CHAPTER IV

3.6 CHARACTERISATION OF ANTIOXIDANT PEPTIDES BY ESI MS/MS

3.6.1 Introduction

Mass spectrometry using ESI is called electrospray ionization mass spectrometry (ESI-MS) or commonly called electrospray mass spectrometry (ESI-MS) and is especially useful in producing ions from macromolecules because it overcomes the propensity of these molecules to fragment when ionized. Mass spectrometry and tandem mass spectrometry (MS/MS) experiments are major tools used in protein identification. Mass spectrometers measure the mass/charge ratio of analytes; for protein studies, this can include intact proteins and protein complexes [322], fragment ions produced by gas-phase activation of protein ions (top-down sequencing) [323] peptides produced by enzymatic or chemical digestion of proteins (mass mapping) and fragment ions produced by gas-phase activation of mass-selected peptide ions (bottom-up sequencing) [324]. The application of mass spectrometry and MS/MS to proteomics takes advantage of the vast and growing array of genome and protein data stored in databases. Applications to analyse smaller quantities of sample are driving the development of more sensitive mass spectrometers, as well as low flow, high resolution separation technologies, to provide structural information on individual components in complex mixtures of thousands of proteins derived from biological samples. Protein identification by mass spectrometry requires an interplay between mass spectrometry instrumentation (how molecules are ionized, activated, and detected) and gas-phase peptide chemistry (which bonds are broken, at what
rate, and how cleavage depends on factors such as peptide/protein charge state, size, composition and sequence). Pharmaceuticals are being increasingly investigated by electrospray ionisation-mass spectrometry (ESI-MS) since the technique was introduced by Yamashita and Fenn [325]. Many pharmaceuticals with a relatively small molecular mass of less than 1000 daltons have been studied primarily using ESI signals corresponding to their [M+H]^+ ions and the corresponding fragment ions. Quinoline [326], antibacterials, antibiotics, steroids, anti-diabetic drugs, anti-tumour drugs, erectile dysfunction agents, anti-epileptic drugs, b-blockers, antiasthmatic drugs and psychoactive drugs are among the drug classes that have been investigated [327]. The main benefits of ESI are that it can be interfaced readily to chromatographic and electrophoretic separation techniques, and has a propensity to generate multiply charged analyte ions thus enabling instruments with modest m/z ratios, such as those with quadrupole, ion trap or magnetic sector analysers, to detect high molecular mass analytes. ESI-MS is as equally suitable for low molecular mass samples as it is for those of high molecular mass protein complexes [328]. Mass spectrometry has become the core technology for protein identification with the peptide mass map, a set of peptide molecular masses obtained from the MS analysis of the proteolytic digestion of a protein, represents a unique fingerprint from which the protein can be identified by comparison with theoretical peptide masses generated by the simulated digestion of protein entries in a database with the same enzyme [329].

3.6.2 Materials and Methods

Identification of peptides by ESI-MS/MS

Identification of peptide by ESI-MS/MS was done by the method of Ren et al., [208].
Reagents

1. Acetonitrile
2. HPLC grade water

Procedure

The highest antioxidative activity peptides after gel filtration chromatography purification was dissolved in a buffer of 75% acetonitrile/25% HPLC grade water, then loaded into FIA type 3200 QTRAP mass spectrometer (Applied Biosystem). The sample was passed at a flow rate of 20 µl/min, which was operated in the positive electro spray ionization (ESI + ve) mode, via the electro spray interface. The Drying (35 psi) and ESI nebulizing gas (45 psi) used was high purity nitrogen. Spectra were recorded over the mass/charge (m/z) range 200 - 1000. About three spectra were averaged in the MS and multiple MS (MS/MS) analyses. The peptide sequencing was performed by manual calculation.

3.6.3 Results

Identification of peptides by ESI-MS/MS

The MS spectrum of purified fractions of muscles and backbones of *N. japonicus* and *E. volitans* are shown in Figure 26a, 27a, 28a and 29a whereas, the MS/MS spectrum of a major charged ion with m/z at 620.3 Da, 451.8 Da, 747.3 Da and 596.9 Da for purified peptides of *N. japonicus* muscle, backbone, *E. volitans* muscle and backbone respectively as illustrated in Figure 26b, 27b, 28b and 29b. The manual analysis gave following possible sequences, viz. Glu-Ser-Asp-Arg-Pro (*N. japonicus* muscle); Gly-His-Met-Ser (*N. japonicus* backbone); Gly-Trp-Met-Gly-Cys-Trp (*E. volitans* muscle) and Leu-Glu-Val-Lys-Pro (*E. volitans* backbone).
3.6.4 Discussion

