3.1 INTRODUCTION

Free radicals are a highly reactive short-lived species generated in vivo or in vitro through physiological reactions, particularly in vertebrates. Normally, they serve in signaling routes and provide defense against infections (Hancock et al. 2001). Any excessive amount of these highly reactive species can result in cellular damage, which, in turn can initiate several diseases including atherosclerosis, arthritis, diabetes and cancer (Gelse et al. 2005). Under normal circumstances, the antioxidant defense system of the body, comprising of an array of enzymatic (superoxide dismutase, glutathione peroxidase) and non-enzymatic (vitamins, trace elements and coenzymes) antioxidative components are enough to counter this free radical load (Johansen, 2005). However, in certain circumstances, like an excessive free radical load or a faulty antioxidative component, this defense system fails. This gives rise to oxidative stress, a condition in which the generation of highly reactive molecules like hydroxide, superoxide and peroxide radicals (collectively termed as reactive oxygen species or ROS) exceed their quenching, resulting in uncontrolled reactions with the cell macromolecules (Sarmadi and Ismail, 2010).

3.1.1 ROS PRODUCTION

Molecular oxygen is fairly unreactive due to its stable electronic configuration and activation requires an electron donation. The primary function of O₂ is to serve as the terminal electron acceptor for cytochrome-c oxidase, the complex IV of the mitochondrial electron transport chain (ETC) that catalyzes the four-electron reduction of O₂ to H₂O. Partially reduced and highly reactive metabolites of O₂ may be formed during these (and other) electron transfer reactions.

One electron reduction of O₂ gives rise to superoxide anion:

\[ \text{O}_2 + e^- \rightarrow \text{O}_2^- \]

It is generally formed as a by-product of ETC, NADPH oxidases and xanthine oxidases under controlled conditions. The radical undergoes spontaneous dismutation to H₂O₂ and \(-\text{OH}\) in a reaction catalyzed by superoxide dismutase (SOD).

Two electron reduction of O₂ gives rise to the peroxide anion:
\[ \text{O}_2 + 2e \rightarrow \text{O}_2^{2-} \]

It is formed by SOD, although the \( \text{H}_2\text{O}_2 \) created is converted to \( \text{H}_2\text{O} \) by a number of agents like glutathione and enzymes such as catalase and peroxiredoxins. Although, \( \text{H}_2\text{O}_2 \) is a weaker oxidizing agent than \( \text{O}_2^- \) under physiological conditions, in the presence of transition metals such as iron or copper, \( \text{H}_2\text{O}_2 \) can give rise to the indiscriminately reactive and toxic hydroxyl radical (\( \cdot\text{OH} \)) via Fenton chemistry.

Three electron reduction of \( \text{O}_2 \) gives rise to the hydroxyl radical:

\[ \text{H}_2\text{O}_2 + e \rightarrow \cdot\text{OH} + \cdot\text{OH} \]

This is produced through Fenton reactions with \( \text{Fe} \) (II) and Haber-Weiss reaction through superoxide radicals. Moreover, the resultant \( \text{Fe} \) (III) can revert back to \( \text{Fe} \) (II) state with the production of further peroxides and the hydroxyl radical formed can react with \( \text{H}_2\text{O}_2 \) in the presence of \( \text{Fe} \) (III) to regenerate superoxide anion.

\[ \text{H}_2\text{O}_2 + \cdot\text{OH} \rightarrow \text{H}_2\text{O} + \text{O}_2^- + \text{H}^+ \]

The oxygen radicals also induce the generation of several other reactive species including \( \text{HOCl}, \text{HOBr}, \text{NO}^- \) and \( \text{NO}_2^- \) (Kirkinezos and Moraes, 2001; Manda et al 2009).

3.1.2 ROS-GENERATING ENZYMES

Several cellular enzymes take part in ROS genesis including the mitochondrial cytochrome family of enzymes; P450, b5 and oxidases, components of ETC along with peroxisomal oxidases and catalases. Plasma membrane-associated oxidases, the best characterized of which is the phagocytic NADPH oxidase, have also been implicated to be involved in growth factor- and/or cytokine-stimulated oxidant production. Auto-oxidation of small molecules like dopamine, epinephrine, flavins, and hydroquinones can also act as an important source of intracellular ROS production (Manda et al 2009).

3.1.3 ROS AND DISEASES

ROS are highly reactive and readily cause oxidative modifications to biomolecules. Due to a short half-life and limited diffusion distance, most ROS cause damage locally near the sites of production. However, \( \text{H}_2\text{O}_2 \) has a relatively long half-life and can travel long distances, which enable this molecule to function as a secondary messenger in signal transduction pathways and to cause damage at distant sites including nuclear DNA. Under physiological conditions, ROS are maintained at required levels by a balance between its generation and elimination. The steady state of ROS would readily change if any step in the ROS production or quenching is disturbed. An increase in ROS
generation, a decrease in antioxidant capacity, or both will lead to oxidative stress (Lu et al 2007; Manda et al 2009). There has been a great deal of research on the role of ROS in the pathogenesis of a number of human diseases such as atherosclerosis, diabetes, viral infection, chronic inflammation and ischemia-reperfusion injury. A multitude of theories exist, attempting to explain the mechanisms of ROS mediated damage and its effects in the progression of diseases. In most diseases, primary mitochondrial involvement is profound and evident (Kirkinezos and Moraes, 2001).

3.1.3.1 ROS AND DIABETES

Oxidative stress, primarily acting through superoxides has been found to be the common pathogenic factor leading to insulin resistance, β-cell dysfunction and impaired glucose tolerance ultimately resulting to type 2 DM. Furthermore, this mechanism has been implicated as the underlying cause of both the macrovascular and microvascular complications associated with T2DM. It follows that therapies aimed at reducing oxidative stress would benefit patients with T2DM and those at risk for developing diabetes (Wright et al 2006).

3.1.3.2 ROS AND APOPTOSIS

ROS-mediated apoptotic signaling is associated with decreased cellular GSH levels and the loss of cellular redox balance, particularly in the mitochondria. Release of mitochondrial cytochrome c due to ROS activates the caspase-9 mediated apoptosis of the cell. Prolonged activation of JNK via ROS can induce tumor necrosis factor-α (TNF-α) induced pro-apoptotic effects. Excess mitochondria-derived ROS is known to induce apoptosis through TNF-α and IL-1 (Watson, 2013).

