"Characterization and Bioactivity Evaluation of Peptides of Indian Squid, (*Uroteuthis duvauceli)* from the Arabian Sea"

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"'Characterization and bioactivity evaluation of peptides of Indian squid, (Uroteuthis duvauceli) from the Arabian Sea"

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Dedicated to my family

Declaration

*I, Remya Kumari K.R. do hereby declare that the thesis entitled "**Characterization and bioactivity evaluation of peptides of Indian squid, (Uroteuthis duvauceli) from the Arabian Sea** is a genuine record of bonafide research carried out by me under the supervision of Dr. Suseela Mathew Principal Scientist and Head, Biochemistry and Nutrition Division, Central Institute of Fisheries Technology, Cochin and has not previously formed the basis of award of any degree, diploma, associateship, fellowship or any other similar titles of this or any other University or Institution.*

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This is to certify that this thesis entitled "Characterization and bioactivity evaluation of peptides of Indian squid, (Uroteuthis duvauceli) from the Arabian Sea" embodies the original work done by Ms. Remya Kumari K.R. (Reg. No. 4196), under my guidance and supervision in the Biochemistry and Nutrition Division of Central Institute of Fisheries Technology, Cochin. I further certify that no part of this thesis has previously been formed the basis of award of any degree, diploma, associateship, fellowship or any other similar titles of this or in any other University or Institution.

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Date:

Cochin-29 **Remya Kumari K.R.**

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Abbreviations

Abs	-	Absorbance
%	-	Percentage
H_2O_2	-	Hydrogen peroxide
°C	-	Degree celsius
µg-	-	Microgram
µl	-	Microlitre
µm	-	Micrometre
ANOVA	-	Analysis of Variance
AOA		Antioxidant activity
AOAC	-	Association of Official Analytical Chemists
BF_3	-	Boron Triflouride
BHA	-	ButylatedHydroxylanisole
BHT	-	ButylatedHydroxytoluene
BSA	-	Bovine Serum Albumin
Ca	-	Calcium
cm	-	Centimeter
CO_2	-	Carbon dioxide
Cu^{2+}	-	Copper
Da	-	Dalton
dF	-	Degrees of Freedom
DH	-	Degrees of Hydrolysis
DNA	-	Deoxyribonucleic acid
DPPH	-	2,2-diphenyl-1-picrylhydrazyl
E/S	-	Enzyme substrate ratio
EDTA	-	Ethylene diaminetetraacetic acid
EPA	-	Eicosapentaenoic acid
ESI-MS/MS	-	Electro Spin Ionization Mass Spectrometry

FA	-	Formaldehyde
FAO	-	Food and Agriculture Organization
FDA	-	Food and Drug Administration
Fe^{2+}	-	Iron
FFA	-	Free Fatty Acid
Fig	-	Figure
FPLC	-	Protein Liquid Chromatography
g	-	Grams
GAGs	-	Glycosaminoglycans
gcp	-	gram per crude protein
GP	-	Glycoproteins
GPC	-	*Sn-glycero-3-phosphocholine*)
GPE-	-	*sn*-glycero-3-phosphoethanolamine
H	-	Hydrogen radicals
h	-	Hours
H_2O	-	Water
H_2O_2	-	Hydrogen peroxide
H_2S	-	Hydrogen sulfide
HCl	-	Hydrochloric acid
HPLC	-	High performance liquid chromatography
IL	-	Interleukin
INF	-	interleukin necrosis factor
ISO	-	International Organization for Standardization
KCl	-	Potassium chloride
kDa	-	Kilodalton
kg	-	Kilogram
KOH	-	Potassium hydroxide
L	-	Liter

LC–MS/MS	-	Liquid Chromatography Tandem Mass Spectrometry
M	-	Molar
m	-	Meter
MFP	-	Myofibrillar protein
min	-	Minute
mL	-	Milliliter
N	-	Normality
Na_2SO_4	-	Sodium sulfate
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
NH_3	-	Ammonia
nm	-	Nanometer
O_2	-	Oxygen
OH	-	Hydroxyl radicals
$OH^{\bullet}$),	-	hydroxyl radicals
PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate buffered saline
ppm	-	Parts per million
PUFA	-	Poly unsaturated fatty acids
R^2	-	Coefficient of determination
rpm	-	Revolutions per minute
RSM	-	Response surface methodology
SAS	-	Statistical analysis software
SD	-	Standard deviation
SDS	-	Sodium dodecyl sulphate
Sec	-	Second
SPSS	-	Statistical package for the social sciences
USFDA	-	Food and Drug Administration

UV	-	Ultraviolet
V	-	Volt
WHO	-	World Health Organization
µ	-	Micro
mm	-	Millimeter
NO	-	nitric oxide
NSAIDs	-	non-steroidal anti-inflammatory drugs
$O2^{-}$,	-	Superoxide
PE	-	petroleum ether
PG	-	Prostaglandins
ROS	-	reactive oxygen species
SPH	-	squid protein hydrolysate
USA	-	United States of America
w/v	-	Weight/ volume
α	-	Alpha
γ	-	Gamma
TNF	-	tumour necrosis factor
$1O_2$	-	singlet oxygen

Chapter 1

Introduction

Contents

1.1 Introduction

Squids are delicious and nutritive seafood which is capable to produce various value added products especially squid gel, surimi and seafood analogues. Commendable level of proximate content indicates its nutritional significance; moreover occurrence of low fat and high proteins content are beneficial to elderly population. Oceanic squids are considered as unexploited resource having immense scope for marketing. Generally, trading of squid will be in fresh, chilled and frozen form. Even though there is well acceptance in the global market, it's therapeutic and nutraceutical properties are not yet established. Moreover, the severity of deadly diseases such as cancer, kidney damage, liver damage etc. led to necessity of establishing novel bioactive and pharmaceutical molecules from marine source. Nowadays, marine sector have immense attention among the researchers for the synthesis of bioactive compounds especially alginate, chitin, chitosan, proteoglycans, glycosaminoglycans, polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA), alkylglycerols, collagen and bioactive peptides etc. Antihypertensive, antioxidant, anticoagulant, anticancer, immunomodulatory and antidiabetic properties of marine peptides are already being investigated, whereas the bioactivities of squid peptides are not fully explored which will

definitely gain wide attention in pharmaceutical, cosmeceutical and nutraceutical industries. Hence, a detailed investigation to establish the antioxidant, antiulcer and anticancer properties of Indian squid have assume importance.

Severe diseases like cancer, ulcer, atherosclerosis, arthritis and diabetes are common in humans due to the action of free radicals. Generally, free radicals and reactive oxygen species (ROS) generated in biological systems by endogenous processes or exposure to external stimuli can cause damage in proteins, mutations in DNA, cellular damage, oxidation of membrane phospholipids and modification in low density lipoproteins (LDL). Oxidation of fats and oils during processing and storage of food products worsen the quality of their lipid content and generate ROS. Consumption of these potentially toxic products can give rise to adverse effect in human beings. Organisms have developed efficient protective mechanisms against excessive accumulation of ROS, which are neutralized by an elaborate antioxidant defense system consisting of enzymes such as catalase, superoxide dismutase and glutathione peroxidase/reductase, and numerous non-enzymatic antioxidants such as vitamin C, vitamin E, vitamin A, pyruvate, glutathione, taurine and hypotaurine. Under normal conditions, antioxidant defense systems can remove reactive species through enzymatic (like superoxide dismutase and glutathione peroxidase) and non-enzymatic antioxidants (such as antioxidant vitamins, trace elements, coenzymes and cofactors). Even though there exists a good range of natural and diverse protective systems, there is considerable evidence to support the hypothesis that oxidative damage is widespread in mammals, plants and microorganisms. However, in certain circumstances the endogenous defense system fails to protect the body against reactive radicals on its own. This damage may arise from an increased rate or

extent of oxidant formation, a failure or decrease in activity of defense systems, or both simultaneously. Under pathological conditions, the balance between the generation and elimination of ROS is disturbed. This altered balance between formation of oxidants and their removal / repair in favour of higher oxidant levels is often termed 'oxidative stress', a condition in which the generation of highly reactive molecules such as ROS and reactive nitrogen species (RNS) exceed their elimination and/or when their elimination is inadequate. In human beings oxidative stress usually plays the role of a promoter rather than an initiator of chronic diseases. Extensive interest has risen about the instrumental role played by oxidants and antioxidants in the etiology of numerous human diseases, preservation of food quality, and efficacy of medicines and antibodies. This uncontrolled generation of free radicals affects membrane lipids, protein and DNA, and interrupt normal proliferation and apoptosis mechanism of the body. This causes uncontrollable cell growth or carcinogenesis in an organism. Chemotherapy and anticancer drugs are the common available treatments for cancer; however it has disastrous side effect and immunotoxicity to normal cells. Hence, development of new generation drugs for the effective destruction of cancer cells with low impact to the normal cells gained more attention among the researchers. Inflammation is another severe problem caused by ROS especially nitric oxide (NO) and superoxide (O^{2-}), released by lysosome which finally accelerate the tissue damage in the affected area. Initial strong pain, redness and swelling shift to vasomotor rhinnorrhoea, arthritis and atherosclerosis at chronic stage. It mainly generates severe health problem to the elder population and find a remedy to this situation has great significance. Peptic ulcer is a common gastric disorder that affects millions of people worldwide, which is caused by the action of ROS. Uncontrollable formation of ROS leads to mucosal damage

and peptic ulcer. Development of natural drug possessing less or no side effects from seafood source gains great attention for the treatment of peptic ulcer.

In order to prevent these diseases and provide better health benefits to the consumer, antioxidants are widely used as ingredients in dietary supplements. Antioxidant is a molecule, which is capable for inhibiting the uncontrolled oxidation of the biomacromolecules by reactive oxygen species. They are known to act at different levels in an oxidative sequence and terminate chain reactions by removing free radical intermediates, and inhibit other oxidation reactions and therefore could control many diseases like cancer, heart disease etc. Antioxidants provide electron density to compounds which are likely to undergo oxidation, thus preventing them from losing electrons. Antioxidant prevents the oxidation by neutralizing the free radicals such as, superoxide anion radical (O^{2-}) and hydroxyl radical (OH) which are unavoidable in aerobic organisms. Recent scientific reports indicated that hydrolysis of proteins from plant and animal sources to acquire the compounds which show antioxidant activity has been productive, such examples include quinoa seed protein, capelin protein egg-yolk protein, soybean, milk case in hoki frame protein, mackerel protein etc. to name a few. However, synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) are restricted to use in food products because of their induction of DNA damage and toxicity. In this context, the focus of research for the synthesis of antioxidant peptide from terrestrial origin has now shifted towards marine sources.

Bioactive peptides are considered specific protein fragments that are inactive within the sequence of the parent protein. After they are released by enzymatic hydrolysis, they may exert various physiological functions. Alcalase,

papain, pepsin, trypsin, a-chymotrypsin, pancreatin, flavourzyme, pronase, neutrase, protamex, bromelain, cryotin F, protease N, protease A, orientase, thermolysin, and validas are the most commonly used enzymes for the hydrolysis of seafood proteins. Protein hydrolysis decreases the peptide size, and thereby making hydrolysates the most available amino acid source for various physiological functions of human body. Based on their structural properties and their amino acid composition and sequences, these peptides may play various roles, such as opiate like, mineral binding, immunomodulatory, antimicrobial, antioxidant, antithrombotic, hypocholesterolemic and antihypertensive functions. Moreover, due to low molecular weight and simple structure of antioxidant peptides they have the capacity for better molecular mobility and diffusivity which improves the interactions with cancer cell components. This invaded peptide destroys cancer cell by damaging lysosomal membrane, altering mitochondrial pathway of apoptosis by caspase cascade, activation of immune modulatory pathway, and inhibition of DNA replication in the cell cycle. Moreover, the peptides which are capable to neutralize reactive oxygen species have great potential to control inflammatory response and peptic ulcer in humans.

Seafood is a good source for the synthesis of natural antioxidant peptides. Recent research have identified a number of bioactive compounds from fish muscle proteins, collagen and gelatin, fish oil, fish bone, internal organs and shellfishes (Je *et al.,* 2005; Jeon and Kim, 2002; Kim *et al.,* 2001). Marine fishes, shrimps and algae have many important bioactive substances, such as peptides, unsaturated fatty acids, polysaccharides, trace elements and natural pigments, etc. These extracted and purified bioactive compounds especially marine bioactive peptides have several applications in biotechnological and pharmaceutical industries. Although numerous bioactive peptides have been reported from fish sources, other marine organisms such as

mollusk and crustacean sources have not been extensively studied. Therefore, development of new technologies in search of novel bioactive compounds from these sources will bring more value and represents unique challenges and opportunities for the seafood industry. There has been a considerable momentum in the field of modern pharmaceuticals towards the use of natural products, or compounds derived from, or inspired to, natural products. This is attributed to the incredible biomedical potential represented by the chemical analysis of the biodiversity of natural organisms. Secondary metabolites contained in these organisms are the result of millions of years of evolution and natural selection, even a single species constitutes a library of metabolites that is validated for the bioactivity. In the light of these observations, this research is focused to identify new bioactive natural compounds from Indian squid and establishes its nutritional significance, antioxidant, anti-cancer, anti-inflammatory and antiulcer properties for human utilization.

1.2 Scope of the study

Seafood is considered as one of the excellent source for bioactive compounds. Oceanic squids were underexploited resource which caters commendable quantities of nutritional and bioactive compounds. However, bioactivities of peptides derived from Oceanic squid's especially Indian squid (*U. duvauceli*) are not fully explored established and their role in the organism is yet not clearly identified. If we establish its antihypertensive, antioxidant, anticoagulant, anticancer, immunomodulatory and anti-diabetic properties, it could be a cheap source to formulate anti-carcinogenic, anti-ulcer and anti-inflammation nutraceutical. Hence, this study paves the way to explore this moderately exploited resource to provide essential nutrients to nutritionally deprived population. Invention of new diving techniques and capture methods help to explore oceanic squids from marine water which promote the research

for the synthesis of bio-molecule and also find out the possibilities for its utilization.

1.3 Relevance of the research

Changing life style and food habits of humans invited several health problems and fast spreading of severe diseases. Moreover, the action of reactive oxygen species and free radicals mutates DNA and damages other cellular components causing dangerous diseases especially cancer, ulcer, inflammation etc. Synthetic drugs and other treatment for these deadly diseases are not effective and also causing several side effects to humans. Moreover, application of most of the synthetic antioxidants such as BHA, BHT and propyl gallate in food is restricted due to their potential health risks to consumers. In this context, researchers showed keen interest to find out effective natural therapeutic agents with less or no side effects for the treatment of the deadly diseases and also to identify and synthesis natural antioxidants especially peptides from natural protein sources. However, utilization of terrestrial resources for the synthesis of bioactive compounds had been utilized to a large extend, and now the researchers are focusing to explore the underutilized marine sector. Hence, the investigation of moderately explored squid peptides as nutraceutical and pharmaceutical agent got great relevance in the modern scenario. Detailed research is essential to identify suitable protease and its optimum hydrolysis condition for the synthesis of squid peptides and to establish its antioxidant, anticancer, antiulcer and anti-inflammation properties for the development of new arena of drugs for cancer, ulcer and inflammation treatment. Moreover, the generation of scientific back up for the nutritional profiling of Indian squid throw light to hitherto unknown nutritional profiling of Indian squid which will open up the scope for its exploration. Hence, appropriate research for the development of efficient

nutraceuticals and pharmaceutical compounds from Indian squid protein has high relevance.

1.4 Objective of the study

- Establish the biochemical composition and nutritional profiling of Indian squid (*U. duvauceli*).
- Optimize the hydrolysis conditions (ie., enzyme/substrate ratio, pH and time) of pepsin, papain and trypsin based on the antioxidant properties of Indian squid protein hydrolysate using response surface methodology (RSM).
- Differentiate and identify suitable protease for the hydrolysis of squid protein at optimized condition based on its antioxidant properties such as DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, superoxide anion radical-scavenging activity, metal chelation ability, inhibition of lipid peroxidation in the linoleic acid model system and reducing power.
- Isolate and purify antioxidant peptide from squid (*U. duvauceli*) protein hydrolysate based on the molecular weight and also determine the antioxidant properties and amino acid sequence of the resultant peptides.
- Investigate anti-cancer, anti-inflammatory and anti-ulcer properties of isolated and purified Indian squid peptides.

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Chapter 2

Review of Literature

Contents

2.1 Squid as seafood

Cephalopods are commercially important and nutritionally valuable seafood (Torrinha *et al.,* 2014). There is an increasing demand for cephalopod in global food market (Guerra and Rocha, 1994). In order to satisfy these demands, culture of cephalopod has been expanded (Sykes *et al.,* 2006). Commercially important cephalopod comprises octopus, cuttlefish and squid (Paarup *et al.,* 2002). Among this, squids were getting immense attention because of the presence of nutritionally important fatty acids, minerals (Torrinha *et al.,* 2014) and essential amino acids (Bano *et al.,* 1992). Among the squids, genus *Loligo* possess better consumer acceptance due to organoleptic characteristics (Sikorski and Kołodziejska, 1986). *Loligo* genera comprise edible species such as *Loligo duvaucelii, L. gahi, L. reynaudii, L. opalescens* and *L. vulgaris* (FAO, 2011). Squids are neritic shallow water species, which are mainly found at the depth of 30 to 170 m and chiefly feed mysiids, euphaussids, ostracods, fish fry's and small squids. Squids are widely distributed in Red Sea, Arabian Sea, Mozambique to the South of China Sea, Philippines Sea and northward to Taiwan. Indian marine water is found to be a major habitat for many economically important squid species such as *L. duvauceli, S. lessoniana, Doryteuthis spp.*, etc. However, available literatures

indicate that oceanic squids are unexploited resources (Xinjun *et al.,* 2007), which are mainly distributed in Central Arabian Sea at a depth above 250 to 300 m (Mohamed *et al.,* 2006; Zuyev *et al.,* 2002).

2.1.1 Nutritional profiling of squid

Squids are widely accepted seafood commodity because of its peculiar palatability, sensory properties and better yield percentage of meat for consumption. Bano *et al.,* (1992) reported the significance of squid resource as an alternative for animal protein. Generally, squids are being marketed as fresh, dried (Kugino *et al.,* 1993), chilled (Hurtado *et al.,* 2001) and in a frozen forms (Paredi and Crupkin, 1997). Moreover, it has the potential to develop various value added products such as squid gel, surimi and seafood analogues (Sánchez-Alonso *et al.,* 2007). However, marketing of squids is risky due to easily perishable nature and quality deterioration especially browning. These changes in squids are accompanied by the biochemical composition (Caili *et al.,* 2006). Biochemical profile of squid is remarkably different from fishes. According to Spitz *et al.,* (2010) ecosystem has great role for the nutritional quality of an organism. Similarly, metabolic activity influences biochemical composition and body mass of an organism (Schmidt-Nielsen, 1984). Most of the squids show isometrical metabolism (Seibel, 2007).

Proximate composition of an organism comprises relative amounts of water, protein, lipid, ash and carbohydrate. Proximate content of European squid (*L. vulgaris*) was reported by Atayeter and Ercoşkun (2011). Santoso *et al.,* (2013) investigated the proximate composition and mineral content in the liver of *Todarodes pacificus* (Japanese common squid) and reported a content of 44.0 g $100g^{-1}$ of fat (dry matter), 13.5 g $100g^{-1}$ of protein (dry matter), 2.11 g 100 g^{-1} of ash (dry matter) and better quantity of macro minerals (sodium,

potassium, magnesium, and calcium) and trace minerals (iron, zinc, cadmium, and copper). Proximate composition of squids varies with species, habitat, season, age of maturation, food and feeding, etc. (Ozogul *et al.,* 2008). Deng *et al.,* (2014) studied the changes of proximate composition of squid fillet during drying.

According to Roper *et al.,* (1984), squids are beneficial to elderly population because of low fat and high protein content. Bano *et al.,* (1992) observed high protein and low fat content in squid species. Significant difference in the protein content of digestive gland and squid muscle was noticed by Lee (1995) and Kunisaki (2000). Bano *et al.,* (1992) analyzed and compared the protein and amino acid content of *Sepiella inermis* and *Sympleclotenllies oualaniensis* and found better protein content in *S. oualaniensis*; whereas, homogenous amino acids content was observed. Amino acids play vital role for the growth and metabolic functions of humans. Amino acid composition and its score determine the nutritional quality of protein (Iqbal *et al.,* 2006). Generally, seafood are a good source for essential amino acids and aromatic amino acids (Adeyeye, 2009). Aspartic acid, lysine, glutamic acid, leucine, serine, arginine, cystine, histidine and tryptophan are found in the protein of squid (Bano *et al.,* 1992). Similar result was published by Konosu *et al.,* (1958). Ali (1987) noticed significant quantity of lysine in squid, which is essential for growth and development. Alanine, glutamic acid and aspartic acid were the major amino acid in the gelatin of splendid squid (*L. formosana*); whereas tyrosine, phenylalanine, histidine and lysine were noticed at lower level (Nagarajan *et al.,* 2012). Gelatin of giant squid (*Dosidicus gigas*) contains 16 to 17% of amino acid (Giménez *et al.,* 2009a; Gómez-Guillén *et al.,* 2002). According to Clarke *et al.,* (1994) nitrogen content in the mantle of *I. argentines* varies with sex and maturity.

Marine lipids are mostly accepted by the consumers because of their high content of omega-3 fatty acids and low content of omega-6 fatty acids (Steffens, 1997). Generally, essential fatty acids have great role to determine the level of nutritional quality of seafood. "International Society for the Study of Fatty Acids and Lipids" recommended the consumption of combined ecosapentaenoic acid (EPA) and docosahexaenioc acid (DHA) at a dose of 500 mg/day, which is beneficial for cardio vascular diseases (ISSFAL, 2004). Apart from this, presence of omega 3 fatty acids, an anti-depressive and anti-aggressive agent provides additional health benefit to the consumer (Hibbeln *et al.,* 2006). Omega-3 fatty acids are essential for neural and retinal development during infancy, which indicates their nutritional significances (Montaño *et al.,* 2001). Squids had commendable quantity of nutritional elements including omega-3 polyunsaturated fatty acids, which were essential for growth and maintenance of body (Ozyurt *et al.,* 2006). Necrosis of liver cell due to the action of carbon tetrachloride could be cured by squid phospholipids (Takama *et al.,* 1999). Fecundity and survival of bream (*Sparus aurata*) had improved by the use of squid oil enriched feeds (Zohar *et al.,* 1995). Reports are available for the fatty acid profile of squid (Sinanoglou and Meimaroglou, 1998; Okuzumi and Fujii, 2000; Ferrara *et al.,* 2001; Phillips *et al.,* 2002) whereas, few literatures are available for the seasonal variation of fatty acid profile of squid (Ozyurt *et al.,* 2006). Presence of significant quantity of fatty acid content was noticed in *Uroteuthis duvauceli*, *L. valgaris* (Salman *et al.,* 2007) and *Sthenoteuthis oualaniensis* (Wang *et al.,* 2008). Similarly, fatty acid profile of *L. vulgaris* was discussed by Navarro and Villanueva (2000) and Passi *et al.,* (2002). Significant difference of total lipid content in squid and cuttlefish was discussed by Okuzumi and Fujii (2000). According to the authors, squid fat contains better phospholipids and

minimum triglycerides than fishes. Phospholipids are the major components of squid lipid (Passi *et al.,* 2002), which comprises 62-84% of total lipids (Okuzumi and Fujii, 2000). Commendable quantity of phospholipids were found in *L. vulgaris* (De-Koning, 1993), squid rhabdome segment (Mason *et al.,* 1973) and *O. bartrami* (Takama *et al.,* 1994). There was a striking similarity between the phospholipids of squid and fish **(**Henderson and Tocher, 1987) and human platelets (Vishnubhatla *et al.,* 1988). *Sn-glycero-3-phosphocholine* (GPC) and *sn*-glycero-3-phosphoethanolamine (GPE) were the major phospholipids found in squid (De-Koning, 1993). Squid phospholipids contribute to essential fatty acids such as DHA (De-Koning, 1993). Suzuki *et al.,* (1992) and Deng *et al.,* (1998) found better concentration of DHA in the integument of *Ommastrephes bartrami*. Studies revealed that cephalopod from Mediterranean Sea was rich in biologically beneficial PUFA especially DHA and EPA (Sinanoglou and Miniadis-Meimaroglow, 1998; Culkin and Morris, 1970).

