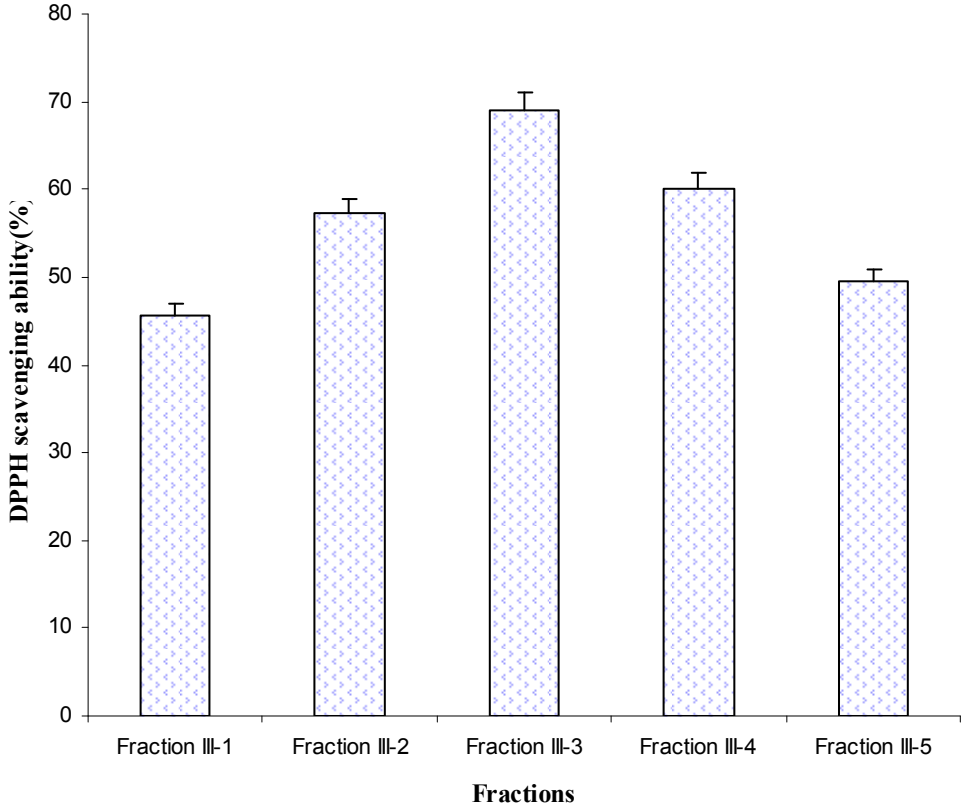
Results reported are means of triplicate samples ± standard deviation. Values in the same column with different superscripts are significant different at $P < 0.05$.

3.4. Peptide Fractionation by RP-HPLC and Antioxidant Activity Assay

From the fraction III RP-HPLC chromatogram results of purification, only five major peaks were obtained as shown in Figure 2a. Each peak was collected and subjected to antioxidant test. Figure 2b shows the DPPH radical scavenging effect of the different fractions obtained after separation. The results in Figure 2b indicate that fraction FIII-3 at 0.2 mg/mL exhibited excellent DPPH radical-scavenging activity (68.96%). The different DPPH radical scavenging patterns for the purified fractions observed from these results may be related to the structure of peptides eluted at different step of the purification [30, 31]. For the reason that FIII-3 exhibited the highest DPPH radical scavenging capacity among all the fractions obtained from purification of FIII, it was chosen for peptide sequences determination by liquid chromatography/ tandem mass spectrometry (LC-MS/MS).



(a)



(b)

Figure 2. *RP-HLPC chromatogram of fraction FIII (a) and the DPPH radical scavenging activity of the eluted peaks (at the concentration, 0.2 mg/mL) (b).*