3.1.3.3 ROS AND CANCER

Studies support the role of oxidative DNA damage as an important mutagenic and apparently carcinogenic factor. ROS can damage DNA and the division of cells with unrepaired or damaged DNA leads to mutations. If the mutation occurs in critical genes such as oncogenes or tumor suppressor genes, initiation/progression of cancer can follow. It is now assumed that ROS are involved both in the initiation and progression of cancer and act at several steps in multistage carcinogenesis (Halliwell, 1999). Tumor suppressor protein p53 occupies a pivotal position in maintaining genomic integrity. In response to ROS-related stress that lead to DNA damage, wild-type p53 orchestrates transcriptions of
numerous genes and directs cells either to cell cycle arrest, senescence, or apoptosis (Watson, 2013).

3.1.3.4 ROS AND ATHEROSCLEROSIS

The major sources of ROS in atherosclerotic vessels are macrophages and smooth muscle cells and they act in myriad ways to influence atherosclerosis. First, the increased production of ROS reduces the production of NO, leading to vasoconstriction, platelet aggregation and adhesion of neutrophils to the endothelium. Oxidative stress also increases phosphorylation state of kinases and activates signaling molecules, leading to further neutrophil adhesion and alteration of vessel permeability. Most importantly, oxidative stress leads to oxidation of LDL (ox-LDL), which is easily taken up by macrophages. Ox-LDL can promote atherogenesis through lipid accumulation and vascular cell dysfunction leading to induction of apoptosis causing rupture and plaque growth. Also, small oxidized lipids components of ox-LDL, such as oxysterols, are potent inducers of ROS production. They have been shown to increase mitochondria ROS generation and to trigger cytochrome c release leading to caspase activation and apoptosis (Vogiatzi et al 2009).

3.1.3.5 ROS AND NEURODEGENERATION

Because of its reduced capacity for cellular regeneration, the brain is particularly susceptible to the damaging effects of ROS. In neurodegenerative diseases like Parkinson's, Alzheimer's and amyotrophic lateral sclerosis (ALS), ROS damage has been reported within the particular brain area that undergoes selective neural damage. Protein oxidation has been reported in the hippocampus and neocortex of patients with Alzheimer's disease, in Lewy bodies in Parkinson's disease and within motor neurons in ALS. Lipid peroxidation has also been identified in the cortex and hippocampus of patients with Alzheimer's disease, substantia nigra of patients with Parkinson's disease and spinal fluid in patients with ALS (Knight, 1995; Klein and Ackerman, 2003).
3.1.4 ANTIOXIDATIVE AGENTS AS PREVENTIVE CURES

Agents that counter oxidative stress may be divided into four categories: prevention of reactive species formation, scavenging and/or quenching of radicals, damage repair and excretion of oxidative agents along with adaptive responses. Also, none of the in vivo antioxidative agents work in isolation; rather all antioxidative agents form an interlinked set of redox antioxidant cycles, termed as the antioxidant network.

There are several in vivo agents including vitamins such as retinol and tocopherol, which can scavenge radicals as well as have a regulatory aspect on ROS-related gene expression. An agent such as tocopherol, after quenching lipid radicals is returned to its native reduced state by interactions with water- and lipid-soluble elements through both enzymatic and non-enzymatic means. Vitamin C is one such element which reduces tocopherol, along with glutathione and lipoic acid. Niacin has been displayed to inhibit lipid peroxidation (Lesgards et al 2005). Among fatty acids, there is report of $\omega-3$-FA being radical scavenging but $\omega-6$-FA to be pro-oxidant (Mazza et al 2007).

Among amino acids, taurine has been suggested to yield a number of cytoprotective effects through bile acid excretion and antioxidant activity. Free amino acids R and H are known to act as antioxidative agents through metal-chelation abilities (Koch et al 2009). Thiol groups are central to most, if not all, redox-sensitive cell signaling mechanisms. Reversible oxidation of thiol groups represents a sensitive redox regulated functional switch, through which the functionality of several proteins are controlled. The small peptide carnosine $\beta$-alanyl-L-histidine occurring mainly in muscles and tissues is an efficient antioxidant. Even proteins like lactoferrin, with the inherent ability to chelate Fe, take part in regulating ROS metabolism.

In a physiological system, none of these systems work alone. All of these agents act synergistically to maintain a steady state between the oxidized and the reduced form of an in vivo antioxidant (Packer et al 2001). Consequently, it leads to protection of tissues against oxidative stress and associated pathologies such as cancers, coronary heart disease and inflammation. In the presence of a faulty component, possibly due to environmental and/or genetic disposition, the antioxidative network fails leading to cell death and consequently, tissue injury. Presence of an antioxidative agent administered from outside can lead to countering the ROS stress and reducing its pathological effects.
3.1.5 PEPTIDES AS ANTIOXIDATIVE AGENTS

Tissue remodelling following wound creation or inflammation requires breakdown and re-synthesis of the ECM basal material. The proteolytic fragmentation of ECM components such as collagen can yield cryptic peptides with divergent physiological activities. One such activity involves quenching free radicals and consequent reduction in ROS-generated stress.

Historically, antioxidative peptides have been isolated from milk-derived peptides and have been found in almost all protein-based life forms. Treatment of rice endosperm, algal protein waste and peanut kernel protein with different proteases has given rise to peptides in the 0.9-5kDa domain that displays antioxidative activity. The peptide components FRDEHKK and KHDRGDEF from rice endosperm and VECYGPNRPQF from algae has been identified to be strong antioxidative agents. In fact, treatment of several plant proteins, e.g. Sunflower leaf, maize zein and corn gluten with the enzyme alcalase has resulted in hydrolysates with strong antioxidant properties. Enzymatic digestion of peanut proteins, tubers and soy protein has also generated hydrolysates with profound bioactivity. Peptides derived from tryptic digest of β-casein and egg yolk protein have been reported to display potent lipid oxidation inhibition (Liu et al 2003; Pihlanto, 2006; Sarmadi and Ismail, 2010).

In the last few years, collagen has also been revealed as a source for antioxidative peptides and peptides as such have been identified in porcine and fish collagen (Kim et al 2001; Li et al 2007; Nagai et al 2007). The necessity of such ‘hidden’ regions in collagen could possibly stem from the need to reduce the ROS stress caused by invading cells and/or during wound healing. Identification of such peptides from collagen could elucidate the necessary presence of such cryptic regions that when fragmented, give rise to antioxidative activities. This study was undertaken to identify cryptic antioxidative peptides from the IEC fractions of the bovine Achilles tendon collagen hydrolysate through antioxidative screening.
3.2 MATERIALS AND METHODS

BHT, DPPH, linoleic acid, chromatographic matrices (sephadex G25, G75 and G100) and molecular weight markers were obtained from Sigma-Aldrich Chemicals Private Limited, Bangalore, India. Ferrozine \(3-(2\text{-pyridyl})-5, 6\text{-bis (4-phenylsulfonic acid)}-1, 2, 4\text{-triazine, monosodium salt}\) was obtained from Himedia, India. All other chemicals used were of analytical grade.