2.2 Ocean, an alternative source for bioactive compounds

Globalization and introversion of new life style evidently influenced the food habit of human, which led to the outbreak of several health related diseases. Combination of healthy lifestyle and neutraceutical diet is pretty advisable in the current scenario. Such diets should have the ability to withstand or lower the onset of such diseases (Harnedy and Fitz-Gerald, 2012). Hence, appropriate research for the development of efficient nutraceuticals for the benefit of consumer is highly relevant. The utilization of nutraceuticals from terrestrial resources had been explored to a large extent; in this juncture researchers are focusing to explore the underutilized marine sector. Marine environment covers wide ranges of thermal, pressure and nutrients and it has extensive photic and non-photic zones. This variability

facilitated extensive specification on all phylogenetic levels, from microorganism to mammals. Oceans are blessed with diverse level of established and non-established bioactive molecules that have the potential to become a strong neutraceutical and pharmaceutical agent. Seventy percent of earth's surface is spread by ocean which is an excellent habitat for several flora and fauna possessing bioactive molecules (Jimeno *et al.*, 2004). Biodiversity in marine environment far exceeds than that of terrestrial environment. Unavailability of ethno medical history and difficulties for the collection of marine samples (Jha and Zi-Rong, 2004), make search for marine natural products as pharmaceutical agent difficult. Invention of new diving techniques and remote operated machines are paving the way to explore the marine samples. Therefore, it grabbed the attention of several researchers to conduct experiments for the synthesis of marine bio-molecule and also find out the possibilities for its utilization (Aneiros and Garateix, 2004; Barrow and Shahidi, 2007). Moreover, available literature for antimicrobial resistance for the deadly diseases such as cancer, kidney damage, liver damage etc. also influences extensive research to find out novel bioactive and pharmaceutical molecules from marine source.

During last few decades numerous bioactive compounds were discovered and isolated from marine sources. Exploration of marine sector for the synthesis of drug had been initiated from 1970s. For instance, marine bioactive products had been issued to 300 patents during 1969 to 1999. During the past decade, over 4200 novel compounds had been isolated from shallow sea waters at a depth of 900 m. In recent years, many bioactive compounds were extracted from various marine animals like tunicates, sponges, soft corals, bryozoans, sea slugs and marine organisms (Harvey, 2000). So far, more than 10,000 compounds had been isolated from marine organisms

(Proksch *et al.,* 2002), but only 10% of over 25,000 plants had been investigated for biological activity.

2.2.1 Marine bioactive compounds

Seafood and its by-products were identified as one of the excellent sources for bioactive compounds (Kim and Mendis, 2006). Major nutraceutical compounds such as alginate, chitin, chitosan, proteoglycans, glycosaminoglycans, polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA), alkylglycerols, collagen, peptides etc. are abundantly found in marine organisms. Alginate is a polysaccharide made up of guluronic and mannuronic acid, which is abundantly present in marine brown algae. Alginate is being explored to develop drug delivery systems, wound healing materials, enzyme encapsulations etc. Chitin and chitosan are the other marine organic compounds abundantly found in the exoskeleton of crabs and shrimps. Chitin is a linear polysaccharide composed of (1-4)-linked 2-acetamido-2-deoxy-b-D-glucopyranose units, whereas chitosan is a derivative of chitin formed by deacetylation process. Both chitin and chitosan have many applications in biomaterials, pharmaceuticals, cosmetics, metal ion sequestration, agriculture, and foodstuff industries (Zargar *et al.*, 2015). Glycosaminoglycans (GAGs) are highly negatively charged polysaccharides with linear structure found in marine organisms and have diverse biological functions with tremendous applications in cosmetic, nutraceutical, industrial and pharmaceutical industries such as anti-inflammatory, anti-cancer, immunostimulant, tissue regeneration etc. Shark fin and cartilage were found to be an alternative option for the extraction of GAG than terrestrial origin. Proteoglycans are specialized proteins in the matrix tissues which is covalently attached to GAG. Their biological function was very much resembling with GAG, hence it could be explored for pharmaceutical and nutraceutical

applications (Yamada and Sugahara, 2008). Sulfated polysaccharides (fucoidans) (polymer of L-fucose) have the ability for anticoagulantion and antithrombotic activities, cell migration, immunostimulation and cell division (Berteau and Mulloy, 2003). Omega 3 fatty acids such as EPA, DHA and omega 6 polyunsaturated fatty acids in seafood have immense bioactive properties and are used as nutraceuticals. DHA directly influence many metabolic processes in the body, it is also vital for retina and brain development (Sinclair *et al.*, 2002). Sterols are well known for its potential health benefits and active ingredients for the synthesis of functional foods and pharmaceuticals. Seafoods are found to be a chief source for wide variety of sterols with potential health benefits (Kim and Van, 2011). Alkyl glycerols were reported to possess various bioactivities such as the immune stimulatory effect, immune modulatory effect, anti-cancer properties, etc. Interestingly, these compounds are commonly found in liver oils of marine origin and have made much attention in neutraceutical and pharmaceutical sector (Ermolenko *et al.*, 2016). Collagen is one of the most important biomolecules abundantly found in terrestrial as well as marine organisms, which exhibited numerous health promoting activities, hence it could be used in pharmaceutical and nutraceutical industry. Collagen is extensively adopted in biomaterial engineering for wound healing and tissue regeneration applications. Apart from this, collagen supplement boosts muscle growth, joint regeneration and cardio protective activities (Pati *et al.*, 2010; King'ori, 2011).

2.3 Bioactive peptides in general

Bioactive peptides might be directly occurring as such in food, or could be released from different dietary proteins of plant or animal origin during gastrointestinal digestion or during the preparation of protein hydrolysates using exogenous enzymes or fermentation process (Korhonen and Pihlanto,

2003). Bioactive peptides are the specific protein fragments not only act as a nutrient source but also capable to improve metabolic and therapeutic activities of the body (Hartmann *et al.,* 2007). Bioactive peptides are usually short in length (ie., 3-20 amino acids residues) and their activity is based on fragment size, sequence and amino acid composition (Kitts and Weiler, 2003; Pihlanto and Korhonen, 2003). Moreover, the ability of peptides to enter intestinal epithelium and dissolve in the blood (Pihlanto, 2006) is signifying their potential application as nutraceuticals and functional food ingredients for health promotion. Even though considerable research has been done on nutritional aspects of protein, the researchers are now focusing to derive bioactive peptides from various sources. Initial report for the synthesis of bioactive peptide from food was done by Mellande during 1950, who isolated peptide from milk casein, which showed the ability to calcification in rachitic infants (Mellander, 1950). Animal and plant sources were immensely exploited for the preparation of bioactive peptides (Yamamoto *et al.,* 2003; Hartmann *et al.,* 2007) including marine protein resources as well (Malve, 2016).

2.3.1 Marine bioactive peptides

Bioactivity of peptides derived from the marine sources was established by several researchers. Bioactivities of peptides are not evident when they are associated with full protein; whereas some peptides possess bioactivities, which are obtained from cleavage of protein. Usually, 3-20 amino acids residues are associated with bioactive peptides. Moreover, antihypertensive, antioxidant, anticoagulant, anticancer, immunomodulatory and anti-diabetic properties of marine peptides gained attention for the application in pharmaceutical, cosmeceutical and nutraceutical industry (Malve, 2016); however, those bioactivities vary with amino acids sequence (Cheung *et al.,*

2015). Available literature revealed that initial research for the extraction and purification of bioactive peptides from marine sources was conducted by Tu (1974) (neurotoxin), Norton *et al.*, (1976) (cardiotonic peptide), Rinehart *et al.*, (1981) (antiviral and antitumor peptide), Bernheimer *et al.*, (1982) (cardiotoxin) and Matsunaga *et al.*, (1985) (antimicrobial peptide).

Ziconotide (derived from cone snail) and Brentuximab Vedotin (peptide derivative) were the commercial pharmaceutical products derived from marine bioactive peptides. The first marine peptide approved by FDA was Ziconotide (Prialt®), which could be used to develop analgesic drug (Olivera, 2006). Later, anti-carcinogenic drugs such as Brentuximab vedotin (marine peptide) and Adcetris® (derived from sea hare) were approved by FDA. Gabolysat PC60®/Stabilium®/Protizen®/Procalm (anxiolytic properties), Seacure® (intestinal health), Nutripeptin®/ Hydro MN Peptide® (postprandial blood glucose control) were the commercially available nutraceuticals derived from fish protein hydrolysate, whereas Katsuobushi oligopeptide is a natural product possessing antihypertensive properties which was synthesized from dried bonito. Plitidepsin (natural product) from *Aplidium albicans*, Glembatumumab Vedotin (derivative) from *Dolabella auricularia*, HTI-286 (derivative) from *Hemiasterella minor*, Kahalalide F (natural product) from *Elysia rufescens* and Elisidepsin (derivatives) are some of the commercially important anti-carcinogenic marine bioactive peptides under clinical experiments (Leal *et al.*, 2014).

2.3.2 Antioxidant activities of marine bioactive peptides

Lipid oxidation and resultant products alter the quality and nutritional properties of food. Unsaturated fatty acids are easily vulnerable to oxidation and produce lipid peroxy radicals, hydroperoxide radicals, reactive oxygen

species (ROS) including superoxide anion radicals (O_2), hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($1O_2$) (Di-Bernardini *et al.,* 2011; Je *et al.,* 2007). Braughler and Hall, (1989) found that polyunsaturated fatty acids in the spinal cord and brain are highly susceptible to oxidation. Usually, formation and elimination of free radicals in the body were controlled by natural defense mechanism. If any unfavorable condition occurs, this system collapse and generate highly unstable free radicals, which accelerate oxidation of lipids, reaction with protein (Viljanen *et al.,* 2004), damaging cell, DNA and membrane lipid (Shahidi *et al.,* 1997) and cause severe diseases such as diabetes mellitus, cancer, aging, neurodegenerative and chronic and inflammatory diseases to humans (Nazeer and Srividhya, 2011; Je *et al.,* 2005; Davalos *et al.,* 2004; Korycka-Dahl and Richardson, 1978) Tobacco smoke, ultraviolet radiation, consumption of alcohol and rancid foods easily liberate ROS (Bunker, 1992; Powers and Jackson, 2008) which cause health risks to the consumers (Gey, 1990). According to Martinez *et al.,* (2003) and You *et al.,* (2002), DNA is highly susceptible to ROS-mediated oxidative damage especially with hydroxyl radicals which lead to severe adverse diseases such as carcinogenesis, parkinson's and alzheimer's disease to humans. Sakanaka *et al.,* (2005) also reported the role of hydroxyl radical for carcinogenesis. Oxygen consumption of prooxidant transition metal ions of the brain initiate the generation of free radicals which also cause carcinogenesis.

Antioxidation is a process to prevent the formation of free radicals during oxidation of lipid (Vimala and Adenan, 1999). Antioxidants could be used as dietary supplements, which suppress the oxidation of lipids by donating hydrogen or electron and maintain the quality of food lipids (Rajaram *et al.,* 2010). Inhibition of lipid oxidation in food by effective antioxidants

gained wider attention among the researchers; as a result several synthetic antioxidants such as butylated hydroxylanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate were developed. Even though they show better antioxidant properties, these synthetic antioxidants are restricted due to hazardous effect to the consumer. Therefore, synthesis of natural antioxidants from marine sources gained more popularity.

Marine peptides protect human health by scavenging free radicals, which are generates during oxidation of lipids. In addition, these compounds have many applications in food and cosmetic industries, and are also used to prevent rubber and gasoline degradation. Several researchers revealed that bioactivities of peptides are suppressed in the parent protein, which will be activated once the peptides are released from the proteins. Bioactivities including antioxidative properties of peptides depend on the structure, sequence and composition of amino acids in the peptides (Chen *et al.,* 1998, Klompong *et al.,* 2009). Hydrophobicity of peptides has great influence on the antioxidant properties of peptides (Chen *et al.,* 1998; Saiga *et al.,* 2003). According to Mendis *et al.,* (2005b) antioxidant property of squid skin gelatin peptides was due to the presence of hydrophilic-hydrophobic amino acids sequence. Similarly degree of hydrolysis, hydrolysis conditions and proteases had immense role for the antioxidant properties of peptides (Klompong *et al.,* 2007; Wu *et al.,* 2003). According to Fang *et al.,* (2012) papain, pepsin and trypsin were the proteases effective for the synthesis of marine antioxidative hydrolysates and peptides. Reports are available for the antioxidant properties of marine peptides (Amarowicz and Shahidi, 1997; Je *et al.,* 2005; Jun *et al.,* 2004; Thiansilakul *et al.,* 2007; Wu *et al.,* 2003). Several authors synthesized antioxidant hydrolysates and peptides from marine organisms such as giant squid (Mendis, *et al.,* 2005b; Giménez *et al.,* 2009a), jumbo flying squid (Lin

and Li, 2006), squid (*Todarodes pacificus*) (Nam *et al.*, 2008), prawn (Suetsuna, 2000; Guerard *et al.*, 2007), alaska pollock (Kim *et al.*, 2001), hoki (Mendis *et al.*, 2005a; Kim *et al.*, 2007), cobia (Yang *et al.*, 2008), sole (Giménez *et al.*, 2009a), conger eel (*Conger myriaster*) (Ranathunga *et al.*, 2006), ornate threadfin bream (Nalinanon *et al.*, 2011), royal jelly (Guo *et al.*, 2009), yellow stripe trevally (*Selaroides leptolepis*) (Klompong *et al.*, 2007), tuna (Jao and Ko, 2002) and mackerel (*Scomber austriasicus*) (Wu *et al.*, 2003). Chen *et al.*, (2010) found better autoxidation inhibition power of squid protein hydrolysate than BHA group. Similarly, Mendis *et al.*, (2005b) reported scavenging properties of the peptides hydrolyzed from jumbo squid skin (*Dosidicus gigas*). Moreover, amino acid composition determines the potential scavenging properties of peptides (Klompong *et al.*, 2009). Occurrence of branched chain amino acids such as valine, leucine and isoleucine were observed in the antioxidative peptides of alaska pollock (Je *et al.*, 2005), yellowfin sole (Jun *et al.*, 2004), hoki (Mendis *et al.*, 2005a) and shellfishes (Jung *et al.*, 2007); whereas Chen *et al.*, (1998) found better antioxidant properties of marine peptides due to the presence of histidine and aromatic amino acids. Whereas, Rajapakse *et al.*, (2005) noticed antioxidant characteristics of giant squid peptides due to the action of lysine and histidine amino acids.

Available literatures revealed antioxidant properties in tyrosine, methionine, histamine, lysine, tryptophan, cysteine, histidine, proline, alanine, leucine and phenylalanine (Yamaguchi, 1971; Suetsuna *et al.*, 2000; Kim *et al.*, 2001; Naqash and Nazeer 2010; Kumar *et al.*, 2011a; Nazeer *et al.*, 2011). According to Chen *et al.*, (1996a) proline-histidine-histidine sequence of amino acids provides better antioxidant properties to the peptides. Similarly, presence of histidine at the second residue of peptides has great role for the

antioxidant properties of peptides (Tsuge *et al.,* 1991). Authors also listed such histidine contained peptides like "alanine-histidine", "valine-histidine-histidine" and "valine-histidine-histidine-alanine-asparagine-glutamine-asparagine". According to Jung *et al.,* (2005) alanine and leucine (non polar aliphatic amino acids) inhibited lipid peroxidation. Radical scavenging abilities of proline, leucine, alanine and tyrosine amino acids were well established by Je *et al.,* (2008) and Mendis *et al.,* (1992). Cystine residue in the peptides indirectly suppresses the free radicals by donating sulfur hydrogen and protects the cell from oxidative stress (Rajapakse *et al.,* 2005; Hernandez *et al.,* 2005); whereas tryptophan, histidine and phenylalanine scavenge free radicals by donating electron. Wang *et al.,* (2007) suggested that the disintegration of imidazole ring of histidine provides scavenging capacity to peptides; whereas Li *et al.,* (2007) and Tsuge *et al.,* (1991) found the proton liberated from imidazole had the ability to scavenge free radical. Glycine, glutamate, tyrosine and aspartic acids inhibit lipid peroxidation by donating hydrogen atom (Je *et al.,* 2007). Amino acids such as leucine, valine, tyrosine and methionine donate protons and scavenge free radicals generated during lipids oxidation (Hsu, 2010). Occurrence of aromatic amino acids in phenylalanine, tyrosine, and histidine inhibits radical-mediated peroxidizing chain reaction (Suetsuna *et al.,* 2000; Rajapakse *et al.,* 2005); whereas antioxidant properties of valine, alanine, proline, leucine and methionine are due to the presence of hydrophobic amino acids (Kim *et al.,* 2006; Hernandez *et al.,* 2005).

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

DPPH is a free radical which accepts electron or hydrogen and form diamagnetic molecule which is stable at ambient temperature (Zhong *et al.,* 2011). Scavenging of free radical such as DPPH by an antioxidant compound indicates its ability to donate electron or hydrogen (Wu *et al.,* 2003; Shimada,

et al., 1992). Hence, potential of peptide or protein hydrolysate to scavenge DPPH radicals reveals its antioxidant property. Moreover, it is cheap, reliable and convenient assay having wide popularity for the determination of antioxidant properties in the protein hydrolysate (Guerard *et al.,* 2007; Peng *et al.,* 2009). DPPH radical scavenging activity of fish protein hydrolysate is usually enhanced with degree of hydrolysis (Slizyte *et al.,* 2009; Thiansilakul *et al.,* 2007). Controversy to this diminishing DPPH scavenging with enhancing degree of hydrolysis was observed in alcalase digested protein hydrolysate (Klompong *et al.,* 2007). According to Wang *et al.,* (2007) DPPH scavenging assay was assumed as primary characterization in antioxidant activity analysis of peptides. In this assay reduction of absorbance indicates scavenging of DPPH radical and the solution turns purple to yellow (Shimada *et al.,* 1992). Yang *et al.,* (2008) observed better DPPH scavenging activity in papain digested protein hydrolysate of cobia skin. According to Chen *et al.,* (2010) protein hydrolysate synthesized from flying squid skin exhibited better scavenging of DPPH than synthetic antioxidants such as BHA and α-tocopherol. Fang *et al.,* (2012) found that flying squid muscle peptides synthesized by different proteases exhibited better scavenging of DPPH radicals.

2.3.2.2 Superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2) radical scavenging activity

Superoxide anion radical ($O2^-$) was one of the free radical formed by the reaction of oxygen and electron (Miller, 1990) during aerobic metabolism (Kim *et al.,* 2007) in mitochondria. This primary reactive oxygen species (ROS) further react with other molecules to form secondary ROS and this reaction is catalyzed by enzyme or metals (Valko *et al.,* 2005). Hydrogen peroxide (H_2O_2) generated from superoxide further converts to hydroxyl

radical (·OH) by the action of superoxide dismutase in the presence of reduced metal ions. According to Pastor *et al.,* (2000) hydroxyl radicals possess strong reactivity, which easily interact with lipids and proteins and also damage purine, pyrimidine and deoxyribose of nucleic acids (Valko *et al.,* 2005). Similarly, superoxide anion radical devastates cell membrane and cause ageing (Zhong *et al.,* 2011). Hence, the ability of protein hydrolysates to scavenge superoxide anion radicals and hydroxyl radicals are highly relevant to establish its bioactivity especially antioxidant activity. This is in well agreement with Athukorala *et al.,* (2003) and Kuda *et al.,* (2005). According to Kim *et al.,* (2007) an antioxidant should be neutralized with superoxide anion radical and hydroxyl radical to prevent oxidation. Scavenging of superoxide and hydroxyl radicals was noticed in the peptides below 2 kDa, which were hydrolyzed from the skin gelatin of jumbo flying squid by using properase and pepsin (Lin and Li, 2006). Scavenging of hydroxyl and superoxide anion free radicals in juvenile Jian carp (*Cyprinus carpio* var. Jian) was reported by Xiao *et al.,* (2011).

Superoxide anion and hydroxyl radical were neutralized by the peptides derived from *Theragra chalcogramma* (Zhuang *et al.,* 2009), tilapia (Ngo *et al.,* 2010) and hoki (Kim *et al.,* 2007). Cartilage extract of chum salmon (*Oncorhynchus Keta*) inhibited oxidation of superoxide anion radical. Similalry, antioxidant peptides derived from *Ctenopharyngodon idellus* (Ren *et al.,* 2008), blue mussel (Jung *et al.,* 2005), horse mackerel (Kumar *et al.,* 2012) and jellyfish (Li *et al.,* 2012) also exhibited hydroxyl radical scavenging activity and this might be due to the occurrence of amino acids such as tyrosine, phenylalanine, glutamic acid and leucine (Suetsuna *et al.,* 2000).