3.5. Molecular Mass and Amino Acid Sequence Analysis

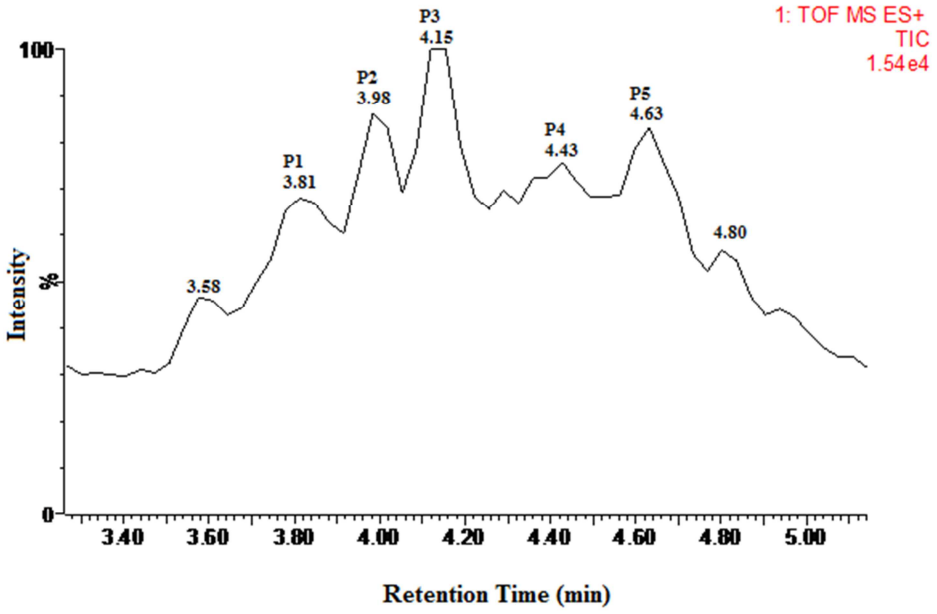
The beneficial effects of antioxidants are preventing oxidative damage by interrupting the radical chain reaction of lipid peroxidation [32]. It is generally considered that the inhibition of lipid peroxidation by an antioxidant may be due to free radical scavenging activity. In the peptide sequences determination of the FIII-3 fraction, five peptides were found, these were further analysed by matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-TOF MS) for the characterisation of their amino acid sequences. Figure 3 presents the five matched peptides from Bio Lynx protein/peptide Editor (Mass Lynx V4.1). The potent antioxidant peptides indicated the sequences of Thr-Val-Glu-Asn-Leu/ Ala-Leu-Gly-Ala-Asp-Cys-Asp-Val/ Tyr-Thr-Met-Phe-Ser-Thr-Ser-Trp-Phe/ His-Asn-Asp-Pro-Glu-Phe/ Thr-Pro-Glu-Cys-Asn-Val (Table 2).

The Bioactive peptides usually contain 2–20 amino acid residues per molecule [9] and the lower their molecular weight, the higher is their chance to cross the intestinal barrier and exert a biological effect [27]. All the identified peptides showed 5 to 9 amino acid residues in their molecule and their molecular weight ranged from 572.99 to 1168.45 Da. This may explain the highest antioxidant capacity of the RP-HPLC fraction and also can lend to the identified peptides a potential to exert a biological effect. In the free radical-mediated lipid peroxidation system, antioxidative activity of peptide or protein is dependent on molecular size and chemical properties such as hydrophobicity and electron transferring ability of amino acid residues in the sequence. As shown in Table 2, the purified peptides containing in their molecule 2 to 4 hydrophobic amino acid residues. Two of the identified peptides contained histidine, tyrosine, tryptophan, and/or phenylalanine with aromatic residues witch can make reactive oxygen species (ROS) stable through direct electron transfer. Three peptides also contained in their sequence, hydrogen donors such as aspartic acid; glutamate and tyrosine, which are able to quench unpaired electrons or radicals by supporting protons. Aromatic amino acid Phe is present within two peptide sequences. Phe has been reported to contribute for peroxidation inhibition by increasing the solubility of peptide in lipid [10, 11]. By donating protons easily to electron deficient radicals and maintaining their stability via resonance structures, Phe has also been shown to act positively as direct radical scavengers [28]. It was reported that acidic and/or basic amino acids play an important role in the chelation of metal ions by carboxyl and amino groups in their side chains [33]. Four of the purified peptides, also showed in their molecule, acidic and/or basidic amino acid residues. In addition, the structures were composed of two cysteine residues, height acidic residues (Asp and Glu) and non-polar residues (Gly, Leu). Further, in the peptide sequences there is the presence of all hydrophobic amino acid residues. Cysteine residues are independently important for antioxidant action, since they can directly interact with radicals. As reported by Kumar et