3.2.1 PREPARATION OF THE HYDROLYSATE

Hydrolysis of collagen was performed as described in section 2.3, Chapter 2. Samples extracted at 3h time intervals were subjected to antioxidative assay to determine the time at which the display of bioactivity was conspicuous.

3.2.2 PURIFICATION OF THE PEPTIDES

Fractions A-F obtained from IEC, were dialyzed against deionized water, lyophilized in a Micromodulyo freeze dryer (Thermo Scientific, USA), weighed and a known amount used for antioxidative assay. Active fractions were subjected to automated gel-permeation chromatography using an ÄKTApure plus unit (GE Healthcare, United Kingdom). The IEC fractions recognized as active were lyophilized, dissolved in acetate buffer at a concentration of \(5\text{mg ml}^{-1}\) and applied to a 1.4 \(\times\) 25cm column of sephadex G100 equilibrated with 50mM acetate buffer, pH 4.5. The column effluent flow rate was maintained at \(1.5\text{ml min}^{-1}\); absorbance was monitored at 214nm (using a zinc lamp) and fractions of 3ml were collected. Fractions representing the major portion of a given peptide peak were pooled and dialyzed against 0.1M acetate buffer.

Following separation each of the fractions were screened for bioactivity. The active fractions were dissolved in the same acetate buffer and applied to a 1.4 \(\times\) 25cm sephadex G25 column for further separation. The column effluent flow rate was maintained at \(0.8\text{ml min}^{-1}\), absorbance was monitored at 214nm and fractions of 3ml were collected. Fractions showing high absorbance values were pooled, dialyzed in acetate buffer, lyophilized and re-subjected to antioxidative assays.
3.2.3 ASSAYING ANTIOXIDATIVE PROPERTIES

3.2.3.1 RADICAL SCAVENGING ACTIVITY

The scavenging effect of the peptides on DPPH free radical was measured according to the method of Mensor et al (2001) with some modifications. Known amounts of the test peptides were dissolved in 1ml methanol, diluted to 3ml with deionized water and 1ml of 0.3mM DPPH in methanol was added to it. Samples were kept in the dark for 30min and the absorbance measured at 517nm. The radical scavenging activity (%) was calculated according to the following equation:

\[
\text{Scavenging activity (\%) = } (\frac{\text{Control + (Blank – Sample)}}{\text{Control}}) \times 100
\]

Blank was set with methanol. 1ml DPPH, 2ml deionized water along with 1ml methanol was used for control. For peptides insoluble in methanol, acetic acid buffered methanol, as suggested by Sharma and Bhatt (2009), was used for dissolution. The controls and the blank were suitably modified. The synthetic antioxidant, BHT was used as positive control.

3.2.3.2 CHELATING ACTIVITY

Ferrous ion chelating activity was determined according to the method of Dinis et al (1994) with some modifications. The test samples were dissolved in 50mM acetic acid in increasing concentrations. 1ml was aspirated from each sample, diluted with 1ml deionized water and added to a 0.5ml solution of 2mM FeSO\textsubscript{4}. The reaction was initiated by addition of 0.1ml of a 5mM ferrozine solution, the mixture shaken vigorously and incubated at 28°C. After 10min, absorbance of the solutions was measured at 562nm. EDTA was used as positive control. BSA and collagen were also checked for chelating ability. All tests were conducted in triplicates. The chelating effect (%) was calculated according to the following equation:

\[
\text{Chelation (\%) = } \left[\frac{(\text{Control} - \text{Test})}{\text{Control}}\right] \times 100
\]

Since the ferrous-dye complex is stable over a pH range from 4 to 8 (Pascal-Reguera et al 1997), the effect of change in pH of the reaction medium on the chelating ability of the peptides were also measured. Different reaction systems with varying pH were set up with suitable controls for the assay.
3.2.3.3 REDUCTIVE ABILITY

For the measurement of reductive ability, the ferric-ferrous transformations in the presence of the peptides were measured according to the method of Oyaizu et al (1986) with some modifications. Known amounts of the test peptides were dissolved in 3ml of 0.2M phosphate buffer, pH 6.6. 2ml of 1% potassium ferricyanide solution was added and the reaction mixture incubated at 50°C for 20min followed by addition of 1ml 20% TCA. 2.5ml of the test solutions was aspirated out, mixed with 2.5ml deionized water, 0.5ml of 0.1% ferric chloride solution and incubated at 28°C for 10 min. The absorbance of the resulting solutions was measured at 700nm. Triplicate tests were conducted for each sample. Increase in absorbance was taken as an indication of greater reductive abilities.

3.2.3.4 LINOLEIC ACID PEROXIDATION INHIBITION ASSAY

The method of Li et al (2007) was followed to measure the extent of lipid auto-oxidation. 1.5ml of 50mM linoleic acid in 99% ethanol was mixed with an equal volume of phosphate buffer of pH 7 and 2ml of the test peptide in phosphate buffer was added. BHT was used as the positive control. The final solution in a screw-cap glass tube was incubated in the dark at 60°C. Triplicate glass tubes were used for each sample. The degree of oxidation was measured at 24h intervals using ferric thiocyanate for color development. To 100µl of the reaction mixture, 4.5ml of 75% ethanol, 100µl of 30% ammonium thiocyanate, 200µl of 1N HCl and 100µl of 20mM ferrous chloride solution in 3.5% HCl was added sequentially and the color developed measured at 500nm.