2.3.2.3 Metal-chelating activity

Transition metals such as iron and copper donates electron and accelerate the formation of alkoxyl radicals from peroxide (Pokorny *et al.,* 2001). Moreover, transition metals such as iron, copper and cobalt accelerate autoxidation and produce highly reactive hydroxyl radicals through Haber–Weiss or Fenton reactions (Nawar, 1996). Some compounds are able to scavenge these metals to prevent further lipid oxidation (Chen *et al.,* 1998; Rajapakse *et al.,* 2005). Kristinsson and Rasco (2000) suggested that an antioxidant should have the capability not only to scavenge oxygen containing compounds but also scavenge metals. According to Sherwin (1990) antioxidative protein hydrolysate or peptides effectively scavenge transition metal ions. Hence, metal chelating property of a protein hydrolysate is a useful indicator to establish its potential antioxidant property (Alemán *et al.,* 2011). This is in well agreement with Pihlanto (2006) and Sherwin (1990). Je *et al.,* (2005) noticed metal chelating properties of the peptides isolated from *N. japonicus* and *E. volitans* were due to the presence of histidine amino acids. Imidazole ring of histidine amino acids have the potential to scavenge metals and lipid radicals (Murase *et al.,* 1993; Park *et al.,* 2001; Uchida *et al.,* 1992). Degree of hydrolysis is directly proportional to metal chelating properties by enhancing exposure of carboxyl and amino groups in the side chains of acidic and basic amino acids (Saiga *et al.,* 2003). Similarly, metal chelating properties have a linear relationship with molecular weight of peptides. Similar findings were reported by Klompong *et al.,* (2007) and Dong *et al.,* (2008). Authors observed an improvement of metal chelating activities with decreasing molecular weight of peptides; however metal chelation was unstable in too small peptides (Megías *et al.,* 2007). Peptides have the ability to bind with iron, so it disrupts the reaction between ferrozine and Fe^{2+} ion

(Thiansilakul *et al.,* 2007). So the metal chelating property of peptides could be established by measuring the scavenging of ferrous ions.

2.3.2.4 Reducing power

Reducing power of protein hydrolysates reveals its ability to donate electrons, which react with free radicals in the lipid to form a stable product; hence it could be used as a suitable indicator to determine the antioxidant property of protein hydrolysate (Yildirim *et al.,* 2000). Duh *et al.,* (1999) suggested that antioxidant activity of protein hydrolysate was directly correlated with reducing power. Je *et al.,* (2009) suggested that reducing power of hydrolysate was mainly due to high content of amino acids which had the potential to release electron or hydrogen. This assay was based on the estimation of ferrous iron from ferric iron (formation of Perl's Prussian blue) by using redox linked colorimetric reaction (Decker *et al.,* 2005; Dorman *et al.,* 2003). Chen *et al.,* (2010) suggested that reducing power of protein hydrolysate synthesized from flying squid skin was comparable with butylated hydroxyanisole. According to Klompong *et al.,* (2007) protease and degree of hydrolysis significantly influence the reducing power of protein hydrolysate. Reducing power was noticed in the protein hydrolysate synthesized from yellow stripe trevally (Klompong *et al.,* 2007) and squid (Alemán *et al.,* 2011).

2.3.2.5 Lipid peroxidation in a linoleic acid model system

Secondary breakdown products of oxidation of fatty acids cause several adverse reaction to the mammalian cells (Qian *et al.,* 2008a). Hence, the capacity of a protein hydrolysate to preserve linoleic acid from oxidation also indicates its antioxidant property (Osawa and Namiki, 1985). Mendis *et al.,* (2005b) reported that bioactive peptides isolated from jumbo squid exhibited better inhibition of lipid peroxidation in linoleic acid model system than ∞-

tocopherol, a widely using natural antioxidative agent. Some reports were available for the protection of linoleic acid from lipid peroxdiaton by the action of peptides synthesized from marine sources such as oyster (Qian *et al.,* 2008b), *Nemipterus japonicas* (Naqash and Nazeer, 2010) and rotifer (Byun *et al.,* 2009). Researchers also suggested that the peptides composed of 5 to 16 amino acids residues are more efficient to preserve linoleic acid from oxidation. According to Mendis *et al.,* (2005b) hydrophobic properties of marine peptides had great role for the protection of linoleic acid from autoxidation. Authors also noticed protection of linoleic acid from autoxidation by jumbo squid (*Dosidicus gigas*) gelatin hydrolysate due to the presence of significant quantity of hydrophobic amino acids.

2.3.2.6 Degree of hydrolysis

Degree of hydrolysis indicates the amount of peptides released from the protein, which was highly influenced by molecular size, polar groups and hydrophobic properties of protein and type of protease being used for hydrolysis (Adler-Nissen, 1986; Kristinsson and Rasco, 2000; Pena-Ramos and Xiong, 2003). According to Bougatef *et al.,* (2010), degree of hydrolysis could be used to differentiate the antioxidant activity of various protein hydrolysates. You *et al.,* (2009) reported that antioxidant properties of protein hydrolysates were highly influenced by the protease used for the hydrolysis and degree of hydrolysis. Hence, identification of suitable protease is highly necessary for the production of antioxidative hydrolysate. Dong *et al.,* (2008) also suggested that degree of hydrolysis had great role for the antioxidant properties of protein hydrolysate. This statement was in well agreement with Bougatef *et al.,* (2010). Degree of hydrolysis was directly proportional with reducing power, metal-chelating ability and antioxidant activities of the resultant hydrolysates (Klompong *et al.,* 2007; Dong *et al.,* 2008). Moreover,

solubility of protein hydrolysate enhances with degree of hydrolysis (Gbogouri *et al.,* 2004; Quaglia and Orban, 1987; Shahidi *et al.,* 1995). Maximum breakdown of fish proteins were observed in the initial 30 min of enzymatic hydrolysis (Benjakul and Morrissey, 1997; Dong *et al.,* 2008; Guerard *et al.,* 2002); whereas enzymatic hydrolysis of tuna cooking juice showed maximum degree of hydrolysis at 360^{th} min of the digestion (Jao and Ko, 2002). Yang *et al.,* (2008) found antioxidant properties of cobia skin peptides had enhanced with degree of hydrolysis.

2.3.3 Anticancer properties of marine peptides

Cancer, a non communicable disease spreads abnormal cells and disrupts the normal function of the body (Roy *et al.,* 2015). Changing life style and food habits of humans are the main reason for fast spreading of this death causing disease (He and Karin, *et al.,* 2011). Relative oxygen species and free radicals generated during lipid oxidation mutates DNA and cause cancer by interrupting normal proliferation and apoptosis mechanism of the body (Nazeer *et al.,* 2012). This causes uncontrollable cell growth or carcinogenesis in an organism (Suarez-Jimenez *et al.,* 2012; You *et al.,* 2002). Martinez *et al.,* (2003) found that hydroxyl radical is highly sensitive to DNA which induces carcinogenesis (Sakanaka *et al.,* 2005). Globally, the number of cancer death cases is increasing at an alarming rate. According to American Cancer Society (2016), cancer is the second most common disease in Europe and USA. Cervical cancer is commonly noticed in women at an age between 19 to 39 and it was recognized as third most common cancer in the world (Jemal *et al.,* 2011). Cervical cancer is persistent or recurrent orremain as a metastatic disease even after radiation (Mountzios *et al.,* 2013).

Chemotherapy and anticancer drugs are the common treatments for carcinogenesis which causes side effect and immunotoxicity to normal cells (Hall *et al.,* 2014). In this context, researchers showed keen interest to find effective treatment to prevent this disease without any adverse effect to the normal cells. Peptides are reliable organic compounds possess anticarcinogenic properties due to low molecular weight, simple structure and specific cytotoxicity (Mulder *et al.*, 2011). Moreover, Jumeri and Kim (2011) suggested that better molecular mobility and diffusivity of low molecular weight peptides improve the interactions with cancer cell components. These invaded peptides induce the cell death by damaging lysosomal membrane, altering mitochondrial pathway of apoptosis by caspase cascade, activation of immune modulatory pathway, and inhibition of DNA replication in the cell cycle (E-Kobon *et al.*, 2016). Cationic peptides react with negatively-charged cell membrane and damage cells by interrupting stability and fluidity, hence the net negative charges of the cancer membrane is a vital factor for the toxicity and selectivity of cationic peptides (Schweizer *et al.,* 2008). Hydrophobicity and amphiphilicity significantly influence the anticancer property of peptides (Dennison *et al.,* 2006; Huang *et al.,* 2011).

Potentiality of marine source to develop cancer therapeutic agents gained more popularity (Zheng *et al.,* 2012), since anticancer properties of traditional medicines were widely investigated (Kim *et al.,* 2015**).** Several reports revealed anticancer properties of marine bioactive peptides (Naqash and Nazeer, 2010). Zheng *et al.,* (2012) and Cheung *et al.,* (2015) isolated anticarcinogenic bioactive peptides from marine source which induced cancer cell death by apoptosis mechanism. Wesson and Hamann (1996) synthesized antitumor peptides from mollusk. Similarly, peptides obtained from jumbo flying squid skin gelatin (*Dosidicus gigas*) after digestion with esperase and alcalase exhibited

antiproliferative/cytotoxic activity on MCF-7 and U87 cells (Alemán *et al.,* 2011). Lee *et al.,* (2013) reported effective antiproliferative properties of peptide extracted from anchovy sauces due to hydrophobic property.

HeLa is a type immortal cervical cancer cell line widely accepted and extensively used for cancer research. Several findings indicated effective application of marine peptides for the inhibition of HeLa cervical cancer cells. Dolastatin C, a depsipeptide purified from *Dolabella auricularia* (mollusk) revealed cytotoxic effect on HeLa S3 cell-line (Sone *et al.,* 1993). Kanzawa *et al.,* (2004) noticed death of HeLa cells after the treatment with achacin (glycoprotein) synthesized and purified from giant African snail. This could be due to the blebbing of cancer cell membrane by the action of hydrogen peroxide, which was liberated from oxidative deamination reaction of achacin (Biddlestone-Thorpe *et al.,* 2013). Peptides isolated from black clam (*Chione fluctifraga*) also showed antiproliferative activities against cervical cancer HeLa cells (Martinez-Cordova and Martinez-Porchas, 2006). Similarly, peptides extracted from sponges such as carteritins A and B (cyclic heptapeptides), lipodiscamides A-C, stylissamide X (proline-rich octapeptide) and callyaerin G from *Stylissa carteri, Discodermia kiiensis, Stylissa* sp. and *C. aerizusa* also inhibited the growth of cervical cancer HeLa cells (Afifi *et al.,* 2016; Tan *et al.,* 2014; Ibrahim *et al.,* 2008).

2.3.4 Anti-inflammation

Inflammation is a strong immunological response of the body which evoked against external or other stimuli, in turn cause several processes such as vasodilatation and recruitment of inflammatory cells to the affected area. It is basically a protective mechanism of the body to fight against microbes and toxins in the injury. But later stage of inflammation is highly undesirable due

to pain development, necrosis at affected area etc (Kumar *et al*., 2010; Mitchell and Cotran, 2000). Inflammatory cells like macrophages produced by inflammatory mediators which provoke symptoms associated with inflammation (Zhou *et al*., 2008). Series of inflammatory mediators are produced during inflammation which include IFN-γ and pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), IL-6 and interleukin (IL)-1beta (Ackerman and Beebe, 1974). These agents accelerate the necrosis and symptoms of the affected area of the body. Major symptoms generated during inflammation are strong pain, redness, swelling, temperature which are quite uncomfortable to the body (Temponi *et al*., 2012). Chronic or acute inflammation leads to more complications to the body such as vasomotor rhinnorrhoea, arthritis and atherosclerosis (Chaudhary, 2001; Henson and Murphy, 1989). Major causalities of inflammations are noticed in aged population and an urge to find remedy to this situation all over the world is having great significance. Severe pain during inflammation is making a big concern over patients. The current therapies to inflammation are mainly focused to alleviate pain and related symptoms developed during inflammation (Stucky *et al*., 2001). Drugs currently employed in treatment for suppression of pain and inflammation symptoms such as non-steroidal anti-inflammatory drugs (NSAIDs), opioids and corticosteroids help to alleviate only symptomatic relief. Frequent usage of these drugs causes many side effects apart from their beneficial effects (Temponi *et al.,* 2012). The non-steroidal anti-inflammatory drugs have many side effects especially gastric irritation leading to development of gastric ulcers (Chandra *et al*., 2012). Hence, researchers are seeking a new solution for safe and effective anti-inflammatory drugs. Natural products have gained much attention towards the development of modern medicine for inflammation due to less or no side effects.

Researchers pointed out the importance of marine products such as shark liver oil against formalin induced paw edema (Mathew *et al*., 2008; Vishnu *et al*., 2015) and collagen peptides to alleviate inflammatory symptoms.

Several biochemical and physiological changes occur during inflammation which in turn causes the development of inflammatory symptoms. One of the major reasons for inflammatory changes are generation of free radicals such as nitric oxide (NO) and superoxide (O2−), released by lysosome which finally accelerate the tissue damage at the affected area (Reddy and Reddanna, 2009). So any compounds which are able to neutralize reactive oxygen species have great potential to control inflammatory response. Several reports were available for the potentiality of marine peptides to scavenge free radicals (Amarowicz and Shahidi, 1997; Je *et al.*, 2005; Jun *et al.*, 2004; Thiansilakul *et al.*, 2007; Wu *et al.*, 2003). Peptides isolated from squid protein exhibited antioxidant properties (Mendis, *et al.*, 2005b; Giménez *et al.*, 2009a; Lin and Li, 2006; Nam, *et al.*, 2008). Hence, antioxidant peptides synthesized from marine source can be included in the list of natural anti-inflammatory compounds.

2.3.5 Antiulcer

Peptic ulcer is a common gastric disorder that affects millions of people worldwide. Statistical report indicates that globally approximately 15 million people are affected by gastric ulcers with a mortality rate of 4.08 million per year (Maity and Chattopadhyay, 2008; Mentis *et al*., 2015). The gastric mucosa is routinely kept in contact with various agents and harmful substances that may activate the symptoms of gastric ulcers (Sumbul *et al*., 2011). However, exact pathology of peptic ulcer is not fully elucidated. Studies suggested that it is mainly developed due to the lack of balance between HCl

and pepsin levels; and defensive mucus secretion and bicarbonate levels (Farzaei *et al*., 2013). The routine use of anti-inflammatory drugs, improper eating habits, alcohol consumption, excess stress and *Helicobacter pylori* infection are the major reasons behind etiology of peptic ulcer (Konturek *et al*., 2004; Vítor and Vale, 2011). Macrophages and neutrophils formed during peptic ulcer infiltration release pro-inflammatory cytokines (IL-1β, TNF-α, IL-17 and INF-γ) and chemokines to the area of stomach and thus cause the development of ulcer symptoms (Arakawa *et al*., 2012).

The drugs used for ulcer mainly target on the inhibition of acid secretion and eradication of *H. pylori* (Sung *et al.,* 2009). However, the available therapy is not ineffective and continuous usage of these drugs can create many side effects (Bassi *et al*., 2014). Symptoms of ulcer mainly developed due to the excessive production of gastric acid and in turn lead to auto digestion, lysis of the gastric mucosa barrier may result in decreased gastric blood circulation, thereby lead to gastric ulcer formation (Zanatta *et al*., 2009). The gastric mucosa play vital role in maintaining the integrity and structure of the stomach. This mucosa acts as a barrier to protect the inner layers of stomach from gastric acid and other chemical irritants (Laine *et al* ., 2008; Tarnawski *et al* ., 2013).

Free radicals and reactive oxygen species (ROS) are major factors in tissue damage which are formed due to lack of balance in the production and neutralization of ROS that will leads to oxidative stress and lipid peroxidation of tissues. Studies have reported the free radicals induced mucosal damage lipid peroxidation. Hence, it is considered as one of the major reasons for peptic ulcer related tissue. According to Repetto and Llesuy (2002) major symptoms like mucosal damage during ulcer peptic progression is accelerated by the action of abnormal production of free radicals.

Mainly synthetic such as steroidal and non-steroidal drugs are employed to treat ulcer but they are not devoid of several side effects (i.e., arrhythmia's, impotence, funaecomastia and haematopoeitic changes) (Akhtar *et al*., 1992). In this scenario natural drugs possessing less or no side effects have great attention as a better alternative for the treatment of peptic ulcer. The role of plant metabolites such as phenols, flavonoids, terpenoids, alkaloids, tannins and oils is well established for reducing the ulcer symptoms since plant metabolites are known for their active role as anti-oxidants. Hence, natural antioxidants especially from medicinal plant sources have got great importance as promising anti-ulcer agents through the inhibitory action on toxic metabolites (Roy *et al.,* 2016). Whereas, antioxidant molecules in marine environment are unexploited, which have vast scope for the production of antiulcer compounds. Reports on antiulcer properties of antioxidant peptides synthesized from marine sources are limited. Glyprolines, a short peptide derived from collagen exhibited antiulcer property. According to Zolotarev *et al.,* (2006) administration of marine peptides at a dose of 1 mg kg^{-1} increased the stability of the gastric mucosa such as ethanol and stress, whereas at low dose (0.1 mg/kg) reduced ulcer lesions. Similarly, antiulcer effects of the tripeptide and its metabolites such as prostaglandins (PG), glycoproteins (GP), proline, and glycine decreased the indomethacin-induced ulcer development in rat model (Zhuikova *et al*., 2004).

2.3.6 Technological approaches for the production of bioactive peptides

Solvent extraction, chemical process, enzymatic hydrolysis, microbial fermentation and the conventional methods such as cooking, pressing, drying and hot oil extraction are the common methods for the extraction of bioactive peptides. Adoption of solvent extraction and fermentation for the synthesis of

peptides are not popular due to poor selectivity, low extraction efficiency, solvent residue and formation of pollutants (Alasalvar *et al.,* 2011). Chemical process for the synthesis of bioactive peptides is a traditional method, which requires strong acid or alkali in a controlled condition to cleave the protein. Fish protein hydrolysates obtained by this method are widely applied for manufacturing of animal feeds, culture media and fertilizers (Hsu, 2010). Uncontrolled proteolysis, unspecific cleavage pattern, loss of tryptophan and cysteine (essential amino acids) (Jaswal, 1990), bitterness due to the exposure of hydrophobic residues in the peptides (Pedersen, 1994) poor functionality, loss of nutritional quality and restricted application as flavor enhancers (Skanderby, 1994; Blenford, 1994; Webster *et al.,* 1982; Loffler, 1986) were the limitations of this method.

Enzymatic hydrolysis for the synthesis of peptides is a well recognized methodology in recent years because it ensures the functional and nutritional properties of resultant peptides (Ngo *et al.,* 2010; Kumar *et al.,* 2011a, Venkatesan *et al.,* 2017). This methodology is widely adopted in neutraceutical and pharmaceutical industry due to purity and quality of the resultant peptide (Kotzamanis *et al.,* 2007; Kristinsson and Rasco, 2000; Marchbank *et al.,* 2008). Moreover, functional properties of peptides are improved by enzymatic hydrolysis (Quaglia and Orban, 1990). Several reports are available for the effective application of natural and commercial proteases for the synthesis of bioactive peptides (Korhonen and Pihlanto, 2003; Shahidi and Zhong, 2008). Types of protease, degree of hydrolysis and hydrolysis conditions such as time, temperature, pH and enzyme/substrate ratio influence the bioactivities of peptides (Jao and Ko, 2002; Jun *et al.,* 2004; Pena-Ramos and Xiong, 2003; Sathivel *et al.*, 2003; Wu *et al.*, 2003). Peptide size and composition of amino acids affect the antioxidative properties of protein

hydrolysates (Wu *et al.,* 2003). Several findings are available for the application of proteases to produce antioxidant protein hydrolysates from jumbo squid (*Dosidicus gigas*) (Mendis *et al.,* 2005b), yellowfin sole (Jun *et al.,* 2004), yellowfin tuna (Jun *et al.,* 2004; Je *et al.,* 2007) and hoki (*Johnius belengerii*) frame protein (Kim *et al.,* 2007). Qian *et al.,* (2008a) studied inhibition of lipid peroxidation, DPPH, hydroxyl, superoxide, peroxyl radical scavenging activity of frog skin peptide hydrolysed using papain, pepsin and trypsin. Researchers recommended proteases such as trypsin, chymotrypsin, papain, pepsin, alcalase and flavourzyme for the hydrolysis of marine protein (Najafian and Babji, 2012; Senevirathne and Kim, 2012; Ngo *et al.,* 2010; Pangestuti and Kim, 2017; Klompong *et al.,* 2007; Yang *et al.,* 2008; Moosmann and Behl, 2002). However, Shahidi and Zhong (2008) suggested that each protease possess specific cleavage pattern for the production of peptides. Moreover, Neves *et al.,* (2004) produced peptides from brackish water fish and shrimps using protease enzymes and found that molecular weight of the resultant peptide depend on the type of proteases and hydrolysis conditions. Trypsin is very effective to cleave carbonyl side of basic amino acids, especially arginine or lysine (Rajapakse *et al.,* 2005; Kim *et al.,* 2001); whereas pepsin, an aspartic protease target on N-terminal of aromatic and acidic amino acids in the protein (Qian *et al.,* 2007b; Je *et al.,* 2008). Rajapakse *et al.,* (2005) successfully used trypsin, pepsin and chymotrypsin for the synthesis of antioxidant peptides from giant squid. Abdulazeez *et al.,* (2013) produced protein hydrolysate from king fish using papain enzyme. Moreover, several reports indicated the popularity of papain hydrolysis for the synthesis of antioxidant marine peptides. Mendis *et al.,* (2005b) derived peptides from jumbo squid skin gelatin using trypsin digestion. Several researchers investigated the efficacy of various proteases for the production of

bioactive peptides from marine origin and found excellent antioxidant properties in papain hydrolyzed peptides (Yang *et al.,* 2008). Fang *et al.,* (2012) produced peptides from flying squid using various proteases such as pepsin, trypsin, papain, alcalase and flavourzyme and found effective elimination of DPPH radicals by papain digested hydrolysate. Yang *et al.,* (2008) synthesized peptides from cobia skin by enzymatic hydrolysis and found maximum antioxidant activity in the papain digested peptides. Chalamaiah *et al.,* (2012) also observed excellent antioxidant properties in the papain digested marine peptides. Similar observation was noticed in papain digested peptides from loach (*Misgurnus anguillicaudatus*) (You *et al.,* 2011) and flying squid (*Ommastrephes bartrami*) (Fang *et al.,* 2012).

2.3.6.1 Purification, identification and characterization of bioactive peptides

Bioactivities of enzyme hydrolyzed peptides depend on the molecular weight and sequence of amino acids (Kim *et al.,* 2006; Kim *et al.,* 2010). Hence, further separation and purification of protein hydrolysate based on the molecular weight and amino acid sequence are highly relevant to establish its antioxidant properties. Je *et al.,* (2005) and Sheu *et al.,* (2006) found enhanced antioxidant properties in the purified protein hydrolysate of horse mackerel. It was found that lower molecular weight protein fragments possess better antioxidant properties (Chen *et al.,* 1995; Suetsuna *et al.,* 2004; Picot *et al.,* 2006). Several reports are available for the purification and separation of lower molecular weight marine antioxidant peptides such as 200 Da from mackerel (Wu *et al.,* 2003); 431 Da from loach (You *et al.,* 2010); 518.5 Da from horse mackerel (Kumar *et al.,* 2011a); 701.9 Da from black pomfret (Jai-Ganesh *et al.,* 2011); 928 Da from conger eel (Ranathunga *et al.,* 2006); 1000 Da from alaska pollak (Je *et al.,* 2005); 1101.5 Da from croaker (Kumar *et al.,* 2011b); 1801 Da from hoki (Kim *et al.,* 2007) and 5000 Da from shrimp (Sumaya-

Martinez, 2004). Ultrafiltration, ion exchange membranes, column chromatography, protein liquid chromatography (FPLC), high performance liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry (LC–MS/MS) are reliable methods for purification and sequencing of marine bioactive peptides (Je *et al.,* 2005; Jeon *et al.,* 1999; Pihlanto and Korhonen, 2003; Hsu *et al.,* 2009; Perkins *et al.,* 1999).