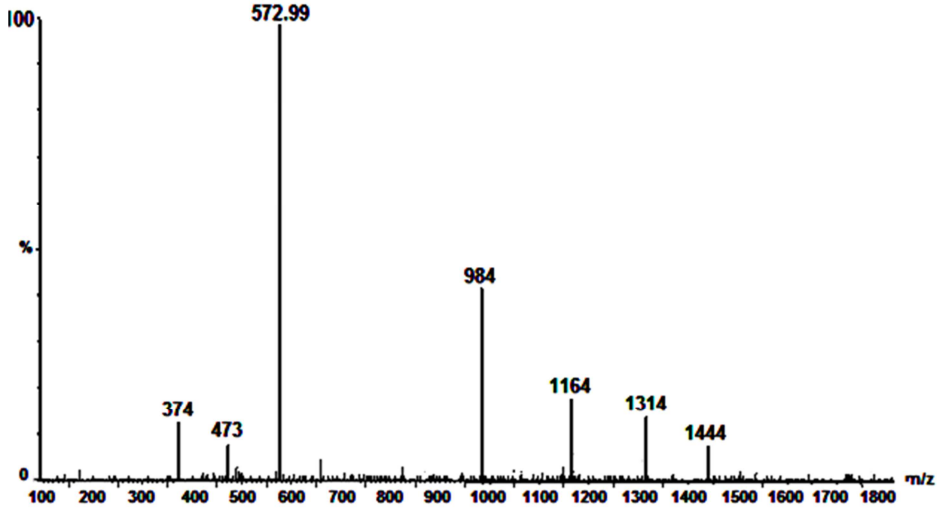
al. [29], the thiol group of cysteine serves a very important role in protecting cells and cellular biomolecules from oxidative stress. Rajapakse et al., Je et al. and Yu et al. [34-36] reported that presence of Asp seemed to play a vital role irrespective of its position as observed in several antioxidative peptide sequences. It was assumed that the presence of both Cys and Asp residues within the sequence of P2, Cys, Glu and Asn within the sequence of P5, Asn, Asp and Glu within the sequence of P4, Glu and Asn within the sequence of P1 and Phe residues within the sequence of P3 seemed to play a vital role for their antioxidant activities. Based on these results, it is suggested that the low molecular weight peptides released from RSPI by enzymatic hydrolysis have potent antioxidant properties. Pepsin is the most efficient enzyme in cleaving peptide bonds between hydrophobic and preferably aromatic amino acids such as phenylalanine, tryptophane, and tyrosine. Trypsin cleaves peptides chains mainly at the carboxyl side of the amino acids lysine or arginine, except when either is followed by proline. In this study pepsin and trypsin proteases were used to digest the protein (trysin was obtained from pancreatin, since pancreatin is a mixture of lipase, amylase and trypsin). All the identified peptides showed in their carboxyl group hydrophobic amino acid and in their amine group no proline, from these results we concluded that there were cleavages between lysine or arginine and the amine group of the identified due to trysin protease. And also, that pepsin cleaved at the carboxyl side of the different identified peptides.