3.2.4 MOLECULAR WEIGHT DISTRIBUTION PROFILE

Molecular weight distribution of the peptides was determined with gel-permeation chromatography using an ÄKTApulse plus unit. A 30cm sephadex G25 bed was prepared in a C10/40 column (GE Healthcare) and equilibrated with 50mM phosphate buffer, pH 7.4 with 20mM KCl. The small molecular weight markers ACTH (4.6kDa), glucagon (3.6kDa), LUC (1.85kDa) and bacitracin (1.5kDa) were individually dissolved in the elution buffer and applied to the equilibrated column with a flow rate of 1.5ml min⁻¹. Absorbance was monitored at 214nm and 2ml fractions were collected. The total volume of elution from the application of each sample till the highest point of the eluted peak was measured for all the markers. Blue dextran was dissolved in the equilibration buffer at a concentration of 2mg ml⁻¹ and 1ml was carefully applied to the gel bed. Void
volume (Vo) was calculated by noting the volume required for blue dextran to elute out. A graph was drawn with the (elution volume of markers/ void volume) along with the logarithmic values of the molecular weight. Known quantities of the purified peptides were dissolved in 50mM acetate buffer, pH 4.5 with 20mM KCl and applied to the calibrated columns with a similar flow rate as the markers. The elution volumes (Ve) of E1 and F3 were calculated and from the (Ve /Vo) values obtained, the molecular weights of the applied samples were determined.

3.2.5 TRICINE-SDS PAGE OF PURIFIED PEPTIDES

The lyophilized purified peptides E1 and F3 along with the hydrolysate were run in a 16% tricine-SDS-PAGE according to Schägger’s protocol (Schägger, 2006). Anode buffer was prepared by adding 1.575g tris-HCL in 100ml deionized water and the pH adjusted to 8.9. Cathode buffer was prepared by adding sequentially 1.215g free tris and 1.792g tricine to a 0.1% SDS solution. Gel buffer was prepared by dissolving 1.215g free tris and 0.01g SDS in 10ml 0.1M HCl and adjusting the pH to 8.43.

For 16% gel preparation, 3.34ml of 60% acrylamide-bisacrylamide mixture, 3.34ml gel buffer, 3.32 ml deionized water, 100µl of 10% APS and 10µl of TEMED was poured carefully into the gel cassette. The gel was allowed to polymerize for 10min. For stacking gel, 0.3ml 60% acrylamide-bisacrylamide mixture, 1ml gel buffer and 2.7ml water were added followed by 90 µl of 10% APS and 9 µl of TEMED. The solution was overlaid on the resolving gel and allowed to polymerize for 30min. The purified peptide fractions were dissolved in a reducing sample buffer comprising of 0.4% SDS, 10% glycerol along with 0.002% Coomassie blue G-250 and 50mM tris buffer (pH 7). The samples were run in a miniVE vertical electrophoresis system (GE Healthcare) with an EPS 301 power pack for 3h. Ultra low range molecular weight markers were also run under similar conditions. After running, the gel was immersed in 1% glutaraldehyde for 5min followed by immersion in Coomassie blue staining solution. The molecular weight of E1 was determined graphically by plotting the logarithms of the molecular weight of markers versus the respective retention factors of the marker bands.

3.2.6 SEQUENCE DETERMINATION

3.2.6.1 TRypsIN DIGESTION

5mg of the peptide E1 was dissolved in 50mM ammonium bicarbonate buffer, pH 8. Mass spectroscopy-grade trypsin was added at an enzyme: substrate ratio of 1:50 and
the sample was digested for 15h at 37°C. The digestion was stopped by freeze-drying the sample.

3.2.6.2 MALDI-TOF MASS SPECTROSCOPY OF TRYPTIC PEPTIDES

For Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry (MALDI-TOF MS), the Bruker Ultraflex TOF/TOF instrument was run in reflective mode with delayed extraction and an acceleration voltage of 25kV to improve signal-to-noise ratio. 50–100 spectra were summed. Flex Analysis 2.0 software was used to analyze the mass spectra. Trypsin autolysis products at 659.48, 803.024, 1113.331, 1431.608 values were removed from the final spectra and the data was exported to Mascot Peptide Mass Fingerprint (http://www.matrixscience.com/). The mass values were matched to the UniProtKB/Swiss-Prot, a curated protein sequence database (http://expasy.org/sprot/). For search, peptides were assumed monoisotopic and oxidized at methionine residues. An ‘other mammalia’ taxonomy restriction was used, a maximum of three missed cleavages were allowed, and a peptide mass tolerance of 0.6 kDa was used for peptide mass fingerprinting.

3.2.7 CIRCULAR DICHROISM OF E1

CD measurements were performed on a Jasco Model J-715 spectropolarimeter. (Jasco, Japan) using a quartz cylindrical cuvette with a path length of 1mm. The peptide was dissolved in solutions varying pH at a concentration of 150µg ml⁻¹. The solvents used for dissolution were 50mM acetic acid, pH 3.4; 50mM acetate buffer, pH 4.5; 50mM phosphate buffer, pH 6 and 7.4. The cuvette was pre-washed with the solvent before usage and filled with 200µl of the solution for each measurement. The CD spectra were obtained by continuous wavelength scans from 240 to 195nm at a scan speed of 50nm min⁻¹ at a temperature of 28°C.

3.2.8 STATISTICAL ANALYSIS

EC₅₀ values were calculated using linear regression lines from the data given in Fig. 3.6. Values of the activities are reported as mean ± standard deviation. The data were analyzed for statistical significance using one way ANOVA and student’s t-test. P values less than 0.05 were considered significant.
3.3 RESULTS AND DISCUSSION

3.3.1 ONSET OF BIOACTIVITY

A curve was plotted with dissociation (%) against time in hours as shown in Fig. 3.1 which gave an estimate of the time from which the cryptic bioactive peptides actually started displaying their activity. A positive correlation was found between the degree of hydrolysis curve and the antioxidant activity curve indicating that the activity increased with increase in dissociation (% Hy). Radical scavenging activity started increasing only after 10h and reached its maximum at about 24h. It was noted that there was a basal chelating activity present in collagen and this activity increased rapidly from 10h onwards till 24h.

![Graph showing correlation between degree of hydrolysis and antioxidant activity](image)

*Fig. 3.1 Correlation of the degree of hydrolysis of collagen along with appearance of peptide bioactivity. The secondary axis denotes the % chelating (□) and scavenging activity (Δ) which increased in accordance with the degree of hydrolysis (▲), mostly during the 10th hour of hydrolysis. Values given are the mean of triplicates ± SD.*

3.3.2 ACTIVITY OF THE IEC FRACTIONS

An initial comparative chelating ability assay was performed with the crude fractions obtained after ion-exchange chromatography (labeled A, B, C, D, E and F). As displayed in Fig. 3.2, overall, fraction E was found to possess the highest antioxidative properties (36.8±0.4% scavenging and 27.2±1.23% chelation activity) followed by F (29.4±0.93% scavenging, 18.1±0.35% chelation activity) and C (19.8±1.42% scavenging and 19.3±0.29% chelation activity) in comparison to collagen (0% scavenging, 8±0.53% chelation activity).
chelation), the hydrolysate (14.6±0.75% scavenging, 12.3±0.49% chelation) and BSA [values not shown in the figure].