Ultrafiltration system is widely used to separate and purify antioxidant peptides depending on the molecular weight (Je *et al.,* 2005; Jeon *et al.,* 1999, Dong *et al.,* 2008; Wang *et al.,* 2007). According to Picot *et al.,* (2010) ultrafiltration could be applied in neutraceutical and feed industry because of its effective purification with enhancing specific activities of peptides. Authors also suggested that this technique is effective for the separation of peptides based on the molecular weight and aromatic property of amino acids. Similarly, Sumaya-Martinez (2004) used ultrafiltration technique for the separation of shrimp frame peptides based on the molecular weight of 30 kDa and 5 kDa. Mendis *et al.,* (2005b) fractioned antioxidant peptides from jumbo squid (*Dosidicus gigas*) skin gelatin hydrolysate using ultrafilteration. Similar study was conducted in grass carp **(**Ren *et al.,* 2008), hoki (*Johnius belengerii*) (Kim *et al.,* 2007), cod (Jeon *et al.,* 1999), alaska pollock (Je *et al.,* 2005), giant squid (Rajapakse *et al.,* 2005) and conger eel (Ranathunga *et al.,* 2006).

Reversed-phase columns are used in HPLC to separate peptides based on the hydrophobic and hydrophilic properties (Shahidi and Zhong, 2008). According to Kuriyama *et al.,* (2004) pore size, particle size, column size and hydrophobicity of the column determines the effective separation of peptides. Hsu *et al.,* (2009) purified antioxidant peptides using HPLC. Mendis *et al.,* (2005b) suggested that RP-HPLC with C18 column was an effective technique for the separation and purification of antioxidant peptides from jumbo squid

(*Dosidicus gigas*). Purification of enzymatically hydrolyzed antioxidant peptides from round sardinella (*Sardinella aurita*) using RP- HPLC was reported by Bougatef *et al.,* (2010). Je *et al.,* (2007) applied reverse phase high performance liquid chromatography with C18 column for the purification and characterization of enzymaticaly hydrolyzed tuna antioxidant peptides. Similarly, Hsu (2010) successfully purified enzymaticaly hydrolyzed tuna antioxidant peptides using two-step high-performance liquid chromatography.

Amino acid sequence of purified peptides could be determined by using liquid chromatography tandem mass spectrometry (LC–MS/MS) method (Perkins *et al.,* 1999). This technique is used to identify amino acid sequence in grass carp muscle hydrolysates (Ren *et al.,* 2008) and sardinelle (*Sardinella aurita*) (Bougatef *et al.,* 2010). Several researchers applied ESI-MS/MS for characterization of antioxidant peptides and observed a sequence of Lys-Thr-Phe-Cys-Gly-Arg-His in the antioxidant peptide (861.6 Da) of croaker (*Otolithes ruber*) (Nazeer *et al.,* 2012), Ala–Cys–Phe–Leu in horse mackerel (*Magalaspis cordyla*) (Kumar, *et al.,* 2011a), 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) in giant squid (Rajapakse *et al.,* 2005), Phe–Asp–Ser–Gly–Pro–Ala–Gly–Val–Leu (880.18 Da) and Asn–Gly–Pro–Leu–Gln–Ala–Gly–Gln–Pro–Gly–Glu–Arg (1241.59 Da) in jumbo squid (*Dosidicus gigas*) (Mendis *et al.,* 2005b).

2.4 Response surface methodology

Response surface methodology (RSM) optimizes process parameters by producing large amount of information from a small number of experimental runs (Khuri and Cornell, 1996) and this is done by selecting appropriate model equations (Edwards and Jutan, 1997). Hence it is considered as a cost effective

methodology for analysis. In another words, response surface methodology is an effective statistical technique to predict the effect of independent variables on the response variables by generating suitable mathematical model (Montgomery, 2005). Hence, RSM could be applied to optimize enzymatic hydrolysis conditions such as enzyme/substrate ratio, time and pH (independent variables) based on the antioxidant properties of the resultant peptides (dependent variable). Several findings indicated that types of protease and hydrolysis conditions such as time, pH and enzyme/substrate ratio significantly influence the bioactivities of peptides (Jao and Ko, 2002; Jun *et al.*, 2004; Pena-Ramos and Xiong, 2003; Sathivel *et al.*, 2003; Wu *et al.*, 2003). Hence, optimization of hydrolytic conditions using RSM is highly relevant. Some researchers used RSM for optimizing the hydrolysis condition for the production of antioxidant peptides in shrimp (Guérard *et al.*, 2007), jellyfish (*Rhopilema esculentum*) (Zhuang *et al.*, 2010), fish gelatin (You *et al.*, 2010), grass carp skin (Wasswa *et al.*, 2008), gold carp (*Carassius auratus*) (Sumaya-martinez *et al.*, 2005) and dogfish (*Squalus acanthias*) (Diniz and Martin, 1996). Fang *et al.*, (2012) produced antioxidant peptides from flying squid (*Ommastrephes bartrami*) using pepsin, trypsin, papain, alcalase and flavourzyme and optimized its hydrolytic conditions using RSM. Authors also found excellent antioxidant properties in papain digested peptides. Similarly, Ren *et al.*, (2008) optimized hydrolytic conditions of proteases for synthesizing antioxidant peptide from grass carp using RSM and reported highest antioxidant activity in papain digested hydrolysate. Pan and Guo (2010) used RSM to optimize the hydrolytic conditions for producing ACE-inhibitory peptides from whey protein. Kim *et al.*, (2001) also applied RSM to optimize hydrolysate condition, such as enzyme/substrate ratio, pH, and time of hydrolysis for producing antioxidant peptides from gelatin hydrolysate of allaska pollock skin.

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Chapter 3

Biochemical Profile and Nutritional Quality of Indian Squid, *Uroteuthis duvauceli*

Contents

3.1 Introduction

Indian squid is widely accepted seafood commodity because of its peculiar palatability, sensory properties and better yield percentage of meat for consumption. Marine lipids are mostly accepted by the consumers due to high content of omega-3 fatty acids and low content of omega-6 fatty acids (Steffens, 1997). Significant content of omega-3 polyunsaturated fatty acids in squids are essential for growth and maintenance of the body (Ozyurt *et al*., 2006). Moreover, it has the potential to develop various value added products such as squid gel, surimi and seafood analogues (Sánchez-Alonso *et al.,* 2007). Generally, squids are marketed as fresh, dried (Kugino *et al*., 1993), chilled (Hurtado *et al.,* 2001) and frozen forms (Paredi and Crupkin, 1997). However, quality changes especially browning are the major concern for the marketing of squid which is greatly affected by biochemical composition (Caili *et al*., 2006). Biochemical profiling of squid is remarkably different from that of fishes. Spitz *et al*., (2010) suggested that ecosystem has significant role for the nutritional quality of an organism. Similarly, metabolic activity has greatly influencing the

biochemical composition and body mass of an organism (Schmidt-Nielsen, 1984). Most of the squids usually showed isometrical metabolism (Seibel, 2007). Available literature indicated that there is lack of information for nutritional composition of squid's especially Indian squid (*Uroteuthis duvauceli*). Hence, this significant study was designed to find out the biochemical and nutritional profiling of Indian squid.

3.2 Objective of the study

Objective of the study was to conduct the nutritional profiling and biochemical composition of Indian squid (*U. duvauceli*) in order to find out the possibilities for the utilization of this modestly exploited resource.

3.3 Material and materials

3.3.1 Raw material

Indian squid (*U. duvauceli*) was collected from the fish landing centre at Fort Cochin, Kerala, India. The samples were transported to the laboratory in iced condition. Raw material had an average length of 570±14 mm and weight 445±15 g. Squid mantle was dissected and used for the analysis.

3.3.2 Chemicals

All reagents and solvents used in this study were of analytical grade. Standards of fatty acid methyl esters, amino acids were purchased from Sigma Aldrich GmbH (Steinheim, Germany).

3.3.3 Determination of moisture

Moisture was determined according to the AOAC (2000) method by drying. A clean and dry petridish was cooled in desiccators and weighed (W_1). Approximately 10-20g of the finely homogenized samples was evenly spread

and weighed (W_2). Petridish with the sample was dried in an oven at 105 °C, cooled in desiccators and weighed (W_3). The process of heating and cooling were repeated to get a constant value. Results were expressed as percentage of wet weight.

$$\text{Moisture } (\%) = \frac{(W_2 - W_3)}{(W_2 - W_1)} \times 100$$

3.3.4 Determination of protein

Total protein content in the homogenized samples (0.2 g) was determined using Kjeldahl method (AOAC, 2000). Weighed and transferred 0.2 g of the sample into a Kjeldhal flask. A pinch of digestion mixture was added followed by 10 mL of concentrated sulphuric acid. Kjeldhal flask was kept over a heater in order to heat the content until the solution became colorless. Cooled and made up to desired volume (100 mL) according to the protein content of the sample. Prepared blank with distilled water. A conversion factor (6.25) was used to convert total nitrogen to crude protein. Results were expressed as percentage on wet weight basis. Placed a conical flask having 10 mL of boric acid at the receiving end of the distillation apparatus and made sure that the tip of the condenser was slightly immersed in the boric acid. Pipetted out 5 mL of the digested sample and transferred in to the distillation apparatus and also added 10 mL of 40% NaOH followed by was distilled water for rinsing. Steam accelerated the liberation of nitrogen from the sample and it distilled and dissolved in the boric acid solution. Depending upon the concentration of nitrogen, colour of the solution turned green from pink.

The solution in the receiving flask was green at this stage. Titrated the content against N/100 sulphuric acid until the original pink colour was

restored. Noted the volume of acid used for titration. One mL of 0.01N sulphuric acid contains 0.14 mg Nitrogen. If the titre value of the sample after subtracting blank was X, then,

$$\text{Protein content } (\text{g}/100\text{g}) = \frac{\text{X} \times 0.14 \times 6.25 \times 100}{1000 \times \text{V}_1 \times \text{W}}$$

Where,

V = Total volume of the digest

V_1 = Volume of the digest for distillation

W = Weight of sample for digestion

3.3.5 Amino acids analysis

Total amino acid composition was determined using Shimatzu amino acid analyzer (Ishida *et al.,* 1981). About 100-150 mg of sample was weighed accurately into a heat sealable test tube. 10 mL of 6 N HCl was added and the tubes were heat sealed after filling pure nitrogen gas. Hydrolysis was carried out in a hot air oven at 110 ^{o}C for 24 hrs. After the hydrolysis, the contents were removed quantitatively and filtered into a round bottom flask through whatmann filter paper No. 42. The contents of the flask were flash evaporated to remove the traces of HCl and the process was repeated for 2-3 times with added distilled water. The residue was made up to 10 mL with 'C' buffer (0.05 N HCl). The sample thus prepared was filtered again through a membrane filter of 0.45 µ size. Of this 20 µl was injected to Shimatzu amino acid analyzer equipped with an ion exchange column (Shodex CX Pak P-421S), fluorescence detector and post-column derivatization chamber. A binary gradient elution programming was used. Buffer A was prepared by dissolving trisodium citrate (13.31 g) in 70 mL ethanol, followed by addition of citric

acid monohydrate (12.8 mL), NaCl (3.74 g) and Brij® (4 mL). Finally the pH was adjusted to ~3.2 with drop wise addition of perchloric acid and volume was made up to 1 L. Buffer B was prepared by dissolving tri-sodium citrate (117.6 g) and boric acid (24.8 g) in 500 mL distilled water followed by addition of 45 mL of 4 N NaOH. Final volume was made up to 2 L with distilled water (~pH 10). Elution programme (flow rate 0.4 mL min^{-1}) was started with 100% Buffer A and was kept constant for 12 min, followed by stepwise gradient to 100% Buffer B at 35^{th} min. Buffer B was kept constant up to 55^{th} min. Next 1 min, again 100% buffer A concentration was achieved. Total run time was 70 min. The oven temperature was set at 60 °C. Samples (100 mg each) were hydrolyzed in 6 N HCl in vacuum sealed tubes at 110 °C for 24 hrs. Following a post-column derivatization with O-phthalaldehyde in the presence of sodium hypochlorite solution, amino acids were detected in fluorescence detector (Excitation 340 nm, Emission 450 nm). Quantification was done with the help of external standard mixture of amino acids (Sigma). The results were expressed in g $100g^{-1}$ of protein.

3.3.6 Determination of tryptophan

About 200-250 mg of sample was hydrolyzed with 10 mL of 5% NaOH at 110 ºC for 24 hrs in a sealed tube filled with pure nitrogen. The hydrolysate was neutralized to pH 7.0 with 6 N HCl using phenolphthalein indicators. The volume was made up to 100 mL with distilled water. The solution was then filtered through whatman filter paper No.1 and filtrate was used for estimation. To a test tube containing 4 mL of 50% H_2SO_4, 0.1 mL of 2.5% sucrose and 0.1 mL of 0.6% thioglycolic acids were added. These tubes were kept for 5 min in water bath at 45-50 ºC and cooled. The sample was then added to the test tubes. A set of (0.1 to 0.8) standard tryptophan (10µg mL^{-1}) was treated similarly. The volume was made up to 5 mL with 0.1 N HCl and allowed to

stand for 5 min. The absorbance was measured using Shimadzu-UV spectrophotometer at 500 nm. The concentration was obtained by drawing standard graph

$$\text{Tryptophan(g/100g meat)} = \frac{\mu\text{g of Tryptophan} \times \text{Volume made up} \times 100 \times 16}{\text{Sample taken for color development} \times \text{Wt of sample} \times 1000 \times 1000 \times 6.25}$$

3.3.7 Estimation of quality of dietary protein

Essential amino acid score was calculated using the following formula (FAO/WHO, 1973).

$$\text{Amino acid score} = \frac{\text{Amount of amino acid per test protein (mg/g)}}{\text{Amount of amino acid per protein in reference pattern (egg) (mg/g)}} \times 100$$

Determination of the total essential amino acid (TEAA) to the total amino acid (TAA), *i.e.*, (TEAA/TAA); total sulphur amino acid (TSAA); percentage of cysteine in TSAA (% Cys/TSAA); total aromatic amino acid (TArAA), etc.; the Leu/Ile ratios were calculated while the predicted protein efficiency ratio (P-PER) was determined using one of the equations developed by Alsmeyer *et al* ., (1974), *i.e.*, P-PER = -0.468 + 0.454 (Leu) - 0.105 (Tyr).

3.3.8 Determination of crude fat (Soxhelet method)

The estimation of crude fat content was carried out by continuous extraction of fat with petroleum ether according to AOAC (2000). 2 g (W_1) of dried sample was weighed into a thimble and a cotton plug was kept on top of it. Thimble was placed in a Soxhelet apparatus and extracted with petroleum ether for 16 hrs. The apparatus was cooled and the solvent was filtered in to a pre-weighed conical flask (W_2). The flask of the apparatus was washed with

small quantities of ether and the washings were added to the above flask. The ether was removed by evaporation and the flask with fat was dried at 80-100 ^{o}C, cooled in a desiccator and weighed (W_3).

$$\text{Fat content (g/100g)} = \frac{(W_3 - W_1)}{(W_1)} \times 100$$

3.3.9 Extraction of total lipids

The total lipid content of the tissues was estimated by the method of Folch *et al.,* (1957). A weighed amount of the samples was minced well and subjected to lipid extraction using chloroform-methanol mixture (2:1). The extraction was repeated twice with fresh aliquot of chloroform-methanol mixture. The lipid extracts were transferred to a separating funnel and added 20% of water into it and left overnight. Next day the lipid extracts were drained through filter paper containing anhydrous sodium sulphate and was collected in round bottom flask and was made up to 10 mL by using chloroform. From this 1.0mL was taken into a pre-weighed vial and allowed to dry in warm temperature to constant weight and total lipid content were calculated from the difference in weight. Sample made up to 10 mL and used for further analysis.

3.3.10 Fatty acid analysis

Fatty acids methyl esters (FAMEs) were analyzed by the modified method of Metcalfe *et al.,* (1966). A fraction weight of lipid, extracted by Folch method was taken into a round bottom flask. Chloroform content was removed by evaporation and added 6 mL of methanolic NaOH and boiling chip in to the sample. Attached condenser, and refluxed under nitrogen until fat globules disappear (usually 5-10 min). Added 5 mL of BF_3 solution from bulb or automatic pipette through condenser and continued boiling for 5 min.

Removed from heat, and added 6 mL saturated NaCl solution. Stoppered the flask and mixed vigorously for 15 seconds while solution was still warm. Transferred it into a 250 mL separating funnel. Washed the round bottom flak with 30 mL distilled water and transferred it into a separating funnel. Added 25 mL of petroleum ether (boiling point was 60-80 °C) to this separating funnel and shook the contents and left it for 5 min under nitrogen for the separation. Lower aqueous layer was transferred to a round bottom flask and the upper PE layer to another separating funnel. Lower aqueous layer in the round bottom flask are extracted twice with 25 mL of PE, and the upper PE layer was pooled with the above one in the separating funnel. Washed thrice the combined PE extracts with 20 mL portions of H_2O, collected the upper PE layer, filtered it through anhydrous Na_2SO_4, and evaporated off solvent under vacuum. Made up the contents in to 1 mL with PE and transferred it into small vials and kept it in freezer till analysis.

Methyl esters of the fatty acids thus obtained were separated by gas chromatography (Thermo Trace GC Ultra) equipped with a capillary column 30 m long and 0.54 mm diameter and a flame ionization detector in the presence of hydrogen and air. The carrier gas was nitrogen and the flow rate was 0.8 mL min^{-1} initial temperature was set as 70 °C and was increased 30 °C min^{-1} until a temperature of 250 °C was obtained. Injector and Detector temperature was kept at 260 °C and 275 °C respectively. Fatty acids separated were identified by the composition of retention times thus obtained by the separation of a mixture of standard fatty acids. Measurement of peak areas and data processing were carried out by Thermochrom card software. Individual fatty acids were expressed as a percentage of total fatty acids.

3.3.11 Determination of ash

Ash content was determined by heating sample for 6 hrs in a furnace at 600 °C (AOAC, 2000). Heated a platinum or porcelain crucible to 600 ºC in a muffle furnace, cooled in desiccators and weighed (W_1). Approximately 5-10 g of the dried sample was weighed and transferred into a platinum crucible (W_2). The crucible along with sample was placed on a clay triangle and heated at a low flame until the material was charred. The charred material was kept inside the previously set muffle furnace and heated at 600 ºC for 3-4 hrs to get white or grayish white ash. The crucible was cooled in desiccators and weighed (W_3). The crucible was again heated for further 30 min, cooled and weighed. Results were expressed as percentage of wet weight.

$$\text{Ash content (\%)} = \frac{(W_3 - W_1)}{(W_2 - W_1)} \times 100$$

3.3.12 Mineral analysis

The minerals were analyzed by dissolving the ash (obtained in ash determination) in dil HCl (6 N) and estimated using atomic absorption spectrophotometer (Spectra AA 220, AAS VARIAN), with Deuterium background correction, acytelene and air supplied in constant ratio for flame and hollow cathode lamp. The wavelengths (nm) of light used for analyzing different minerals are 285.2 for magnesium, 213.9 for zinc, 766.5 for potassium, 328.4 for copper, 279.5 for manganese , 248.3 for iron, 240.7 for cobalt, 670.8 for lithium , 228.8 for cadmium, 217 for lead.

3.4 Result and discussion

3.4.1 Proximate composition

Proximate composition of seafood determines the nutritional and edible characteristics in terms of energy units. Moisture, protein, crude fat and ash content of the sample are presented in Figure 3.1. Proximate composition of *U. duvauceli* showed a content of 80.47% for moisture, 17.5% for protein, 0.52% for fat and 1.13% for ash, respectively. Similar finding was observed in the tentacles of European squid (*L. vulgaris*) (Atayeter and Ercoşkun, 2011). Santoso *et al.,* (2013) investigated the proximate composition of Japanese common squid (*Todarodes pacificus*) and reported the content of 44.0 g $100g^{-1}$ of fat, 13.5 g $100g^{-1}$ of protein and 2.11 g $100g^{-1}$ of ash in dry matter. Relatively high moisture content was observed in *U. duvauceli* as compared to its total fat content and also revealed better content of protein and ash. Roper *et al.,* (1984) suggested that low fat content and high protein concentrations in cephalopods make them appropriate for human consumption especially for elderly population. Bano *et al.,* (1992) analyzed and compared the protein content of *Sepiella inermis* and *Sympleclotenllies oualaniensis* and found better protein content in *S. oualaniensis*. The present findings are in corroboration with the findings of Thanonkaew *et al.,* (2006). Authors suggested that low fat and high protein content were beneficial in the formulation of protein supplements for targeted populations. Lipid was also essential in diet, which absorb fat soluble vitamin A, D, E and K from food and also regulate cholesterol metabolism. Studies had shown that proximate composition of cephalopods varies with habitat, age of maturation, food and feeding, species and season etc. (Ozogul *et al.,* 2008).