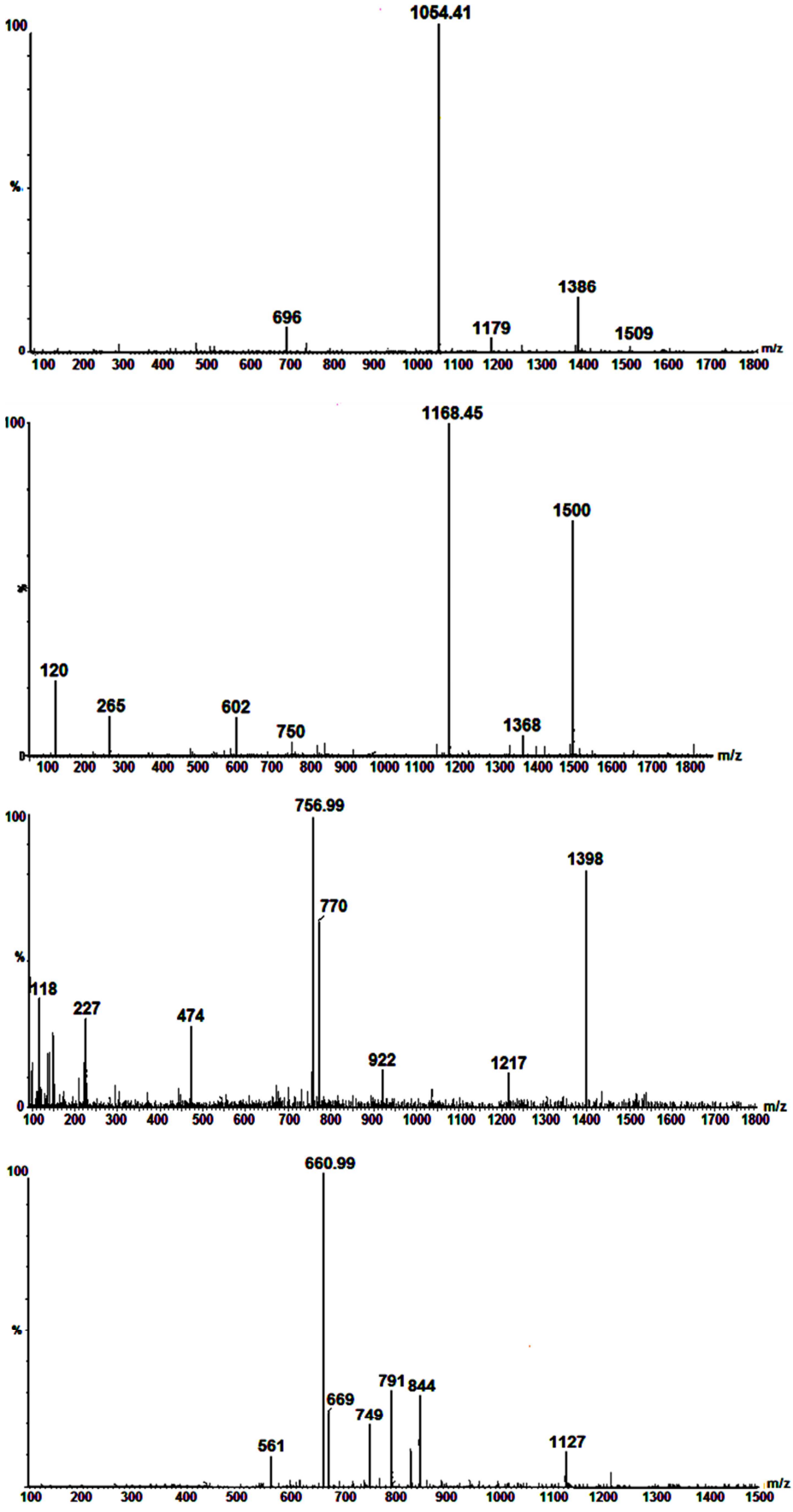
Table 2. *Peptide mass and the matched amino acid sequences.*

	Mass	AA sequences
p_1	572.99	Thr-Val-Glu-Asn-Leu
p_2	1054.41	Ala-Leu-Gly-Ala Asp-Cys-Asp-Val
p_3	1168.45	Tyr-Thr-Met-Phe-Ser-Thr-Ser-Trp-Phe
p_4	756.99	His-Asn-Asp-Pro-Glu-Phe
p_5	660.99	Thr-Pro-Glu-Cys-Asn-Val



(a)





(b)

Figure 3. *Chromatogram of HPLC Fraction FIII-3 in Bio Lynx protein/ peptide editor (a) and the matched peptides mass (b).*

4. Conclusion

This study confirmed that strong antioxidant activities of the extracted protein isolate from Roselle seed can be successfully provided through enzymatic digestion and fractionated into bioactive peptides. In addition, five purified peptides are mainly responsible for the potent free radical scavenging of the fraction and the hydrolysate. Thus, they could be included as antioxidant constituents in the food industry due to their role in food quality preservation and would be expected to protect against oxidative damage in living systems, in relation to aging and carcinogenesis. However, further detailed studies on the peptides as regards to antioxidant activities *in vivo* are needed. The molecular mass of the purified peptides determined by ESI/MS spectroscopy was in excellent agreement with theoretical mass calculated from the sequence.

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