Fig. 3.2 Bioactivity displayed by the six fractions obtained from IEC. The primary Y-axis is common for both chelation and scavenging activity while the secondary axis represents the absorbance obtained for reductive ability.

EDTA, used as a positive control for chelation assay, was found to possess lower chelating ability (9.4±0.75%) at the amounts used. BHT, used as positive control for scavenging and (reductive ability assay displayed higher activities (54.3±2.4%) than the peptide fractions.

3.3.3 ACTIVITY OF PURIFIED PEPTIDES
3.3.3.1 SCREENING OF PEPTIDES

Based on the comparative bioactivity, fractions E and F were chosen for further resolution through gel permeation chromatography. The fraction E was applied to a sephadex G100 column where it resolved into three peptide fractions E1, E2 and E2b as displayed in Fig. 3.3a. E1 and E2 were found to be ‘just resolved’ with a value of 1.4; on the other hand, resolution of E2 and E2b was higher with \( R \) being equal to 3. E2b appeared as a broad peak and when run in a sephadex G25 column, resolved into two peaks, E3 and E4 (Fig. 3.3c) with the \( R \) value of 1.7. To assess purity, all four peptides, i.e., E1, E2, E3 and E4 were run individually in a sephadex G25 column where each displayed a single peak.
Fig. 3.3 Purification of bioactive peptides from IEC fraction E.

**a.** Elution profile of E in sephadex G100. Three fractions were obtained and marked E1, E2 and E2b from left to right in order of decreasing molecular weight. Y-axis is in units of milli-absorbance. The fractions were desalted, lyophilized and subjected to antioxidiant screening.

**b.** Results of antioxidative screening assay {Chelation activity (☉), reductive ability (□) and scavenging activity (■)}; E1 and E2b displayed high activity. The values given are the mean of triplicates ± SD.

**c.** Elution profile of E2 in sephadex G25; it resolved into two peptides, marked E3 and E4. Y-axis is in units of milli-absorbance. The fractions were desalted, lyophilized and subjected to antioxidative screening.

**d.** Results of the screening assays; E3 showed a higher response. The values given are the mean of triplicates ± SD.
Fig. 3.4 Purification of bioactive peptides from IEC fraction F.

a. Elution profile of F in sephadex G75. Two fractions were obtained and marked F1 and F1b, from left to right in order of decreasing molecular weight. Y-axis is in units of milli-absorbance. The fractions were desalted, lyophilized and subjected to antioxidant screening.

b. Results of antioxidative screening assay {Chelation activity (■), reductive ability (□) and scavenging activity (■)}; F1b displayed considerably high activity. The values given are the mean of triplicates ± SD.

c. Elution profile of F1b in sephadex G25; it resolved into two peptides, marked F2 and F3, from left to right with decreasing molecular weights. Y-axis is in units of milli-absorbance. The fractions were desalted, lyophilized and subjected to antioxidative screening.

d. Results of antioxidative screening; F3 was noted to be a comparatively better antioxidant. The values given are the mean of triplicates ± SD.
As displayed in Fig. 3.3c and d, the peptides E1 showed maximum radical scavenging activity (34.167±1.167%), chelating ability of 33.8±0.85% and higher reductive ability.

The fraction F upon loading onto a sephadex G75 column resolved into two fractions as shown in Fig. 3.4a; peaks were distinct but with a low resolution of 1.1. Both fractions were pooled, dialyzed, lyophilized and individually applied to a sephadex G25 column for further purification. F1 was found to display a single peak while F1B was resolved into two distinct peaks, F2 and F3 (Fig. 3.4b), with R value of 2.9. F3 showed maximum activity, (radical scavenging activity 33.8±0.65%, chelating ability 25.4±0.92% and higher absorbance for reductive assay), followed by F2 and F1 as displayed in Fig. 3.4c and d.

The assays used for screening the peptide fractions were chosen based on the definition of an antioxidative agent, which should preferably display the following characteristics: (i) ability to quench free radicals, (ii) ability to chelate reactive metals that might otherwise catalyze production of ROS and (iii) ability to donate electron pairs to reduce a high-oxidative reactive compound.

For the first condition, DPPH, a free radical stable at room temperature was used. Free radicals generally have unpaired electrons in an open shell configuration and hence highly reactive. When DPPH radicals encounter a proton-donating substance, such as an antioxidant, the radical is quenched or scavenged; as a result, the delocalization is disturbed leading to a decrease in absorbance. The extent of decrease gives a quantitative estimation of the scavenging ability of the antioxidant.

For the second condition, ability of the peptides to chelate Fe ion was checked. General chelating agents are organic or inorganic compounds capable of coordinating a metal ion to form complex ring like structures termed as ‘chelates’. Chelating agents possess metal binding groups that can form one or more linkages to the metal, essentially ‘trapping’ it and inhibiting metal-catalyzed reactions. In general, functional groups like –SH, –NH₂, =NH, >C=O, -OH and –OPO₃H are known effective metal-chelators. Five membered chelate rings are especially stable with Dn-X-Y-Dn, where Dn is the donor group and X and Y represents the intervening groups. Ferrous ions can catalyze Haber-Weiss reaction leading to the production of hazardous hydroxyl radicals, which can react with biomolecules and induce oxidative stress. The chelation of the metal ions can lead to a reduction in the hydroxyl radical production and therefore, the measurement of chelating ability is important for evaluating free radical scavenging activity. Ferrozine, a
bi-dentate ligand can chelate selectively with ferrous ions to form a tris-ferrozine iron complex. In the presence of other chelating agents, the complex formation is disrupted resulting in a decrease of the red color. Measurement of this reduction in color can give a quantitative estimate of the metal chelating ability of the added test sample. Optimally effective chelation can be achieved by means of combinations of the basic properties of the ligand, chelating agent and the resulting complex. A chelating agent that will occupy more of the coordination positions of a metal ion will generally (but not always) give a complex of greater stability than otherwise. Similarly, whereas the net ionic charge of the chelator defines its availability, the distribution and ability to reach the metal ion for binding and the net ionic charge of the complex decides the fate of the metal. Thus, it is important that a chelator satisfy criteria that allow it to: (1) transport across physiological barriers into compartments where the metal ion is concentrated, (2) form a stable complex with the metal after removing it from the physiological chelator and (3) form a chelation complex whose properties render it non-toxic and/or unavailable temporarily (Flora and Pachauri, 2010).