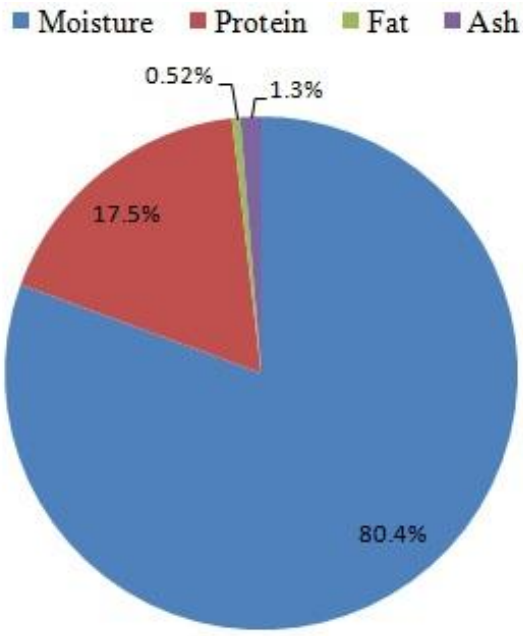

Fig. 3.1: Proximate composition of (% wet weight) *U. duvauceli*

3.4.2 Amino acid composition

Amino acids play vital role for growth and metabolic functions of humans. Amino acid composition and its score determine the nutritional quality of protein (Iqbal *et al.*, 2006). Total amino acid content in *U. duvauceli* was 12.46 g $100g^{-1}$ and amino acid profile of the sample is presented in Figure 3.2. Giménez *et al.*, (2009b) and Gómez-Guillén *et al.*, (1998) revealed 16 to 17% of amino acids in giant squid (*Dosidicus gigas*) gelatin. Present study showed a better content of glutamine, followed by aspartine, tryptopan, leucine, alanine and glycine. Glutamic acid and aspartic acid, the acidic amino acids occupied highest concentrations compared to other amino acids. Nagarajan *et al.*, (2012) reported better content of alanine, glutamic acid and aspartic acid in the gelatin of splendid squid (*L. formosana*); whereas tyrosine, phenylalanine, histidine and lysine noticed at lower level. In the present study leucine and tryptophan were found to

be highest essential amino acids (EAA) content in the sample. Nevertheless, amino acid such as phenylalanine, proline, threonine, serine and isoleucine were also observed in commendable quantity. Lysine at a content of 0.15 g $100g^{-1}$ was noticed in the sample. Ali (1987) reported significant quantity of lysine in squid, which was essential for growth. Glutamic acid is considered as one of the vital amino acids for metabolic activities especially for the synthesis of nucleic acid. Bano *et al.,* (1992) reported the presence of aspartic acid, lysine, glutamic acid, leucine, serine, arginine, cystine, histidine and tryptophan in the protein of squid. Similar findings were reported by Konosu *et al.,* (1958). Generally, fish is a good source of essential (leucine), non essential (glutamic acid) and aromatic amino acids (phenylalanine) (Adeyeye, 2009). Present study has better similarity to what was reported by Adeyeye (2009). This indicates the similarities of amino acid composition of cephalopods with fishes. Aromatic amino acids (tyrosine and phenylalanine) were essential for the synthesis of peptide hormones especially epinephrine and thyroxine (Robinson, 1987). Glutamic acid, glycine and aspartic acid possess wound healing ability (Chyun and Griminager, 1984). Aromatic amino acids (*ie.,* tyrosine, histidine, and phenylanine) and hydrophobic amino acids (*ie.*, valine, alanine, proline and leucine) were able to scavenge free radicals (Suetsuna *et al*., 2000; Rajapakse *et al*., 2005; Kim *et al.,* 2006). Among the nutrients, supplementation with amino acids, and branched chain amino acids in particular, could reduce fat accretion and maintain lean body mass, and therefore had a fundamental role in maintaining insulin sensitivity and counteracting obesity-induced metabolic syndrome **(**Giuseppe, 2014**)**. Sulfur-containing amino acids play indispensable role in biological activities including protein synthesis, methylation, biosynthesis of polyamines and glutathione (Nozaki *et al*., 2005). Bano *et al.,* (1992) compared amino acid content of *Sepiella inermis* and

Sympleclotenllies oualaniensis and found no significant difference for amino acids. However, nitrogen content in the mantle of *I. argentines* varies with sex and maturity (Clarke *et al.*, 1994).

Table 3.1 shows the concentrations of total amino acid (TAA), total essential amino acid (TEAA), total acidic amino acid (TAAA), total neutral amino acid (TNAA), total sulphur amino acid (TSAA) and total aromatic amino acid (TArAA). Content of TEAA with histidine in *L. duvauceli* was 272.00 mg gcp^{-1} and it was reasonably comparable with egg reference protein (566 mg gcp^{-1}) (Paul *et al.*, 1980). This nutritional profile of sample was par with *Zonocerus variegates*, 351 mg gcp^{-1} (Adeyeye, 2005), *S. budgetti*, 389 mg/gcp and *H. fasciatus*, 394 mg gcp^{-1} (Abdullahi and Abolude, 2002).

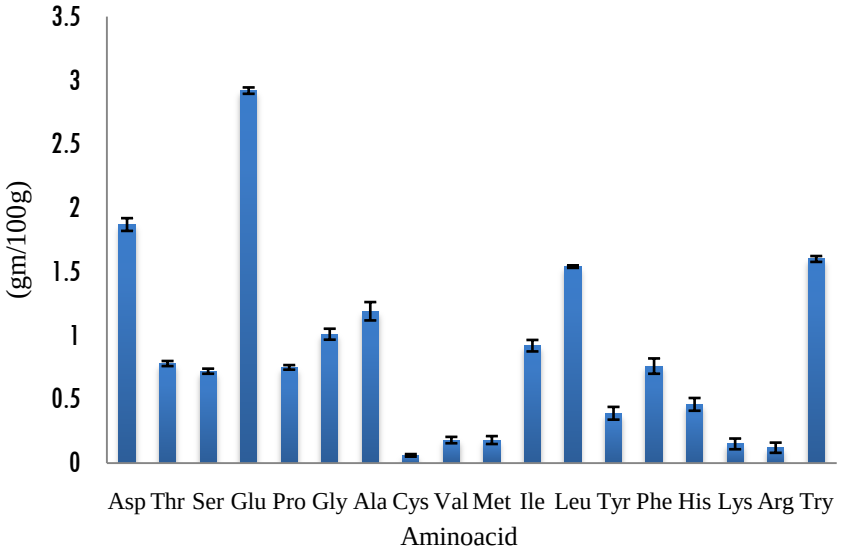

Fig.3.2: Amino acid composition (g $100g^{-1}$ of meat) *of U. duvauceli*

Table 3.1: Concentrations of essential, non-essential, acidic, neutral, sulphur aromatic amino acid composition (mg g^{-1} crude protein) of *U. duvauceli*

Amino acid	*U. duvauceli* (gcp)
TAA	731.23
TNEAA	459.23
%TNEAA	62.80
TEAA with His	272.00
TEAA with out His	248.29
%TEAA with His	37.20
%TEAA with out His	33.96
TNAA	100.59
%TNAA	13.76
TAAA	246.84
%TAAA	33.76
TBAA	16.21
%TBAA	2.22
TSAA	12.35
%TSAA	1.69
% of CYS in TSAA	590.97
TArAA	75.36
%TArAA	10.31
P-PER	3.22
Leu/Ile ratio	1.68

Values are expressed as mean±SD for three replicates.

3.4.3 Fatty acid composition

Fatty acid profile of *U. duvauceli* was shown in Figure 3.3. Among saturated fatty acids, palmitic and stearic acid contributed highest quantity; whereas, DHA, EPA and arachidonic acid were the major unsaturated fatty acids in the sample. Suzuki *et al.*, (1992) and Deng *et al.*, (1998) reported better concentration of DHA in the integument of squid *Ommastrephes bartrami*. Studies showed that cephalopod from Mediterranean Sea was rich in biologically beneficial PUFA (Sinanoglou and Miniadis-Meimoroglow, 1998)

and DHA and EPA were the dominant PUFA (Sinanoglou and Miniadis-Meimaroglow, 1998; Culkin and Morris, 1970). Significant level of essential fatty acids was observed in the present study (Table 3.2). Occurrence of significant quantity of fatty acid content in *U. duvauceli*, *L. valgaris* (Salman *et al.,* 2007) and *Sthenoteuthis oualaniensis* (Wang *et al.,* 2008) was reported. According to Ozyurt *et al.,* (2006) squids had commendable quantity of nutritional elements including omega-3 polyunsaturated fatty acids, which were essential for growth and maintenance of body. DHA, EPA, arachidonic acid and palmitic acids were the dominant fatty acids in cephalopods (Stowasser *et al.,* 2006). Fatty acid profile of *U. duvauceli* had striking similarity with *Loligo valgaris* (Salman *et al.,* 2007) and egg of *Sthenoteuthis oualaniensis* (Wang *et al.,* 2008). Generally, fat content of squid species was low; however, majority of fat contains nutritionally important long polyunsaturated fatty acids. PUFA were nutritionally vital for growth and development of integral part of metabolic activities in higher animals and their consumption was well appreciated (Halilo ğ lu *et al.,* 2004). Prostaglandins and thromboxanes the potent arachidonic derivatives play significant role in blood clotting and healing process (Bowman and Rand, 1980). EPA and DHA had crucial role in preventing cardiovascular problems and side effect caused due to prostaglandin mediated inflammation (Connor, 2000). Long chain fatty acids are essential for growth and developments of infant. Omega -3 PUFA like DHA plays vital role in developmental stages of brain and retina during pre and post pregnancy (Rogers *et al.,* 2013). Result of the present study reveals the significance of fatty acid profile of *U. duvauceli*.

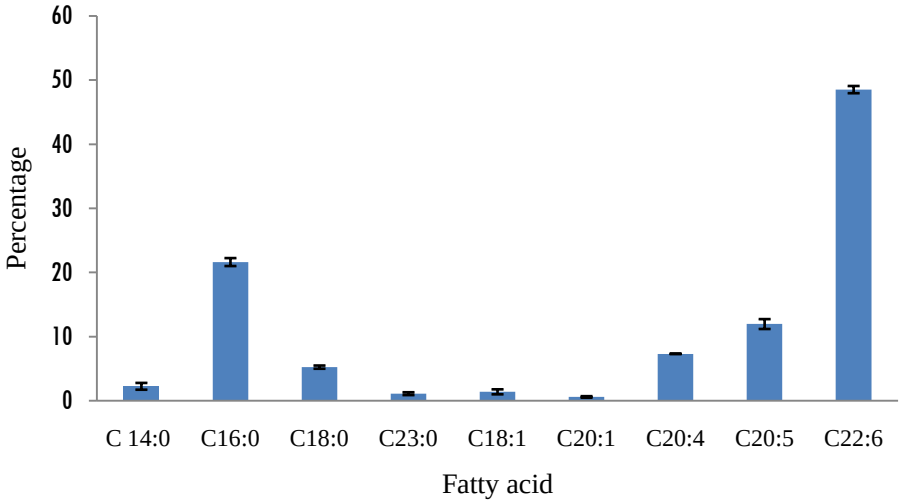

Fig.3.3: Fatty acid composition (% of fatty acids in terms of total fatty acids) of *U. duvauceli*

Table 3.2: Fatty acid n-3/n-6 and polyunsaturated/saturated fatty acid ratio of *U. duvauceli*

Species	n-3:n-6 ratio	P/S ratio
U. duvauceli	8.27	2.24

P/S, polyunsaturated/saturated fatty acid ratio. n-3 -omega 3 fatty acid n-6- omega 6 fatty acid

3.4.4 Mineral composition

Minerals are necessary for physicochemical processes as well as metabolic activities of the body (Soetan *et al*., 2010). Macro and micro mineral profile of the sample is presented in Table 3.3 and Table 3.4, respectively. Among the macro minerals, potassium showed highest content followed by sodium and calcium. Sodium and potassium ATPase is actively involved in sodium and potassium transport across the cells. Calcium and phosphorous are essential for energy metabolism, signal transduction and bone homeostasis (Ha and Bhagavan, 2011). As in the case of micro minerals,

magnesium content showed highest proportion and copper showed least quantity. Santoso *et al.,* (2013) reported better quantity of macro minerals (sodium, potassium, magnesium, and calcium) and trace minerals (iron, zinc, cadmium, and copper) in Japanese common squid (*Todarodes pacificus*). However, trace minerals content in seafood depends on various factors such as nourishment sources, biological differences, seasonal factors and environmental conditions (Fallah and Rahnama *et al*., 2009).

Table 3.3: Macro minerals profile (wet basis) (g $100g^{-1}$) of *U. duvauceli*

Micro minerals	***U. duvauceli***
Sodium	0.102±0.05
Pottasium	0.189±0.03
Calcium	0.015±0.001

Values are expressed as mean±SD for three replicates

Table 3.4: Trace minerals profile (wet basis) (mg $100g^{-1}$) of *U. duvauceli*

Trace minerals	***U. duvauceli***
Iron	2.885±0.8
Zinc	1.032±0.09
Copper	0.121±0.02

Values are expressed as mean±SD for three replicates

3.5 Conclusion

Biochemical profiling of Indian squid (*U. duvauceli*) revealed significant composition of essential nutrients such as proximate contents (80.47% of moisture, 17.5% of protein, 0.52% of fat and 1.13% of ash), essential amino acids (leucine and tryptophan) and essential minerals (sodium, calcium, magnesium and potassium). Generally, essential fatty acids had great role for the determination of the levels of nutritional quality of seafood. In this study essential fatty acids such as DHA, EPA and arachidonic acid were observed at significant level. Moreover, amino acids such as essential amino

acid, acidic amino acid, neutral amino acid, sulphur amino acid and aromatic amino acid also indicates its nutritional significance. Commendable quantities of biochemical and nutritional content in *U. duvauceli* signify the appropriateness of this moderately exploited resource as a source of essential nutrients for nutritionally deprived population.

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Chapter 4

Optimization of Hydrolysis Conditions for the Production of Antioxidant Hydrolysate from Indian Squid (*Uroteuthis duvauceli*) using Response Surface Methodology

Contents

4.1 Introduction

Squid peptides revealed its capability for bioactivities, however it was suppressed in parent protein; once the peptides released from proteins, depending up on the structure, sequence and amino acids composition, peptides exhibit various bioactivities including antioxidation. Moreover, degree of hydrolysis, type of proteases and hydrolysis conditions influence the antioxidant properties of peptides (Klompong *et al.,* 2007). Enzymatic hydrolysis for the synthesis of peptides is a well recognized methodology in recent years because it ensures the functional and nutritional properties of resultant peptides (Ngo *et al.*,2010; Kumar *et al.*,2011a). This is in well agreement with Venkatesan *et al.,* (2017) and the authors also suggested that efficacy of enzymatic hydrolysis for the synthesis of bioactive peptides from marine organisms. However, Shahidi and Zhong (2008) confirmed the each protease possesses specific cleavage pattern for the production of peptides.

Amount of peptides and amino acids liberated from protein varies with specificity of proteases, which also influences the antioxidant property (Wu *et al*., 2003). Fang *et al*., (2012) reported that papain, pepsin and trypsin are the effective enzymes for the production of antioxidant peptides.

Response surface methodology (RSM) optimizes process parameters by producing large amount of information from a small number of experimental runs (Khuri and Cornell, 1996) and this was done by selecting appropriate model equations (Edwards and Jutan, 1997). Hence it was considered as a cost effective methodology for analysis. Several reports indicated that types of protease and hydrolysis conditions such as enzyme-substrate ratio (E/S), pH, and time of hydrolysis greatly influence the antioxidant properties of the resultant peptides (Pena-Ramos and Xiong, 2003; Sathivel *et al.,* 2003; Wu *et al*., 2003). Hence, selection of suitable enzyme for the production of antioxidant peptides and optimization of its hydrolysis conditions are highly relevant. Several reports are available for the application of RSM for optimizing the hydrolysis conditions for the production of antioxidant peptides in shrimp (Guerard *et al.,* 2007), jellyfish (*Rhopilema esculentum*) (Zhuang *et al.,* 2009), gold carp (*Carassius auratus*) (Sumaya-martinez *et al.,* 2005) and dogfish (*Squalus acanthias*) (Diniz and Martin, 1996).

4.2 Objective of the study

In the present study, it was designed to optimize the hydrolysis conditions such as enzyme/substrate ratio, pH and time for pepsin, papain and trypsin hydrolysis based on the antioxidant properties of Indian squid protein hydrolysate using response surface methodology and also to distinguish the

effective protease for the synthesis of antioxidant hydrolysate from Indian squid.

4.3 Materials and methods

4.3.1 Raw materials

Indian squid (*U. duvauceli*) was collected from the fish landing centre at Fort Cochin, India. Samples had an average length of 570±14 mm and weight 445±15 g. The samples were transported to the laboratory in iced condition. The squid mantle was dissected and used for the analysis.

4.3.2 Chemicals

Pepsin, trypsin, papain, gallic acid 2, 2-diphenyl-1-picryhydrazyl (DPPH) and linoleic acid were purchased from Sigma–Aldrich (Shanghai, China). All other reagents used for the study were of analytical grade from standard sources.

4.3.3 Extraction of muscle protein

The muscle protein was obtained according to the method of Sathe and Salunkhe *et al.*, (1981) with a slight modification. After washing, the squid meat was minced by a blender (DS-1, Exemplar and Mould Instruments Co., Shanghai,China). The mince was mixed with isopropanol at a ratio of 1:4 (w/v), and then the mixture was homogenized at a speed of 12,000 rpm for 10 min. After supernatant was drained, the residue was degreased at 75 °C for 120 min using isopropanol at a ratio of 1:4 (w/v). The muscle protein was vaccum-dried at 25 °C after the removal of the supernatant. The obtained

squid muscle protein was stored under −20 °C for further use. Yield was calculated.

$$\text{Yield (\%) wet basis} = \frac{\text{Weight of protein powder (g)}}{\text{Weight of fish minced used (g)}} \times 100$$

4.3.4 Analysis of amino acid composition

Determination of total amino acid composition of the sample was elaborated in Chapter 3, Section 3.3.5.

4.3.5 Preparation of squid muscle hydrolysates

The squid muscle protein obtained was subjected to hydrolysis using various protease enzymes (*ie.*, pepsin, trypsin and papain). Muscle protein (100 g) was mixed with proper deionized water and homogenized before hydrolysis. The obtained mixture were evenly divided into three fractions, each of which was adjusted to the required pH with 0.01 mol L^{-1} NaOH or 0.05 mol L^{-1} HCl, then added required amount of each protease and heated in water bath at 37 °C for pepsin, trypsin and 55 °C for papain at required time interval. Each mixture was incubated with stirring and then heated in a boiling water bath for 10 min to inactivate the enzyme. The resulting hydrolysate was centrifuged at 8000 × g for 10 min and supernatants were freeze dried and used for further analysis.

4.3.6 Experimental design and optimization

Box-Behnken response surafce design with three process variables with three levels and three points at the center was used for the experiment at design formulation (Myers and Montgomery, 2002). Experimental data from 15 experimental runs on AOA of each protease were measured. Second order

response surface regression model was used to predict and optimize the response variable in terms of E/S ratio(X_1),pH(X_2) and time(X_3) for the hydrolysis of squid protein. The functional form of the model is given in Equation 1, which was used to break up the total variability into variability due to linear, quadratic and interaction effect of process parameters and error (Myers and Montgomery, 2002).

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_i \beta_{ii} X_i^2 + \sum_{i \neq j} \beta_{ij} X_i X_j, \; i \neq j = 1,2,3 \ldots\ldots\ldots\ldots\ldots (1)$$

Where, Y is the dependent variable (AOA activity).β_0 is an intercept. β_i, β_{ii}, and $\beta_{ij}$are the linear, quadratic, and interaction regression coefficients, respectively and X_i are independent variables. Ridge analysis was carried out to predict the response variable at different radius from the center of the design region. The optimization of response variable was done based on the evaluation of ridge score, response surface plot and desirability function. A validation study was conducted at optimized hydrolysis condition for each protease. The AOA of different proteases at optimized condition was analyzed using one-way ANOVA. Once ANOVA was found to be significant at 5% level ($p < 0.05$), Duncan test was performed to compare the mean values of AOA of different proteases. All the statistical analysis was done using SAS version 9.3 (SAS Institute, 2012).

4.3.7 DPPH radical scavenging activity

DPPH radical scavenging activity of SPH was carried out based on the slight modification of Kitts *et al.,* (2000) method. Sample solution was

prepared by mixing 1.8 mL of DPPH solution (0.1 mM in 80% ethanol) with 0.20 mL of SPH solution (at 20 mg mL^{-1} final assay concentration in 50% ethanol). 0.20 ml of 50% ethanol was kept as control; whereas sample control was made for each SPH by mixing 0.20 ml SPH solution with 1.8 mL of 80% ethanol. All the samples were incubated at room temperature for 30 min and its absorbance was measured at 517 nm. Radical scavenging capacity of SPH was calculated as follows:

$$\text{DPPH Scavenging activity (\%)} = \frac{(\text{absorbance of blank} - \text{absorbance of samples})}{\text{absorbance of blank}} \times 100$$

4.4 Result and discussion

4.4.1 Yield percentage of squid protein concentrate and its amino acid profile

Yield percentage of protein concentrate extracted from the Indian squid muscle was 10.2% and its amino acid composition was presented in the Figure 4.1. Lowest concentration was noticed for proline (0.26%), both amino acids possess antioxident activity. Amino acids such as aspartic acid, glutamate, lysine,leucine, valine, phenylalanine, histidine, methionine, alanine, tyrosine and cystine have the ability to scavenge free radicals (Je *et al.,* 2007; Nazeer *et al.*,2011; Rajapakse *et al.*, 2005) and showed a concentration of 14.08%, 13.94%, 6.98%, 6.79%, 6.5%, 6.05%, 5.95%, 1.43%, 1.34% and 0.37%,respectively among the isolated amino acids.

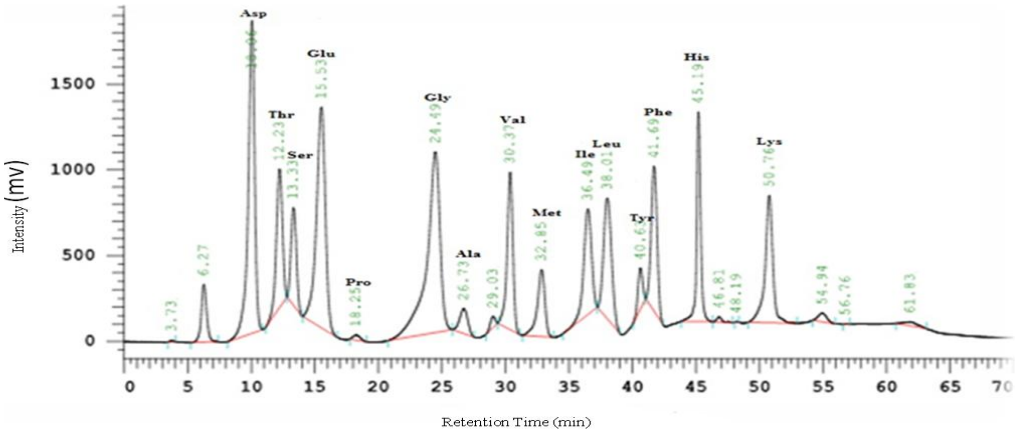

Fig. 4.1: Aminoacid composition of Indian squid protein concentrate

4.4.2 Effect of hydrolysis conditions on the antioxidant activity of pepsin hydrolysate

Second order response surface regression was found to be significant with an R^2 value of 0.83 to explain the total variability of antioxidant activity of pepsin hydrolysate in terms of linear, quadratic and interaction effect ofhydrolysis conditions of squid muscle protein. Linear effect of E/S ratio, pH and time showed a decreasing antioxidant activity of pepsin hydrolysate, whereas it increased with quadratic effect of process conditions except time. Similarly, interaction effect of independant variables revealed an increasing trend except E/S ratio and time (Equation 1). The effect of E/S ratio, pH and time on the DPPH scavenging activity of the pepsin hydrolysate is presented in Table 4.1. Response surface plot of predicted values of antioxidant activity of pepsin hydrolysate with respect to changes in the level of E/S ratio and time at fixed pH (2), time and pH at fixed E/S ratio (1.25%) and pH and E/S ratio at fixed time (3.15 hrs) are depicted in Figure 4.2 (A), 4.2 (B) and 4.2 (C), respectively. Changes in the pH and E/S ratio at 3.15 hrs (fixed condition) revealed a significant reduction of AOA of pepsin hydrolysate for E/S ratio

and pH up to 2.2, and then it showed an increasing trend. At fixed pH of 2, the AOA enhanced with time, whereas, it reduced with E/S ratio up to 1.5 concentrations. Similarly, at fixed 1.25% E/S concentration, AOA of pepsin hydrolysate revealed a slightly increasing trend with time, whereas it significantly reduced up to pH level of 2, then it enhanced with the pH.