Identification of peptides by ESI-MS/MS

The peptide sequences for *N. japonicus* muscle, backbone, *E. volitans* muscle and backbone was found as Glu-Ser-Asp-Arg-Pro; Gly-His-Met-Ser; Gly-Trp-Met-Gly-Cys-Trp; and Leu-Glu-Val-Lys-Pro respectively. The results of the observations showed that the sequences of antioxidant peptides derived from *N. japonicus* and *E. volitans* peptides have hydrophobic amino acids (Pro and Val) which are expected to favour oxidation inhibition [330]. Further, presence of hydrophobic amino acid residues such as, Leu, Gly and Val are presumed to inhibit lipid peroxidation by increasing solubility of peptides in lipid and thereby facilitating better interaction with radical species [202]. It is commonly believed that His, Met and Cys are very important to the radical scavenging activity of peptides due to their special structure of characteristics: the imidazole group in His has the proton-donation ability [331]; Met is prone to oxidation of the Met sulfoxide [332]; Cys donates the sulfur hydrogen [332]. Therefore, these results suggest that antioxidative activity of the isolated peptides was dependent on their amino acid residue and molecular weight. The results were supported by Jun *et al.* [201], isolated the antioxidative peptide of 10 amino acid residues, Arg-Pro-Asp-Phe-Asp-Leu-Glu-Pro-Pro-Tyr (1300 Da) from yellowfin sole frame protein normally discarded as waste. Je *et al.*, [203] reported purified antioxidant peptide with amino acid sequence as Leu-Pro-His-Ser-Gly-Tyr (MW 672 Da) from Alaska pollack frame protein generally considered as industrial by-product in the fish plant. Moreover, Rajapakse *et al.*, [202] reported two antioxidant peptides, Asn-Gly-Leu-Glu-Gly-Leu-Lys (747 Da) and Asn-Ala-Asp-Phe-Gly-Leu-Asn-Gly-Leu- Glu-Gly-Leu-Ala (1307 Da) from giant squid muscle protein. Ranathunga *et al.*, [205] sequenced the purified peptide of conger eel muscle by ESI MS/MS as Leu-Gly-Leu-Asn-Gly-Asp-Asp-Val-Asn. Li *et al.*, [333] identified the peptide Gln-Gly-Ala-Arg, which exhibited the highest antioxidant activity from porcine skin collagen hydrolysates.
Figure 26 (a) MS spectrum of the chromatographic fraction of *N. japonicus* muscle and (b) MS/MS spectrum of the ion *m/z* 620.3. By manual calculation the sequence of this peptide was Glu-Ser-Asp-Arg-Pro.
Figure 27 (a) MS spectrum of the chromatographic fraction of *N. japonicus* backbone and (b) MS/MS spectrum of the ion $m/z$ 451.8. By manual calculation the sequence of this peptide was Gly-His-Met-Ser.
Figure 28 (a) MS spectrum of the chromatographic fraction of *E. volitans* muscle and (b) MS/MS spectrum of the ion *m/z* 747.3. By manual calculation the sequence of this peptide was Gly-Trp-Met-Gly-Cys-Trp.
Figure 29 (a) MS spectrum of the chromatographic fraction of \textit{E. volitans} backbone (b) MS/MS spectrum of the ion \textit{m/z} 596.9. By manual calculation the sequence of this peptide was Leu-Glu-Val-Lys-Pro.
The results were moreover, supported by Ren et al., [208] reported Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val (966.3 Da) sequence from the grass carp muscle peptide produced by alcalase hydrolysis using RP-HPLC connected on-line to an electrospray ionization mass spectrometry. Byun et al., [210] isolated two antioxidant decapetides with sequences Leu-Leu-Gly-Pro-Gly-Leu-Thr-Asn-His-Ala (1076 Da) and Asp-Leu-Gly-Leu-Gly-Leu-Pro-Gly-Ala-His (1033 Da) from marine rotifer as characterised by Q-TOF tandem mass spectrometer. Liu et al., [230] isolated three peptides with antioxidant properties from aqueous extract of Cornu bubali (water buffalo horn) after consecutive chromatographic methods including gel filtration chromatography, ion-exchange chromatography and high performance liquid chromatography. The sequences of the three peptides were identified to be Gln-Tyr-Asp-Gln-Gly-Val (708 Da), Tyr-Glu-Asp-Cys-Thr-Asp-Cys-Gly-Asn (1018 Da) and Ala-Ala-Asp-Asn-Ala-Asn-Glu-Leu-Phe-Pro-Pro-Asn (1271 Da) by matrix assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry (MALDI-LIFT-TOF/TOF MS). Ngo et al., [308] isolated a peptide possessing antioxidant properties from Nile tilapia (Oreochromis niloticus) scale gelatin and reported peptide sequence as Asp-Pro-Ala-Leu-Ala-Thr-Glu-Pro-Asp-Pro-Met-Pro-Phe (1382.57 Da). Lee et al., [228] reported antioxidant peptide from duck processing by-products with peptide sequence Asp-Val-Cys-Gly-Arg-Asp-Val-Asn-Gly-Tyr (1096 Da). Hence, the results demonstrate the importance of amino acid composition, sequence and size in determining the antioxidative potential of peptides as reported in earlier investigations performed in other species.