For the measurement of reductive ability the reduction of ferric ions to ferrous was investigated. The reducing power of bioactive compounds has been reported to be due to the ease of electron pair donation, which is in turn, associated with their antioxidant activity. The peptides were incubated with ferricyanide ions and ferric ions are added to the solution. The ferrous ion formed upon reduction by the peptides, forms a blue-green complex with ferricyanide ions which is measured at 700nm. Increased absorbance produces greater color indicating greater reductive ability. A final criterion of an efficient antioxidative agent is partial lipid solubility. ROS stress occurs in cellular macromolecules along with other cellular components, including cell membrane. Free radicals can react with fatty acids in lipid bilayer to produce reactive lipid peroxides ultimately leading to severe membrane damage.

The auto-oxidation of lipids proceeds through three sequences. The presence of double bonds in fatty acids weakens the C-H bond on the C atom adjacent to the double bond making H• removal easier. Initiation of the sequence occurs when a reactive radical abstracts a proton from a methylene group resulting in a reactive carbon center. Through rearrangement, the molecule forms a conjugated diene and reacts with O2 to form peroxy radicals, which abstracts H• from a different molecule thus propagating the reaction.
Fig. 3.5 Lipid peroxidation inhibitory activity of the peptides. Purified peptides E1 (■) and F3 (□) along with the hydrolysate (▲) was used for the assay. Linoleic acid (LA) was used as the control (♦) and BHT was used as the positive control (◊). As evident from the figure, the peptides reduced the auto-oxidation of linoleic acid, resulting in reduction of absorbance. Values in the figure are mean of triplicates ± SD.

There are several levels at which antioxidative agents may act to inhibit lipid peroxidation. BHT and tocopherol are known to be efficient quenchers of redox species. BHT displays high lipid solubility owing to its hydrophobic methyl groups and the benzene ring. Tocopherol is the major lipophilic antioxidant in vivo and the presence of a large phytyl group allows it to be incorporated in the lipid layers. Both antioxidants are known to inhibit lipid peroxidation at the propagation stage (Niki et al. 2005). BHT can further scavenge even the radicals responsible for the initiation reaction, lowering lipid peroxidation to a minimum value as visualized in the figure. Peptides E1 and F3 were successfully able to reduce the linoleic acid auto-oxidation for a period of 6 days as shown in Fig. 3.5. The results were found significant (p≤0.001) at 95% confidence level based on one way ANOVA.
Fig. 3.6 Effect of amount of the peptides E1 (□), E3 (◆), F2 (×) and F3 (♦) on the following activities: a. %Chelation, b. %Scavenging and c. Reductive ability. EDTA was used as the positive control for chelation assay and BHT was used as the positive control (△) for scavenging and reductive ability assay. Values given are the mean of triplicates.
3.3.3.2 EFFECT OF AMOUNT ON BIOACTIVITY

As displayed in Fig. 3.6, there was an almost hyperbolic increase in radical scavenging and chelating activity with increasing amounts of all the test peptides. The peptides acted as potent antioxidative agents at lower concentrations but the activity asymptotically approached a maximum of 70% at higher concentrations, probably due to the inherent recoiling nature of collagen type I peptides. Upon comparison of the EC_{50} values the scavenging activity was found to be in the given order, E1 followed by F3, E3 and F2. The EC_{50} values for chelating ability were in the given order, F3 followed by E1, F2 and E3. The results are summarized in Table 3.1.

BHT was used as the standard for scavenging activity and achieved its maximum 74% with a concentration as low as 0.01mg ml^{-1}. EDTA was used as the positive control for chelation assay. At amounts lower than 0.06mg ml^{-1}, the chelating ability of the peptides were four times greater than that of EDTA. However at amounts more than 0.2mg ml^{-1}, the chelation ability of EDTA reached 100% and was double the %chelation values for E1 and F3 and 2.5 times that of E3 and F2.

3.3.3.3 EFFECT OF pH VARIATION ON CHELATING ABILITY

A change in pH was found to alter the peptide’s bioactivity quite drastically. The results, as depicted in Fig. 3.7, show a general decrease in activity of the four peptides with increasing pH until nil activity and further increase at higher pH.

![Fig. 3.7 Effect of pH variation upon the chelating ability. The four purified peptides, E1 (■), E3 (■), F2 (□) and F3 (□) were assayed for chelating ability at a range of pH from 3.4 to 7.4.](image)
Chelation of a metal ion by an agent depends on the presence of charged residues capable of donating its bonding pair of electrons to the metal, forming a coordinate bond. The chelating ability of peptides is strictly dependent on the number of charged residues with the ability to donate electrons in a particular reaction system. Chelation of a positively charged metal ion is generally performed through residues with negatively charged functional groups. A minor method of metal chelation also occurs via positively charged residues through an intermediate water molecule. The charged state of a functional group is dependent on its environment being acidic, basic or neutral, i.e., on the pH of the medium. As a consequence the chelation activity also varied in accordance with the change in pH.

E1 and E3 were found to have higher chelating activity at acidic pH while F2 and F3 had higher activity in slightly alkaline pH. So although E1 and E3 gave better results with radical scavenging assay and reductive ability assay, it is F2 and F3 which could be more potent chelating agents in physiological pH. The results were significant (p≤0.001) at 95% confidence level based on one way ANOVA.

### 3.3.4 IDENTIFICATION AND CHARACTERIZATION OF THE CRYPTIC PEPTIDE E1

Among the several requirements for being a bioactive peptide, possession of a low molecular weight is fairly important as this will assist the bioactive peptides to evade digestion and increase their bioavailability. Gel permeation chromatography is an established method for molecular weight determination of proteins and peptides. Separation of proteins is based on the ability of the eluant molecules to enter the pores of the resin. Proteins smaller than a specific size can be retained by the gel but larger proteins are excluded from entering the resin pores and as a consequence, elutes out faster. The molecular weights of unknown proteins are determined by comparing the factor (Ve/Vo) for the protein to a range of (Ve/Vo) obtained for standard marker proteins. The factor (Ve/Vo) is without units and thereby, independent of column size and other conditions. The void volume of a matrix gel is determined by elution of a large molecule like blue dextran (2000kDa). A calibration curve is drawn with the Ve/Vo values of markers versus the log of their molecular weight, as displayed in Fig. 3.8. The (Ve/Vo) of the unknown peptide is plotted onto the calibration curve and the molecular weight determined.
Fig. 3.8 Calibration curve of sephadex G25 with markers. SD values were omitted for clarity.