AOA of pepsin = $309.26-25.18X_1-249.07X_2^{**}-0.82X_3+3.79X_1^2+55.53X_2^{2*}-0.03X_3^2 +5.77X_1X_2-0.08X_1X_3+1.69X_2X_3, R^2=0.83$.....(1)

4.4.2.1 Multiple response evaluation

Response contour graph for the effect of E/S ratio and pH at fixed time (3.15 h), E/S ratio and time at fixed pH (2), time and pH at fixed E/S ratio (1.25%) on the antioxidant activity of pepsin hydrolysate of squid protein is presented in Figure 4.3 (A), 4.3 (B) and 4.3 (C), respectively. Antioxidant activity of pepsin hydrolysate was reduced with the effect of E/S ratio and pH, whereas pH above 2.2 the activity was enhanced (Figure 4.3 A). Similarly, the effect of E/S ratio and time on the antioxidant activity of pepsin hydrolysate indicated an enhancing trend (Figure 4.3 B); similar trend was observed for the antioxidant activity of pepsin hydrolysate by changing the time and pH values (Figure 4.3 C). Fit diagnostics for AOA of pepsin hydrolysite is depicted in Figure 4.4. Based on the ridge analysis, the optimum combinations for pepsin hydrolysis of squid protein were 1:100 E/S ratio, 2 pH and 3.15 hrs hydrolysis time with respect to its highest antioxidant activity (29.49%).

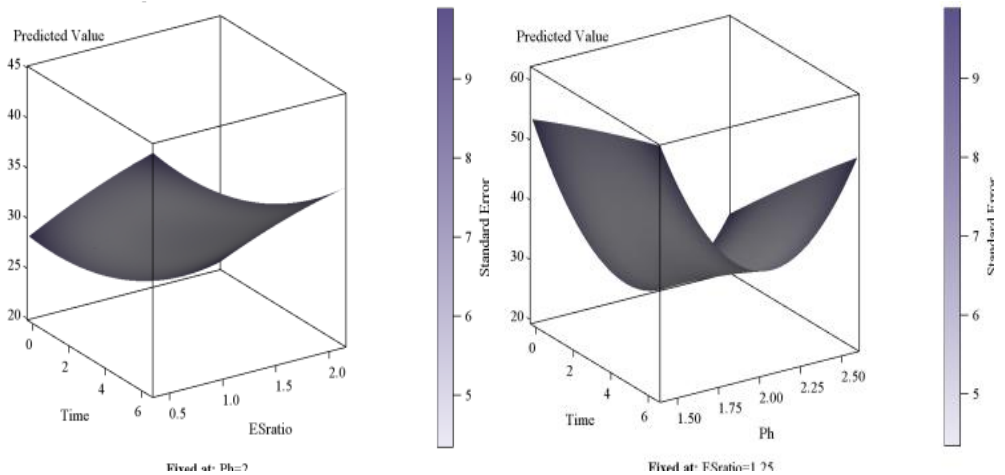

Fig. 4.2 (A) Fig. 4.2 (B)

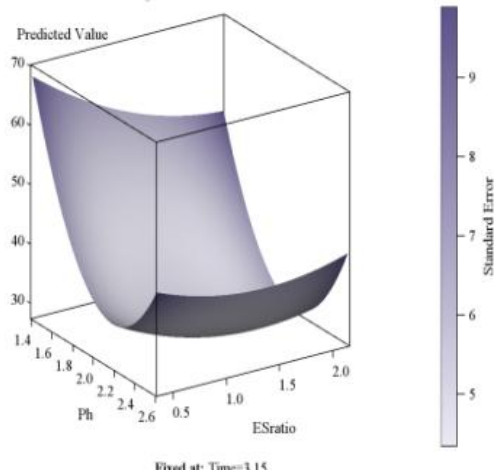

Fig. 4.3 (C)

Fig. 4.2 (A): Response surface plot for the effect of time and E/S ratio at fixed pH (2) on the AOA of pepsin hydrolysate. **Fig. 4.2 (B)**: Response surface plot for the effect of time and pH at fixed E/S ratio (1.25%) on the AOA of pepsin hydrolysate. **Fig. 4.2 (C)**: Response surface plot for the effect of pH and E/S ratio at fixed time (3.15 hrs) on the AOA of pepsin hydrolysate.

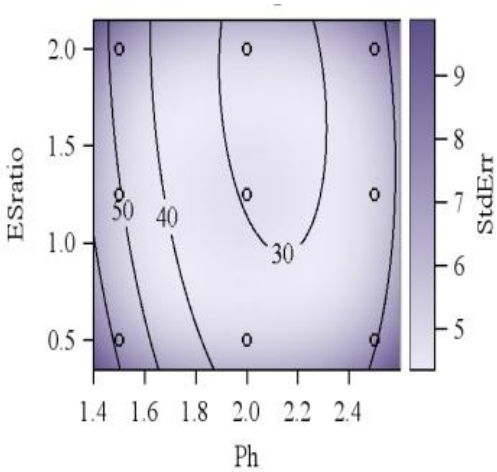

Fig. 4.3 (A)

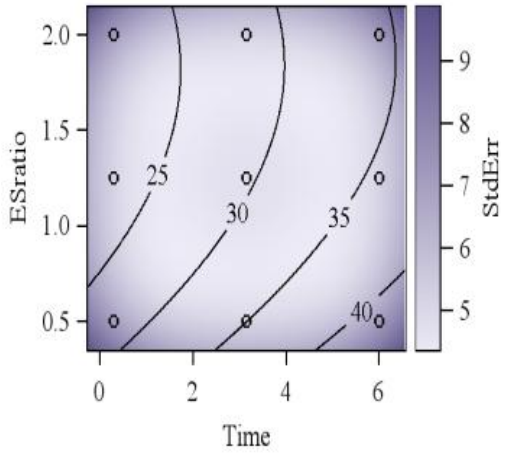

Fig. 4.3 (B)

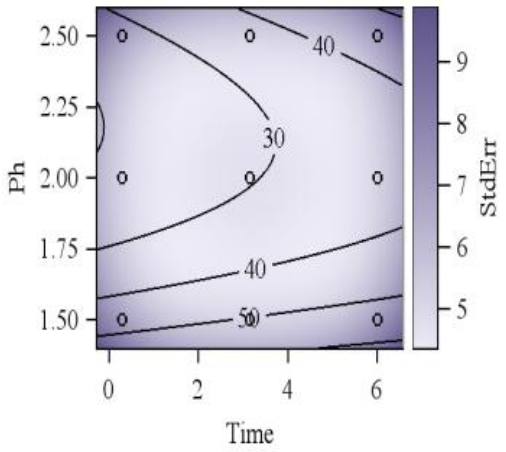

Fig. 4.3 (C)

Fig. 4.3 (A): Response contour plots for the effect of pH and E/S ratio at fixed time (3.15 h) on the AOA of pepsin hydrolysate. **Fig. 4.3 (B)**: Response contour plots for the effect of time and E/S ratio at fixed pH (2) on the AOA of pepsin hydrolysate. **Fig. 4.3 (C)**: Response contour plots for the effect of time and pH at fixed E/S ratio on the AOA of pepsin hydrolysate (1.25%)

Table 4.1: The Box-Behnken design for optimizing the hydrolysis conditions of pepsin hydrolysate with DPPH activity

Run number	Coded levels of variable			Experimental values			Response value
	X_1	X_2	X_3	E/S ratio (%)	pH	Time (h)	AOA (%)
1	0	-1	+1	1.25	1.5	6.00	58.01
2	-1	+1	0	0.50	2.5	3.25	45.67
3	-1	-1	0	0.50	1.5	3.25	63.28
4	-1	0	+1	0.50	2.0	6.00	32.58
5	0	0	0	1.25	2.0	3.25	28.97
6	0	0	0	1.25	2.0	3.25	30.62
7	+1	-1	0	2.00	1.5	3.25	41.01
8	-1	0	−1	0.50	2.0	0.30	25.51
9	0	-1	−1	1.25	1.5	0.30	43.79
10	0	+1	+1	1.25	2.5	6.00	47.25
11	+1	0	−1	2.00	2.0	0.30	30.47
12	+1	+1	0	2.00	2.5	3.25	32.05
13	0	+1	−1	1.25	2.5	0.30	23.4
14	0	0	0	1.25	2.0	3.25	28.89
15	+1	0	+1	2.00	2.0	6.00	36.87

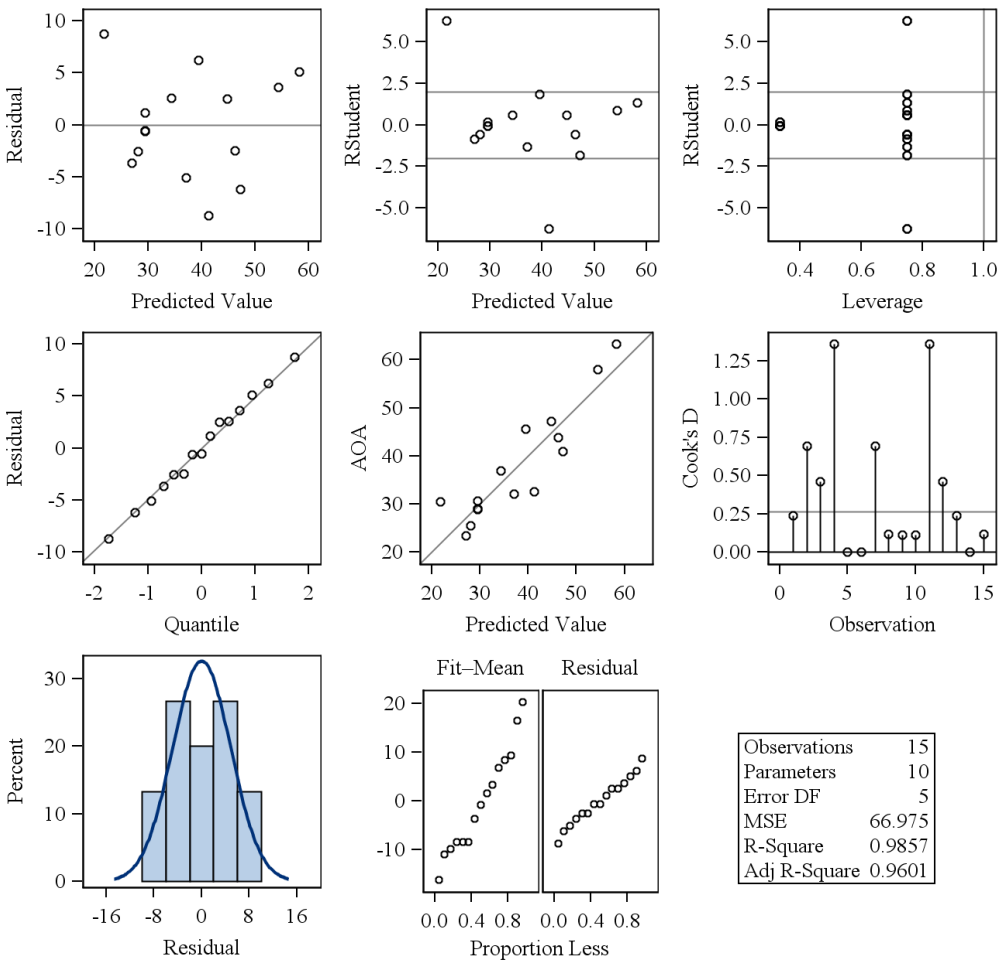

Fig. 4.4: Fit diagnostics for AOA of pepsin hydrolysite

4.4.3 Effect of hydrolysis conditions on the antioxidant activity of papain hydrolysate

Significant regression coefficient (R^2 value 0.84) for the second order response surface regression model indicated its suitability to explain the total variability of AOA of papain hydrolysate in terms of linear, quadratic and interaction effect of hydrolysis conditions on squid muscle protein. Antioxident activity of papain hydrolysate was reduced with linear effect of independent variable, whereas it increased with quadratic effect of hydrolysate

conditions except time. However, interaction effects revealed a reducing trend of AOA activity for the papain hydrolysate except E/S ratio and time (Eq-2). Response values of the AOA with respect to changes in the independent variables are presented in the Table 4.2. Predicted values of AOA of papain hydrolysate with respect to changes in the hydrolysis condition are depicted as response surface plots in Figure 4.5 (A), 4.5 (B) and 4.5 (C), respectively. Antioxidant activity of papain hydrolysate revealed a slightly enhancing trend with pH, whereas it declined with E/S ratio up to 1.5% of concentration at fixed time of 3.15 hrs. At constant pH (6), there was a slight downward parabolic trend of AOA observed with changes in time of the experiment; whereas there no activity was noticed in sample with changing E/S ratio up to a concentration of 1%, after that it enhanced. Similarly, at fixed E/S ratio (1.25%), the AOA papain hydrolysate was showed a slightly increasing trend with time, whereas an upward parabolic trend revealed for the AOA of the sample with changes in the pH.

AOA of papain = $99.34-47.62X_1-0.70X_2-5.51X_3+8.39X_1^2+0.035X_2^2-0.45X_3^2-0.66X_1X_2+6.34X_1X_3-0.96X_2X_3$, $R^2=0.84$ .....(2)

4.4.3.1 Multiple response evaluation

Effect of E/S ratio and pH at fixed time (3.15 h), E/S ratio and time at fixed pH (6), time and pH at fixed E/S ratio (1.25%) on the antioxidant activity of papain hydrolysate of squid protein was presented as response contour graph in Fig 4.6 (A), 4.6 (B) and 4.6 (C), respectively. There was a decreasing trend of AOA activity of the sample noticed with effect of E/S ratio and pH; whereas the effect of pH and time on the AOA activity of papain hydrolysate indicated an increasing trend at fixed E/S concentration (1.25%). Antioxidant activity of papain hydrolysate of the sample decreased with increasing E/S ratio and it increased with time. Fit diagnostics for AOA of papain hydrolysite is depicted in

Fig 4.7. Based on the ridge analysis, it found that 1.5:100 E/S ratio, 6.25 pH and 5.15 hrs were optimum for the production of papain hydrolysate from squid protein with better antioxidant property (66.34 %).

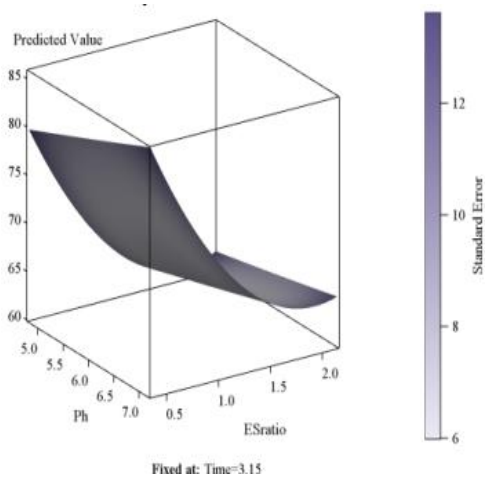

Fig. 4.5 (A)

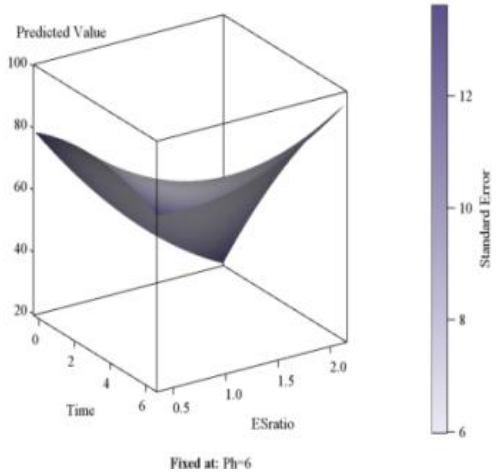

Fig. 4.5 (B)

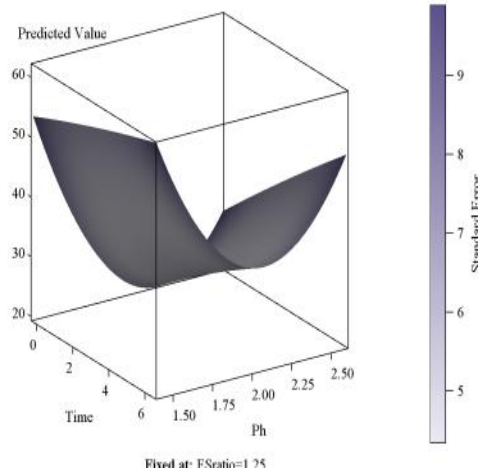

Fig. 4.5 (C)

Fig. 4.5 (A): Response surface plots for the effect of pH and E/S ratio at fixed time (3.15 hrs) on the AOA of papain hydrolysate; **Fig. 4.5 (B)**: Response surface plots for the effect of time and E/S ratio at fixed pH (6) on the AOA of papain hydrolysate; **Fig. 4.5 (C)**: Response surface plots for the effect of time and pH at fixed E/S ratio (1.25%) on the AOA of papain hydrolysate.

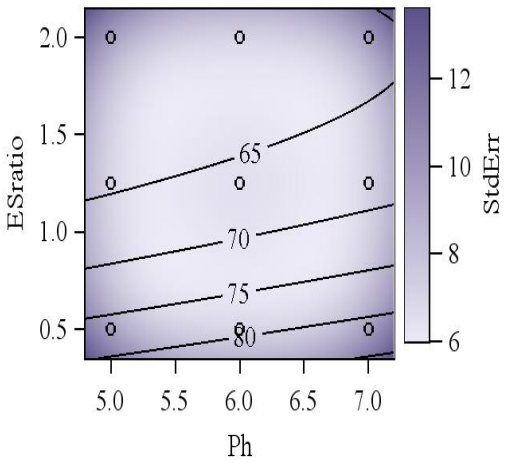

Fig. 4.6 (A)

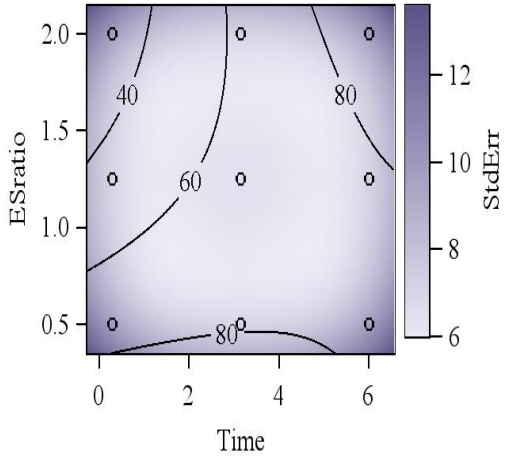

Fig. 4.6 (B)

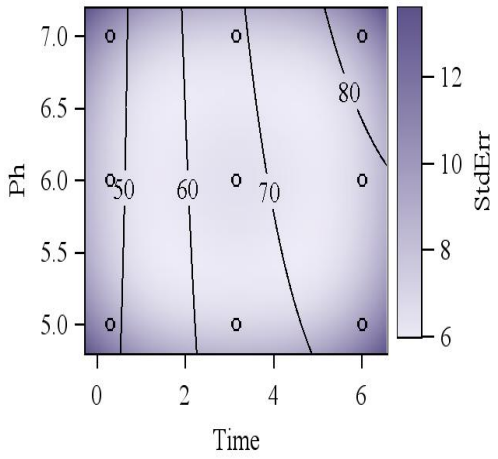

Fig. 4.6 (C)

Fig. 4.6 (A): Response contour plots for the effect of pH and E/S ratio at fixed time (3.15 h) on the AOA of papain hydrolysate. **Fig. 4.6 (B)**: Response contour plots for the effect of time and E/S ratio at fixed pH (6) on the AOA of papain hydrolysate. **Fig. 4.6 (C)**: Response contour plots for the effect of time and pH at fixed E/S ratio (1.25%) on the AOA of papain hydrolysate.

Table 4.2: The Box-Behnken design for optimizing the hydrolysis conditions of papain hydrolysate with DPPH activity

Run number	Coded levels of variable			Experimental values			Response value
	X_1	X_2	X_3	E/S ratio (%)	pH	Time (h)	AOA (%)
1	+1	0	−1	2.00	6.0	0.30	28.51
2	-1	-1	0	0.50	5.0	3.25	66.34
3	0	-1	+1	1.25	5.0	6.00	81.57
4	0	+1	+1	1.25	7.0	6.00	82.63
5	0	0	0	1.25	6.0	3.25	61.39
6	0	0	0	1.25	6.0	3.25	65.54
7	+1	0	+1	2.00	6.0	6.00	77.97
8	+1	+1	0	2.00	7.0	3.25	74.85
9	0	0	0	1.25	6.0	3.25	72.08
10	0	+1	−1	1.25	7.0	0.30	38.42
11	-1	+1	0	0.50	7.0	3.25	79.41
12	-1	0	−1	0.50	6.0	0.30	83.96
13	0	-1	−1	1.25	5.0	0.30	48.32
14	-1	0	+1	0.50	6.0	6.00	79.24
15	+1	-1	0	2.00	5.0	3.25	63.76

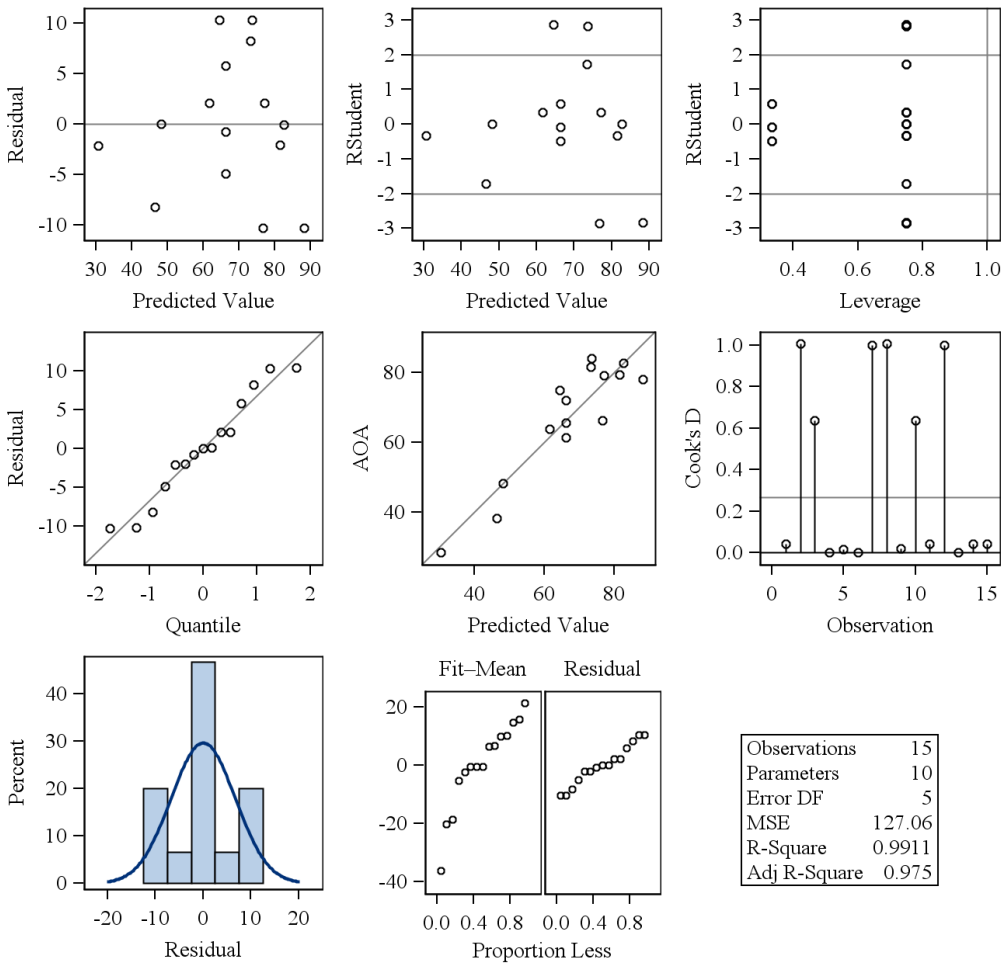

Fig. 4.7: Fit diagnostics for AOA of papain hydrolysite

4.4.4 Effect of hydrolysis conditions on the antioxidant activity of trypsin hydrolysate

Second order response surface regression model was fitted with better regression coefficient (R^2value of 0.85) to explain the total variability of antioxidant activity of trypsin hydrolysate in terms of linear, quadratic and interaction effect of hydrolysis conditions on squid muscle protein. Antioxidant activity of trypsin hydrolysate was reduced with linear effect of hydrolysate conditions except E/S ratio, whereas it increased with quadratic effect of E/S

ratio, pH and time. Similarly, interaction effects of independent variables also revealed an increasing trend of AOA activity of the trypsin hydrolysate except E/S ratio and pH (Eq-3). The effect of E/S ratio, time and pH (independent variables) on the antioxidant activity (dependent variable) of trypsin hydrolysate is presented in Table 4.3. Response surface plots for the E/S ratio and pH at fixed time (3.15 h), E/S ratio and time at fixed pH (7.5), and time and pH at fixed E/S ratio (1.25%) on the predicted values of antioxidant activity of trypsin hydrolysate are depicted as in Figure 4.8 (A), 4.8 (B) and 4.8 (C), respectively. Antioxidant activity of trypsin hydrolysate of the sample revealed an upward parabolic trend with pH, whereas it reduced with increasing E/S concentration. Effect of time and E/S ratio on the antioxidant activity of trypsin hydrolysate indicated an upward parabolic trend with time, whereas it increased with E/S concentration. Similarly, upward parabolic trend of antioxidant activity of trypsin hydrolysate was noticed for the effect of time and pH, however higher AOA was noticed for changes in the pH of the experiment.