Fig. 3.9 shows the electrophoretic pattern of the hydrolysate along with the purified peptides E1 and F3. The hydrolysate appears as a long dark streak and the peptides appear as distinct single low molecular weight bands. F3 and E1 matched with the 1.4kDa and 3.5kDa band respectively, in the marker lane. The retention factor (Rt) values of the peptides were plotted onto the calibration curve prepared with markers, as displayed in Fig. 3.10 and the molecular weights calculated there from. Averaging the molecular weights obtained from column chromatography and electrophoresis, E1 and F3 were found to be 3kDa and 1.4kDa respectively.

These data support the fact that most of the peptides exhibiting bioactivity are those with low molecular weight, as noted in previous studies (Pihlanto 2006). Tricine-SDS PAGE is preferentially used for the optimal separation of proteins <30kDa and provides better resolution for low molecular weight peptides (1-5kDa) than that of Laemmli SDS PAGE. The trailing ion, tricine has a pk_a that is close to that of the pH of the resolving buffer (8.3) and affects the stacking conditions of the proteins, ultimately leading to better resolution (Schagger and von Jagow, 1987).

Since E1 displayed an overall better antioxidative ability than F3, it was further characterized by sequencing and CD spectral analysis. Biological activities of the peptides are dependent on the molecular weight, the amino acid composition and also positioning of the residues in the peptide. Sequencing reveals the amino acids present along with the pattern of arrangement, leading to a sophisticated understanding of the peptide bioactivity.
Fig. 3.9 The electrophoretic pattern of the hydrolysate along with the purified peptides E1 and F3 in 16% tricine SDS PAGE.

Fig. 3.10 Retention factors of low molecular weight markers and determination of molecular weight of the peptides.
Table 3.1. Details of the four cryptic bioactive peptides including their molecular weight, \( EC_{50} \) values and their maximum antioxidative activity (\( C_{\text{max}} \) and \( RS_{\text{max}} \)).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Purified Peptides</th>
<th>Elution with NaCl</th>
<th>Mol. Wt. (kDa)</th>
<th>Chelating ability</th>
<th>Radical Scavenging activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>( EC_{50} ) (mg ml(^{-1}))</td>
<td>( C_{\text{max}} ) (%)</td>
</tr>
<tr>
<td>E</td>
<td>E1</td>
<td>0.2M</td>
<td>3</td>
<td>0.163</td>
<td>80.4 ±1.22</td>
</tr>
<tr>
<td></td>
<td>E3</td>
<td>0.2M</td>
<td>0.95</td>
<td>0.232</td>
<td>71.5 ± 1.0</td>
</tr>
<tr>
<td>F</td>
<td>F3</td>
<td>0.3M</td>
<td>1.4</td>
<td>0.147</td>
<td>73.9 ±2.15</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>0.3M</td>
<td>2.4</td>
<td>0.211</td>
<td>63.6 ±1.56</td>
</tr>
</tbody>
</table>

\( EC_{50} \) values are calculated from the regression lines obtained from the figures and \( ^{b} \) stands for ion-exchange chromatography.

The amino acid sequence obtained after mass peak identification of E1 (Fig. 3.11) was found to match significantly with position 1066 to 1101 of collagen \( \alpha-1(I) \) chain precursor P02453 (CO1A1_BOVIN).

[URL:http://www.uniprot.org/uniprot/P02453]
GETGPAGPAGPAGPVGARGPAGPQGRDGETGEQ

An analysis of the sequence enabled the peptide to be theoretically divided into two parts; beginning from the N terminal G to the 17\(^{th}\) residue and from the 18\(^{th}\) residue to the C terminal. The N terminal half is primarily hydrophobic in nature with only one charged residue, E, at the beginning and two hydrophobic residues; I and V. The remaining C-terminal half comprises of comparatively more number of charged residues and this half would be primarily hydrophilic in nature. Since charged amino acid residues are involved in antioxidative reactions of the peptide, probably the C terminal portion is responsible for the chelation and scavenging activity, while the N terminal portion may have more of a structural role. The hydrophobicity of the residues suggests that the C-terminal portion is indeed more accessible and would, therefore be the key player responsible for its activities.
Fig. 3.11 MALDI-TOF spectrum of the trypsin generated fragments of E1.
The peptide possessed two strictly hydrophobic residues (V and I) and four A residues, 3 of which were present in the N terminal half. In all probability, it is this half which could have been responsible for the lipid solubility and, consequently, inhibition of lipid peroxidation activity of E1. The mechanism behind the activity of E1 would probably be very different from that of BHT or tocopherol. Both these molecules harbor a phenolic hydrogen, located at the water-lipid interface and radical formation on such molecules are stabilized by resonance (Niki et al 2005). E1 on the other hand is deprived of any phenolic groups but was efficient at lipid peroxidation inhibition.

It is presupposed that the presence of certain amino acids such as Y, F, W, H, M and C are crucial for the antioxidative activity of peptides (Ren et al 2008; Sheih et al 2009). Most of these amino acids are aromatic in nature and/or have electron pairs which can be donated to free radicals or used to chelate ions. In spite of lacking the above mentioned amino acids, E1 was able to exhibit a strong antioxidative capacity. This is altogether not impossible, as recent data suggests that the presence of certain amino acids like L, Q, E and D (Rajapakse et al 2005; Alemán et al 2011) and certain sequences such as QG and GP (Byun et al 2009) can render a peptide with antioxidative properties. An analysis of the amino acids in E1 showed it to have six GP sequences, one QG sequence along with charged amino acids K, D, E and R, whose active participation in charge/electron transfer reactions would result in antioxidant properties. E1 possessed most of the engaging residues near the hydrophilic C-terminal, particularly in the sub-sequence GPAGPQGPRGDK.

The amino acids Q and R, in their free form, have been known to induce antioxidative effect. The presence of K in antioxidative peptide is important as it renders a ‘sacrificial’ attribute to it. WBC-derived HOCl and HOBr, although primarily involved in pathogen deactivation, cause collateral damage to proteins by reaction with K and Y forming halolysines and halotyrosines. The reaction proceeds via halogenation of K followed by transfer of the halogen group to Y. Indeed, the presence of these halogenated Y could act as biomarkers for assessment of oxidative damage. In the presence of K and absence of Y, the oxidants form a major product, lysyl nitrile, which being stable does not allow the reaction to proceed further and reduces collateral damage. Presence of K (and absence of Y) in a bioactive antioxidant peptide could similarly, lead to reaction with HOCl and HOBr, with the formation of lysyl nitrile thus acting as a ‘sacrificial antioxidant residue’ (Sivey et al 2013). The presence of P in the sequence imposes conformational restraints on the secondary structure and may have an indirect effect on
the bioactivity. More than their presence, the order and sequence in which these amino acids are arranged can affect the structure, reactivity and solvent accessibility of E1, which in turn, can dictate its inherent bioactivity (Elias et al. 2008).

A comparison with other bioactive collagen peptides showed E1 to have a moderate antioxidant activity. For radical scavenging activity, the EC50 of the peptide E1 was found to be the lowest (338µg ml⁻¹) among the four bioactive peptides obtained. It was lower than the peptides DPALATEPDPMPF (EC50 = 660µg ml⁻¹) obtained from Nile tilapia scale gelatin (Nagai et al. 2007), and RSGH-Pc, a cobia gelatin peptide (scavenging activity of 60.7% at a concentration of 10mg ml⁻¹) (Yang et al. 2008). At a concentration of 11mg ml⁻¹, pepsin hydrolysate of porcine skin collagen had a radical scavenging activity of 87.18±1.84% (Li et al. 2007). The EC50, however was more than that of the peptide LEELEEELEGCE, isolated from bullfrog skin (EC50=16.1µM) (Qian et al. 2008). The papain hydrolysate of tuna backbone protein also showed a DPPH scavenging activity of 36.72% (Je et al. 2007).

For chelating activity, the peptide E1 had an EC50 of 163µg ml⁻¹, which was quite low when compared to collagen peptides from other sources. Peptides from porcine skin and squid collagen displayed a chelating activity of 37.4±1.5% at a concentration of 11mg ml⁻¹ (Li et al. 2007) and 80% activity at 0.2mg ml⁻¹ (Giménez et al. 2009) respectively. Chelating ability was also compared with peptides obtained from other helical fibrous proteins; Pacific hake muscle hydrolysate peptides possessed chelating ability ranging from approximately 7 to 46% at a concentration of 5mg ml⁻¹ (Samaranayaka and Li-Chan, 2008) and round scad protein muscle hydrolysate possessed an activity of about 60% (Thiansilakul et al. 2007).

The molecular weight of E1 was found to be 3.2805kDa, which matched closely with the results obtained from gel permeation chromatography and tricine-SDS-PAGE. The peptide displayed the regular G-X-Y arrangement of amino acids, so typical of collagen, as is evident from the sequence. CD spectroscopy was done to determine the secondary structural characteristics and whether the conformation of the peptide affected its bioactivity.
The CD data of E1, as displayed in Fig. 3.12, comprised of a large negative peak at 196-205nm and a small positive peak around 220nm, similar to collagen (Pêcher et al 2008). Both peaks responded to a change in pH. The negative band minimum at 196nm was seen to shift towards higher wavelengths with increase in pH. At physiological pH, the negative minimum reached a wavelength of 205nm, indicating a possible random coil configuration (Machling et al 1996). The positive peak around 220nm, on the other hand, decreased with the pH, indicating a reduction in polyproline-II (PP-II) helical content and a subsequent increase in random coil conformation (Pêcher et al 2008).

![Fig. 3.12 CD spectra of purified peptide E1 at pH 3.4 (∧)، pH 4.5 (●●●●)، at pH 6 (――) and at pH 7.4 (●―). The curve progressively shifts towards random coil conformation as pH increases. The four spectra were recorded at 28°C.](image-url)
Although native collagen is triple helical, earlier reports have stated that single α-chain of collagen takes up a PP-II conformation in solution (Mezei et al 2004; Vila et al 2004). Overall, the CD data of the peptide supported the fact that at lower pH, it attains a PP-II helix conformation and changes to random coil at alkaline pH. This is strongly indicative of the fact that the conformation of the peptide does indeed affect the bioactivity.

The higher solvent accessibility of the PP-II conformation (Mansiaux et al 2011) would allow the functional groups of the amino acids to interact better with the reactive radicals. In alkaline conditions, the peptide shifts to the random coil conformation, leading to probable structural changes in the reacting groups. In addition, increasing pH brings about a change in the charge state of the reactive functional groups. The cumulative action of these two factors might explain why the activity of E1 varies markedly over a broad range of pH.

A BLAST search (Altschul et al 1997; Altschul et al 2005) of the sequence against UniProtKB/Swiss-Prot protein sequences revealed that other than the RGD motif, the peptide did not contain any putative conserved domains (Query ID: 11468). However, the sequence, with minor variations was found to be present in other mammals including humans (94% identical) as shown in Table 3.2. It was interesting to note that in most of the sequences, the peptide was present in at almost the same position; in the vicinity of residue number 1060 to 1100, lying near the telopeptide region. It was also noted that the sub-sequence GPAGPQGPRGDK was common for all the sequences except for Rana catesbeiana where the 8th residue is a V instead of P.

The mammalian collagenases cleave collagen into two fragments and most probably, wouldn’t be able to release the active peptide immediately. But once this first stage of collagen degradation is complete, other proteases can cleave further until such bioactive peptides are either ‘exposed’ or released and can act as *in vivo* antioxidant in case of a free radical overload.
Table 3.2. pBLAST results. The sequence of E1 compared with closely matching collagen sequences from other species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Type</th>
<th>Sequence</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1</strong> Bos taurus</td>
<td>I α1</td>
<td>1066-1101</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GETGPAGPAGPGVPVGARGPAGPQGPRGDKGEQ</td>
<td></td>
</tr>
<tr>
<td><strong>2</strong> Canis lupus familiaris</td>
<td>I α1</td>
<td>1063-1098</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GETGPAGPAGPGVPVGARGPAGPQGPRGDKGEQ</td>
<td></td>
</tr>
<tr>
<td><strong>3</strong> Rattus norvegicus</td>
<td>I α1</td>
<td>1056-1091</td>
<td>97</td>
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<td></td>
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<tr>
<td><strong>4</strong> Mus musculus</td>
<td>I α1</td>
<td>1056-1091</td>
<td>97</td>
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<td>1094-1128</td>
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<td>970-1005</td>
<td>75</td>
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