AOA of trypsin = $183.85+7.18X_1-30.75X_2-10.68X_3+2.07X_1^2+2X_2^2+1.01X_3^2-2.78X_1X_2+2.11X_1X_3+0.74X_2X_3$, $R^2=0.85$. .....(3)

4.4.4.1 Multiple response evaluation

Response counter plot for E/S ratio and pH at fixed time (3.15 h) on the AOA of trypsin hydrolysate is depicted in Figure 4.9 (A). In the figure it's clearly evident that at lower E/S concentration and pH gives better AOA for the sample. Effect of E/S ratio and time at fixed pH (7.5) condition on the AOA of trypsin hydrolysate is presented as response contour graph in Figure 4.9 (B). E/S ratio at a range of 0.5 to 1.3% and 6 hrs gives higher AOA for the sample. Similarly, lower pH and higher experimental time reveals higher AOA for trypsin hydrolysate at a constant E/S ratio (Figure 4.9 C). Fit diagnostics for AOA of trypsin hydrolysate is depicted in Figure 4.10. Ridge analysis

revealed a combination of E/S ratio at 1.4:100, pH at 7.25 and 6h hydrolysis time were optimum for the production of trypsin hydrolysate from squid protein with better antioxidant property (54.24%).

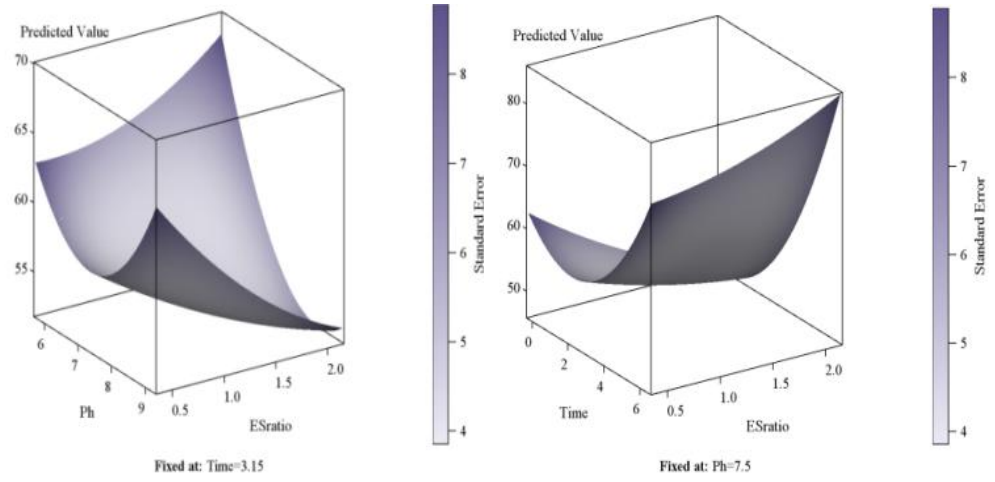

Fig. 4.8 (A) Fig. 4.8 (B)

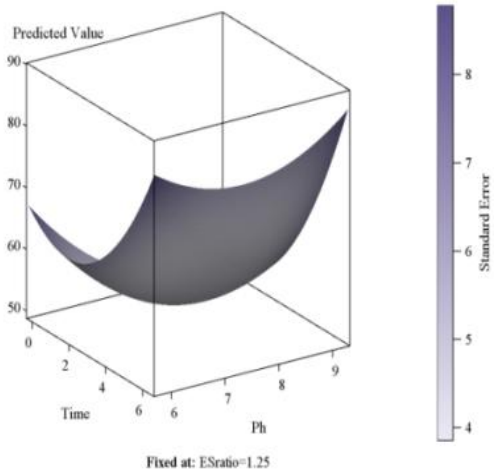

Fig. 4.8 (C)

Fig. 4.8 (A): Response surface plots for the effect of pH and E/S ratio at fixed time (3.15 h) on the AOA of trypsin hydrolysate; **Fig. 4.8 (B)**: Response surface plots for the effect of time and E/S ratio at fixed pH (7.5) on the AOA of trypsin hydrolysate; **Fig. 4.8 (C)**: Response surface plots for the effect of time and pH at fixed E/S ratio (1.25%) on the AOA of trypsin hydrolysate.

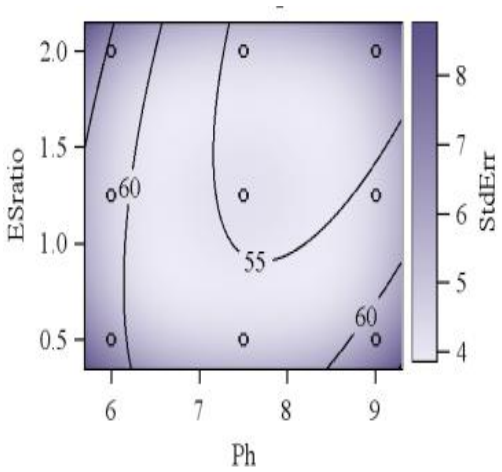

Fig. 4.9 (A)

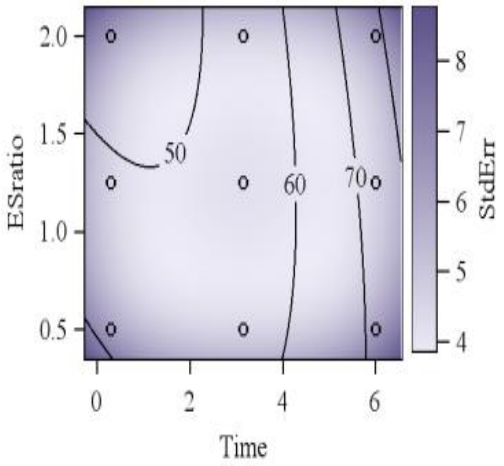

Fig. 4.9 (B)

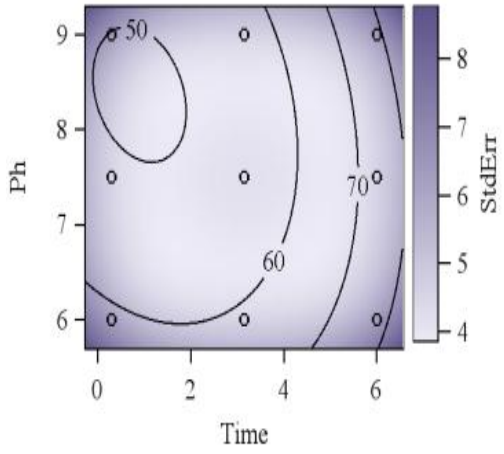

Fig. 4.9 (C)

Fig. 4.9 (A): Response contour plots for the effect of pH and E/S ratio at fixed time (3.15 h) on the AOA of trypsin hydrolysate. **Fig. 4.9 (B)**: Response contour plots for the effect of time and E/S ratio at fixed pH (7.5) on the AOA of trypsin hydrolysate. **Fig. 4.9 (C)**: Response contour plots for the effect of time and pH at fixed E/S ratio (1.25%) on the AOA of trypsin hydrolysate.

Table 4.3: The Box-Behnken design for optimizing the hydrolysis conditions of trypsin hydrolysate with DPPH activity

Run number	Coded levels of variable			Experimental values			Response value
	X_1	X_2	X_3	E/S ratio (%)	pH	Time (h)	AOA (%)
1	0	-1	+1	1.25	6.0	6.00	75.64
2	0	0	0	1.25	7.5	3.25	56.14
3	0	0	0	1.25	7.5	3.25	58.90
4	0	-1	−1	1.25	6.0	0.50	63.35
5	0	+1	+1	1.25	9.0	6.00	76.91
6	0	+1	−1	1.25	9.0	0.30	51.91
7	+1	0	+1	2.00	7.5	6.00	75.00
8	+1	-1	0	2.00	6.0	3.25	69.28
9	-1	0	+1	0.50	7.5	6.00	77.97
10	0	0	0	1.25	7.5	3.25	47.67
11	-1	+1	0	0.50	9.0	3.25	56.78
12	-1	-1	0	0.50	6.0	3.25	56.57
13	+1	0	−1	2.00	7.5	0.30	40.25
14	-1	0	−1	0.50	7.5	0.30	61.23
15	+1	+1	0	2.00	9.0	3.25	56.99

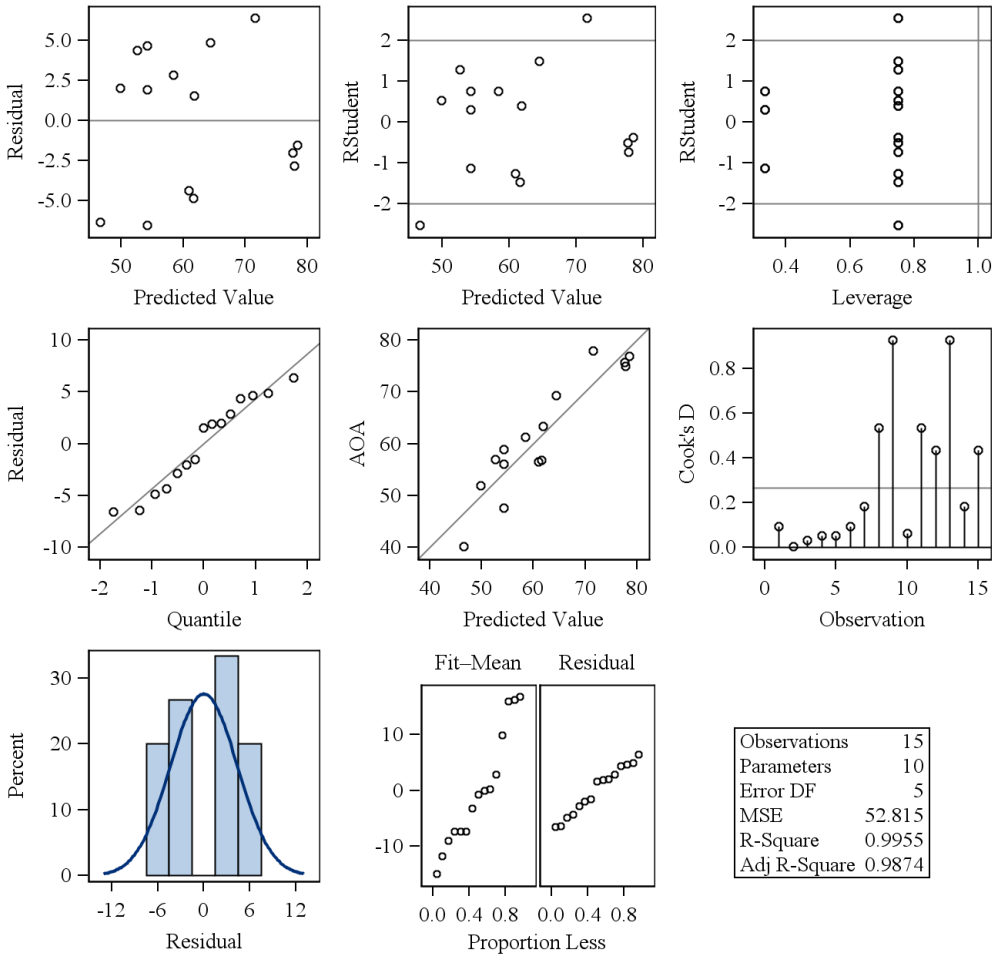

Fig. 4.10: Fit diagnostics for AOA of trypsin hydrolysate

4.4.5 Validation of the experiment

In validation study, the experimental values of the antioxident activity of papain, trypsin and pepsin hydrolisate processed at optimum hydrolysis condition was 65.8%, 53.6%, and 28.4%, respctively (Figure 4.11), which is in well agreement with the predcited values obtained from the second order response surface regression model. Hence, it clearly revealed the suitbaility of the generated model to explain the hydrolysis conditions of the proteases from the squid muscle protein with respect to its antioxidant activity. Moreover, it is

clearly evident that among the protein hydrolysates, papain showed higher percentage of DPPH scavenging activity followed by trypsin and pepsin. Fang *et al.*, (2012) optimized the hydrolysis condition of pepsin, trypsin, papain, alcalase and flavourzyme for the production of antioxidant peptides from flying squid using RSM and also reported excellent antioxidant properties in papain digested peptides. Similarly, Ren *et al.*, (2008) optimized hydrolysis condition of proteases for synthesizing antioxidant peptide from grass carp using RSM and authors also found highest antioxidant properties in papain digested hydrolysate.

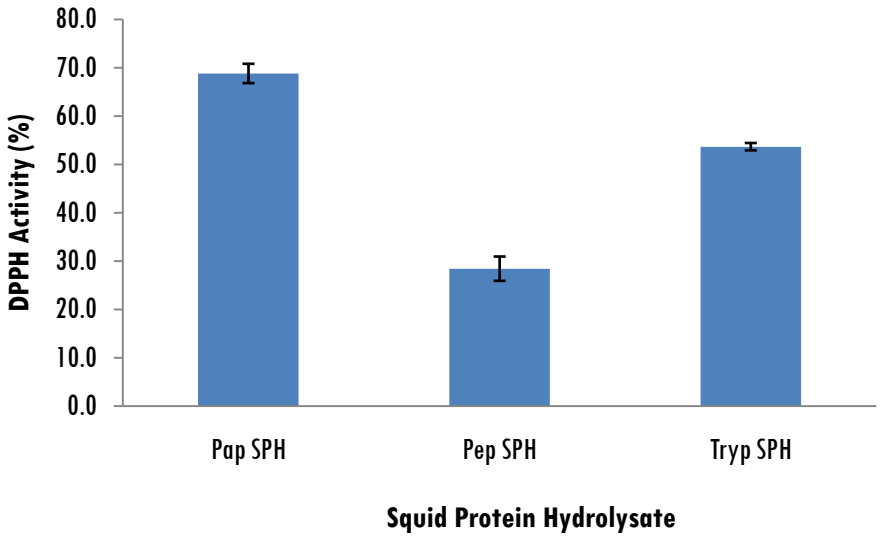

Fig. 4.11: DPPH radical scavenging activity of protein hydrolysates from squid.

4.5 Conclusion

Hydrolysis conditions of proteases were significantly altered the antioxidant properties of the obtained hydrolysate from Indian squid protein. The second order response surface regression model was significant enough to explain the total variability of antioxidant activity of the protein hydrolysate in terms of linear, quadratic and interaction effect of hydrolysis conditions.

Higher coefficient of determination (R^2) indicated the suitability of the model to explain the response variable with respect to independent variables. Based on the antioxidant activity of the protein hydrolysate, the ridge and multiple response analysis revealed an optimum combination for the hydrolysis of Indian squid protein concentrate with papain as 1.5:100 (E/S ratio), 6.25 (pH), 5.15 hrs (time), for pepsin as1:100 (E/S ratio), 2 (pH) and 3.15 hrs (time) and for trypsin as E/S ratio at 1.4:100, pH at 7.25 and 6 hrs hydrolysis time, respectively. Predicted values for the antioxidant activity of the protein hydrolysate was well fitted with experimental values, hence it indicates the suitability of the generated model for predicting the hydrolysis condition of proteases for the production of better antioxidant hydrolysate from Indian squid muscle. Moreover, papain digested protein hydrolysate possessed highest antioxidant activity.

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Chapter 5

Comparison of Antioxidant Activity of Protein Hydrolysates of Indian Squid (*Uroteuthis duvauceli*) Hydrolyzed at Optimized Processing Conditions

Contents

5.1 Introduction

Lipid oxidation and the resultant peroxidation products such as free radicals significantly alter the quality, nutritional value and shelf life of seafood. Moreover, consumption of rancid food causes several health risks especially diabetes mellitus, cancer, coronary heart diseases, inflammation, and alzheimer's to the consumers (Gey, 1990). Antioxidation is a process of preventing the formation of free radicals during oxidation of lipid, hence it inhibits the oxidation of lipids in the membrane, and degeneration of protein and DNA, as a result it prevents neurodegenerative and inflammatory diseases (Vimala and Adenan, 1999). Hence, inhibition of lipid oxidation of food by effective antioxidants was gained greater attention among the researchers; as a result several synthetic antioxidants were developed. Even though there is better efficiency of these artificial antioxidants to retard the lipids oxidation and the formation of free radicals, application of these antioxidants especially

butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate were restricted due to its potential health risks to the consumer. Therefore, in recent time, researchers are focusing to identify and synthesis antioxidant peptides from natural protein sources. Rajapakse *et al*., (2005) reported the ability of peptides from various protein sources for the inhibition of lipid oxidation. Authors also suggested better antioxidation efficiency of the peptides hydrolyzed from seafood proteins.

Superoxide anion radical (O_2^-) and hydroxyl radical (OH) act as free radicals which liberate as the byproducts of aerobic metabolism accelerated by aerobic microbes (Kim *et al*., 2007). These free radicals are highly unstable and susceptible for several reactions in the body which cause cell and tissue damage and initiate chronic diseases (Je *et al*., 2005; Davalos *et al*., 2004). Moreover, transition metals such as Fe and Cu accelerated the formation of peroxides by donating electron (Pokorny *et al.,* 2001). Hence, an antioxidant should have the capability not only to scavenge oxygen containing compounds but also to possess the chelating capacity of metals (Kristinsson and Rasco, 2000). Scavenging of stable free radical such as DPPH by an antioxidative compound indicates its ability to donate electron or hydrogen (Wu *et al.,* 2003). Hence, scavenging of DPPH by a peptide or protein hydrolysate reveals its antioxidant ability. Moreover, DPPH assay is a cheap and reliable method to determine the antioxidant activity of a protein hydrolysate. The capacity of a protein hydrolysate to preserve the linoleic acid from oxidation also indicates its antioxidation ability (Osawa and Namiki, 1985). Reducing power assay is another method to determine the antioxidant activity of a protein hydrolysate by estimating the capability to donate an electron or hydrogen (Yildirim *et al*., 2000). This assay is based on the estimation of ferrous iron formation from ferric iron by using a redox linked colorimetric reaction (Decker *et al*., 2005;

Dorman *et al*., 2003). You *et al*., (2009) reported that antioxidant properties of protein hydrolysates were highly influenced by the protease applied for the hydrolysis and degree of hydrolysis. Qian *et al*., (2008a) studied the inhibition of lipid peroxidation, DPPH, hydroxyl, superoxide, peroxyl radical scavenging activity of the peptide hydrolysed by using papain, pepsin, trypsin from frog skin.

5.2 Objective of the study

Objective of the experiment was to differentiate and identify suitable protease for the hydrolysis of squid protein at optimized condition with respect to its antioxidant properties such as DPPH radical scavenging activity, superoxide anion radical-scavenging activity, metal chelation ability, inhibition of lipid peroxidation in the linoleic acid model system and reducing power.

5.3 Materials and methods

5.3.1 Raw Materials

Indian squid (*U. duvauceli*) was collected from the fish landing centre at Fort Cochin, India. Samples had an average length of 570±14 mm and weight 445±15 g. The samples were transported to the laboratory in iced condition. The squid mantle was dissected and used for the analysis.

5.3.2 Chemicals

Standards for pepsin, trypsin, papain, gallic acid 2, 2-diphenyl-1-picryhydrazyl (DPPH) and linoleic acid were purchased from Sigma–Aldrich (Shanghai, China). All other reagents used for the study were analytical grade from standard sources.

5.3.3 Extraction of muscle protein

Extraction of muscle protein from Indian squid was described in Chapter 4, Section 4.3.3.

5.3.4 Preparation of Indian squid muscle hydrolysates

The squid muscle protein was subjected to hydrolysis at optimized conditions such as 1.5:100 (E/S ratio), 6.25 (pH), 5.15 h (time) for papain, 1:100 (E/S ratio), 2 (pH) and 3.15 h (time) for pepsin and E/S ratio at 1.4:100, pH at 7.25 and 6 h hydrolysis time for trypsin, respectively. Procedure of preparation of Indian squid muscle hydrolysates is described at Chapter-4, Section 4.3.5.

5.3.5 Determination of DPPH radical scavenging activity

The procedure for determination of DPPH radical scavenging activity is described in Chapter 4, Section 4.3.7.

5.3.6 Determination of superoxide radical scavenging activity

The superoxide radical scavenging activity was estimated as per Marklund and Marklund (1974) with slight modifications. 0.1 mL of sample *ie.*, SPH in Tris-HCl-EDTA buffer (0.1 mol/L, pH 8.0) mixed with 0.1 mL of 60 m mol/L pyrogallol solution and incubated at 25 °C for 10 min. Changes of absorbance during 0.5 min to 4 min were recorded at 325nm using spectrophotometer. The absorbance of deionized water was measured as a blank value. The scavenging activity of superoxide radicals was calculated as following:

$$\text{Superoxide radical Scavenging activity (\%)} = \frac{(\text{absorbance of blank} - \text{absorbance of samples})}{\text{absorbance of blank}} \times 100$$

5.3.7 Determination of metal chelation activity

The chelation of Fe^{2+} was measured for SPH by using Decker and Welch (1990) method. The mixture of 1 mL of sample solution (SPH) and 3.7 mL of distilled water was allowed to react with 0.1 mL of 2 mM $FeCl_2$ and 0.2 ml of 5 mM 3-(2-pyridyl)-5,6- bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) for 20 min at room temperature. The control was prepared in the same manner except sample; same quantity of distilled water was added. The absorbance was noticed at 562 nm and chelating activity (%) was calculated as follows:

$$\text{Metal chelating activity (\%} = \frac{(\text{absorbance of Blank} - \text{absorbance of samples})}{\text{absorbance of Blank}} \times 100$$

5.3.8 Determination of reducing power

Reducing power was estimated as per the procedure described by Oyaizu (1986). The sample solution (0.5 mL) was mixed with 0.2 M phosphate buffer (2.5 mL) (pH 6.6) and 1% potassium ferricyanide (2.5 mL). The mixture was incubated at 50 °C for 20 min, then added 2.5 mL of 10% trichoroacetic acid and centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with 2.5 ml of distilled water and 2.5 ml of 0.1% ferric chloride and the absorbance was red at 700 nm. Enhanced absorbance indicated increasing reducing power.

5.3.9 Determination of lipid peroxidation in a linoleic acid model system

The antioxidant activity of SPH was estimated as per Nilsang *et al*., (2005) methodology with slight modification. One milligram of SPH was dissolved in 1.5 mL of 0.1 mol L^{-1} phosphate buffer (pH 7.0), and added to 5 mL of linoleic acid solution (linoleic acid dissolved in 95% ethanol in order to

get 2 mg mL^{-1} concentration) in a glass test tube which was sealed tightly with silicon rubber cap and kept in the dark room for 7 days at 37 °C. The degree of oxidation was evaluated by measuring the ferric thiocyanate values. The sample solution (100 µL) incubated in the linoleic acid model system described above was mixed with 4.7 mL of 75% ethanol, 0.1 mL of 30% (w/v) ammonium thiocyanate, and 0.1 mL of 0.02 mol L^{-1} ferrous chloride dissolved in 1 mol L^{-1} HCl. After 3 min the color variation occurred due to oxidation of linoleic acid and it was measured by using spectrophotometer at 500 nm. The absorbance of the solution without the hydrolysate samples was taken as blank reading.

5.3.10 Determination of degree of hydrolysis (Ortho-phthalaldehyde)

Degree of hydrolysis (DH) of SPH was measured by Spellman *et al*. (2003), with slight modifications for the reaction of amino nitrogen with ortho-phthalaldehyde (OPA). 10µL of the SPH was mixed with 3.4 mL of OPA reagent, and kept the solution at 25 °C for 2 min. Subsequently, the absorbance was read at 340nm and DH was calculated based on the following equation:

$$\mathrm{DH}\ (\%) = \frac{(\mathrm{ABS} \times 1{,}934 \times \mathrm{d})}{\mathrm{c}}$$

Where "ABS" is the absorbance of samples, "d" represents dilution factor and "c" is the protein concentration of the sample (g L^{-1}).

5.3.11 Analysis of amino acid composition

Analysis of amino acid composition of protein hydrolysate is described in Chapter 3, Section 3.3.5.

5.3.12 Statistical analysis

One way ANOVA was used to find the significant effect of hydrolysis conditions on AOA of the samples. SAS version 9.3 (SAS Institute, 2012) was used for statistical analysis. Once ANOVA was found to be significant at 5% level ($p<0.05$), Duncan test was applied to differentiate the significance.

5.4 Result and discussion

5.4.1 DPPH scavenging activity of protein hydrolysates

DPPH is a free radical which accepts electron or hydrogen and stable at ambient temperature (Zhong *et al*., 2011). DPPH scavenging activity of papain, pepsin and trypsin digested protein hydrolysate of Indian squid muscle is presented in Table 5.1. Analysis of variance revealed significant effect of proteases on the DPPH scavenging activity of the hydrolysate ($p <0.05$). Xiao *et al.,* (2010) and Fang *et al.,* (2012) reported that protein hydrolysate synthesized using various proteases from flying squid had showed excellent scavenging of DPPH radicals. It was clearly evident that, among the samples, standard (gallic acid) showed 75.33±0.22% of DPPH scavenging activity; whereas, peptides obtained from the hydrolysis of Indian squid protein by papain showed highest DPPH scavenging activity (25.25±1.12%) than trypsin digested hydrolysate (15.54±1.25%) and pepsin digested hydrolysate (25.25±0.94%). Yang *et al*., (2008) found better antioxidant activity in the papain digested protein hydrolysate of cobia skin.

5.4.2 Superoxide anion radical-scavenging activity of protein hydrolysates

Superoxide anion radical is a free radical species which is produced during oxidative stress and it causes aging by damaging the cell membranes

and also play a key role for the oxidation of lipids (Korycka-Dahl and Richardson, 1978). Hence, the ability of protein hydrolysates to scavenge superoxide anion radicals was highly relevant to establish its bioactivity especially antioxidant activity. All the peptides showed superoxide anion radical-scavenging properties. However, analysis of variance on superoxide anion radical-scavenging activity of protein hydrolysates showed significant difference ($p < 0.05$). Peptide synthesized from Jian carp scavenged hydroxyl and superoxide anion free radicals (Xiao *et al.,* 2010). Similar findings were reported in *Theragra chalcogramma* (Zhuang *et al.,* 2009), tilapia (Ngo *et al.,* 2010) and hoki (Kim *et al.,* 2007). In the present study, standard revealed superoxide anion radical-scavenging activity of 90.54±0.45%, whereas among the protein hydrolysates, papain SPH exhibited highest potential to scavenge superoxide anion radical (32.56±1.09%), than trypsin SPH (30.21±1.22%) and pepsin SPH (16.23±0.35%) (Table 5.1).

5.4.3 Metal chelation activity of protein hydrolysates

Presence of available reactive oxygen species significantly influences the autoxidation and breakdown of hydroperoxides to volatile compounds and it could be accelerated by transition metals such as Fe, Cu and Co (Pokorny *et al.*, 2001). Peptides had the ability to bind with iron, so it disrupts the reaction between ferrozine and Fe^{2+} ion (Thiansilakul *et al.,* 2007). So the metal chelating property of peptides could be established by measuring the scavenging of ferrous ions. Kristinsson and Rasco (2000) suggested that an antioxidant should have the capability not only to scavenge oxygen containing compounds but also scavenge metals. Hence, metal chelating ability is a useful indicator to establish the potential antioxidant activity of a protein hydrolysate. Metal chelation property of protein hydrolysates obtained from papain, pepsin and trypsin is presented in Table 5.1. Highest chelation of metals was

observed for papain SPH (32.56±1.09%) followed by trypsin (30.21±1.22%) and pepsin (23.63±1.22%), whereas standard (EDTA) showed a value of 77.86±0.54% of metal chelation activity. Significantly different metal chelation activity was noticed for each protein hydrolysate at 5% level ($p < 0.05$). According to Je *et al.*, (2005) metal chelating properties of peptides isolated from *N. japonicus* and *E. volitans* were due to the presence of histidine amino acids. Imidazole ring of histidine amino acids had the potential to scavenge metals and lipid radicals (Murase *et al.,* 1993; Park *et al.,* 2001; Uchida *et al.,* 1992). Degree of hydrolysis and molecular weight of peptides influences metal chelating properties of peptides (Saiga, 2003; Klompong *et al.,* 2007; Dong *et al.,* 2008).

5.4.4 Reducing power of protein hydrolysates

Reducing power of protein hydrolysates reveals its ability to donate electrons, which react with free radicals in the lipid to form a stable product; hence it could be used as suitable indicator for the potentiality of protein hydrolysate to scavenge free radicals. Moreover, Je *et al*. (2009) revealed that reducing power of hydrolysate was mainly due to high content of amino acids which had the potential to release electron or hydrogen. Duh *et al*., (1999) suggested that the antioxidant activity of the protein hydrolysate was directly correlated with its reducing power. In the present study, reducing power of protein hydrolysates was determined based on the formation of ferrous (Fe^{2+}) compound by reducing ferric cyanide complex (Fe^{3+}). Reducing power of protein hydrolysates obtained from the digestion of papain, pepsin and trypsin is depicted in Table 5.1. Reducing power was noticed in the protein hydrolysate synthesized from yellow stripe trevally (Klompong *et al.,* 2007) and squid (Alemán *et al.,* 2011). There was no significant difference ($p > 0.05$) on the reducing power of protein hydrolysates except that in the case of

standard ($p < 0.05$) (Table 5.1). Among the samples, highest reducing power (0.123±0.93 mg mL^{-1}) was observed in papain hydrolysate than trypsin (0.109±0.85 mg mL^{-1}) and pepsin hydrolysate (0.101±0.65 mg mL^{-1}). Xiao *et al.,* (2010) suggested that reducing power of protein hydrolysate synthesized from flying squid skin was comparable with butylated hydroxyanisole. According to Klompong *et al.,* (2007) protease and degree of hydrolysis significantly influence the reducing power of protein hydrolysate.

Table 5.1: Antioxidant activities of squid protein hydrolysate

	DPPH scavenging activity[a] (%)	**Superoxide anion radical-scavenging activity [b] (%)**	**Metal chelation activity[a] (%)**	**Reducing power[c]**
Trypsin SPH	18.23±1.25[a]	22.35±0.63[a]	30.21±1.22[a]	0.109±0.02[a]
Pepsin SPH	15.54±0.94[b]	16.23±0.35[b]	23.63±1.22[b]	0.101±0.05[b]
Papain SPH	25.25±1.12[c]	26.35±1.05[c]	32.56±1.09[c]	0.123±0.03[c]
Standard	75.33±0.22[d]*	90.54±0.45[d]	77.86±0.54[d]**	1.633±0.06[d]*

Treatment means having common lower case in rows are homogenous. Each value represents the mean ± SD (n= 3), $p < 0.05$

Hydrolysate concentration: [a`] 2mg mL^{-1}, [b] 500µg mL^{-1} and [c] 1.5 mg L^{-1}. STD - *Gallic acid and ** EDTA.

5.4.5 Inhibition of lipid peroxidation in the linoleic acid model system

Secondary breakdown products of oxidation of fatty acids cause several adverse effects on mammalian cells (Qian *et al*., 2008a). Hence, the ability of protein hydrolysates to protect polyunsaturated fatty acids like linoleic acid from peroxidation indicates its antioxidant property (Osawa and Namiki, 1985). Mendis *et al*., (2005b) reported that bioactive peptides isolated from jumbo squid exhibited better inhibition of lipid peroxidation in the linoleic acid model system than ∞-tocopherol, a widely used natural antioxidative agent. It was clearly evident that all the protein hydrolysates effectively prevented linoleic acid from peroxidation (Figure 5.1). Some reports were available for the protection of linoleic acid from lipid peroxdiaton by the

action of peptides synthesized from marine sources such as oyster (Qian *et al.*, 2008b), *Nemipterus japonicas* (Naqash & Nazeer, 2010) and rotifer (Byun *et al.*, 2009). Mendis *et al.*, (2005b) reported protection of linoleic acid from autoxidation by jumbo squid (*Dosidicus gigas*) gelatin hydrolysate due to the presence of significant quantity of hydrophobic amino acids. Highest absorbance was observed for control sample (without antioxidant) indicating highest degree of oxidation, whereas the reference sample (∞-tocopherol) showed lowest oxidation. Among the protein hydrolysates, papain digested hydrolysate exhibited the highest inhibition of lipid peroxidation than trypsin and pepsin digested hydrolysate.

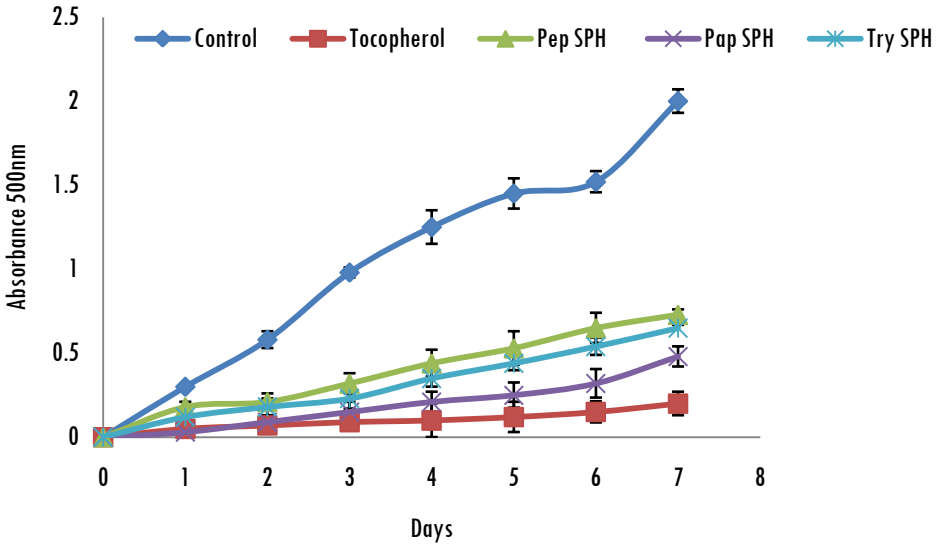

Fig. 5.1: Inhibitory effect of Squid Protein Hydrolysate on lipid peroxidation measured in a linoleic acid model system. Each value represents the mean ± SD (n= 3)

5.4.6 Degree of hydrolysis of protein hydrolysates

Degree of hydrolysis indicates the amount of peptide released from the protein, which was highly influenced by molecular size, polar groups and hydrophobic properties of protein (Adler-Nissen, 1986; Kristinsson and Rasco,

2000; Pena-Ramos and Xiong, 2003). Amount of hydrolysate released from squid protein by digesting pepsin, papain and trypsin at different intervals of time is presented as degree of hydrolysis in Figure 5.2. Degree of hydrolysis had great role for the antioxidant properties of protein hydrolysate (Dong *et al*., 2008). Degree of hydrolysis indicates the amount of peptides released from the protein, which was highly influenced by the type of protease being used for the hydrolysis (Pena-Ramos and Xiong, 2003). Hence, identification of suitable protease was highly necessary for the production of antioxidative hydrolysate to a large extent from protein (Klompong *et al*., 2009). In the present study, all the proteases showed a significantly increase of degree of hydrolysis with time. Yang *et al.*, (2008) found antioxidant properties of cobia skin peptides enhanced with degree of hydrolysis. However, higher rate of hydrolysis of squid proteins was observed in papain digestion process. Result showed good accordance with Fang *et al*., (2012). Degree of hydrolysis of the sample by pepsin was reduced after 2 h of the digestion. Probable reason for this might be due to the reduction of peptide bonds available for hydrolysis, enzyme deactivation, and/or the inhibition of the enzyme by the products formed at high degree of hydrolysis. However degree of hydrolysis varies with species. Maximum breakdown of fish proteins was observed in the initial 30 min of enzymatic hydrolysis (Benjakul and Morrissey, 1997; Dong *et al.*, 2008; Guerard *et al.*, 2002); whereas enzymatic hydrolysis of tuna cooking juice showed maximum degree of hydrolysis at 360^{th} min of the digestion (Jao and Ko, 2002). Degree of hydrolysis was directly proportional with reducing power, metal-chelating ability and antioxidant activity of the resultant hydrolysates (Klompong *et al*., 2007; Dong *et al*., 2008). It was clearly evident that among the proteases, papain revealed highest degree of hydrolysis, reducing power and metal-chelating ability; whereas pepsin revealed lowest degree of hydrolysis, reducing power and metal-chelating ability.

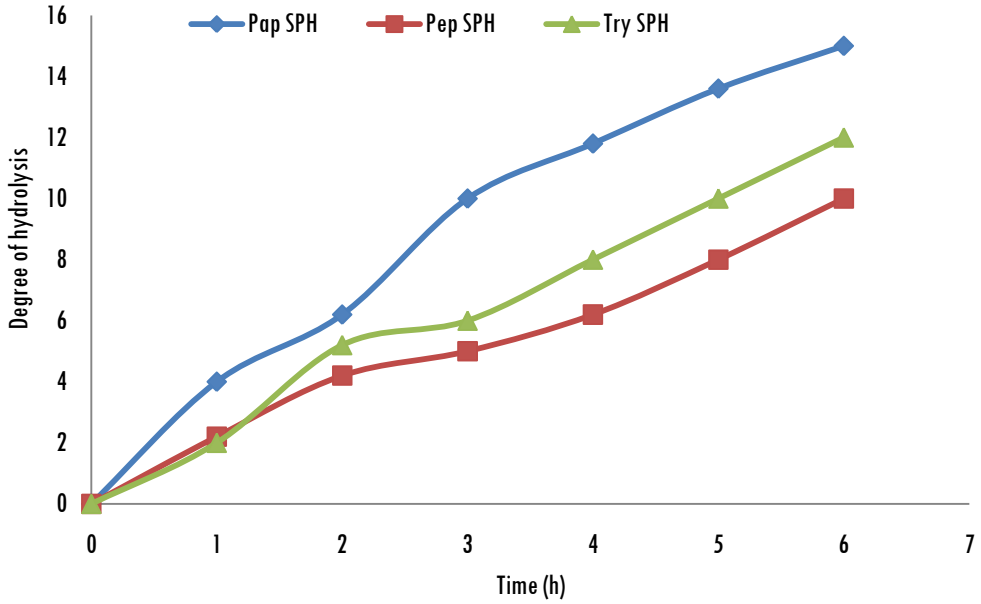

Fig. 5.2: Degree of Hydrolysis of squid protein hydrolysates; each value represents the mean ± SD (n= 3)

5.4.7 Amino acid profile of papain hydrolysate

Antioxidant properties of peptide were established by the sequence and composition of amino acids (Klompong *et al*., 2009). Amino acid profile of papain digested protein hydrolysate of Indian squid is depicted in Figure 5.3 and it was clearly evident that among the identified amino acids, glutamate (14.94%) was the most dominant amino acid, whereas least quantity was observed for arginine (0.72%). According to Young *et al.*, (2007) glutamate inhibits lipid peroxidation by donating hydrogen atom. In the present experiment, the concentration of histidine and tyrosine (aromatic amino acids) was 7.80% and 1.61% respectively. Occurrence of aromatic amino acids such as tyrosine and histidine inhibits radical-mediated peroxidizing chain reaction (Suetsuna *et al.*, 2000; Rajapakse *et al.*, 2005). Tsuge *et al.*, (1991) reported the presence of aromatic amino acids at the second residue of peptides had great role for the antioxidant properties of peptides. Wang *et al.*, (2007)

suggested that the disintegration of imidazole ring of histidine provides scavenging capacity to peptides; whereas Li *et al.*, (2007) found the proton liberates from imidazole had the ability to scavenge free radical. Majority of the amino acids in the hydrolysate was contributed by hydrophobic amino acids (49%) such as glysine, leucine, valine, isolucine, phenyl alanine, alanine, lysine, methionine and proline with a concentration of 10.58%, 8.78%, 7.42%, 7.06%, 6.65%, 3.51%, 5.22%, 2.55% and 1.1%, respectively. All these amino acids had the potential to scavenge free radicals (Suetsuna *et al*., 2000; Rajapakse *et al*., 2005; Kim *et al*., 2006; Hernandez *et al.*, 2005). Moreover, it was reported that antioxidant activities of peptide was improved with hydrophobic properties of amino acids (Saiga *et al*., 2003). Probable reason for this might be due to the solubility of peptides in lipids which is enhanced with hydrophobicity, which accelerates antioxidation (Wang *et al*., 2006). Aspartic acid content in the hydrolysate was 12.48%, which had the capability to release hydrogen ions (protons); it improves the scavenging of free radicals from lipid (Young *et al*., 2007). Concentration of threonine and serine in the sample was 5.69% and 3.94%, respectively.

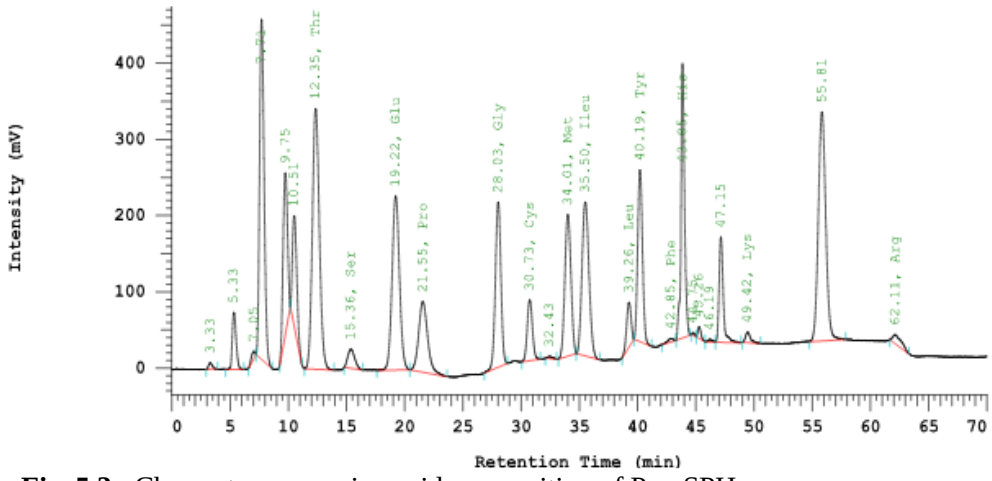

Fig. 5.3: Chromatogram amino acid composition of Pap-SPH;
Each value represents the mean ± SD (n= 3)

5.5 Conclusion

Protein hydrolysates were prepared from Indian squid muscle by digesting papain, pepsin and trypsin at optimized conditions. All protein hydrolysates revealed significant antioxidant properties ($p < 0.05$), however among the samples, papain digested hydrolysate exhibited excellent DPPH radical scavenging activity, superoxide anion radical-scavenging activity, metal chelation ability, inhibition of lipid peroxidation in the linoleic acid model system, reducing power and degree of hydrolysis. Similar findings were noticed in flying squid, cobia skin and loach (*Misgurnus anguillicaudatus*). Several researchers investigated the efficacy of various proteases for the production of bioactive peptides from marine origin and found excellent antioxidant properties in papain hydrolyzed peptides. Chalamiah *et al.,* (2012) also observed excellent antioxidant properties in the papain digested marine peptides. It was found that antioxidant properties such as reducing power and metal-chelating ability of the protein hydrolysates were enhanced with degree of hydrolysis. Moreover, potent antioxidant properties of papain digested protein hydrolysate were justified with the presence of significant quantities of antioxidant amino acids especially hydrophobic amino acids. Hence, it can be concluded that papain enzyme was highly efficient for producing antioxidant hydrolysates from Indian squid muscle.

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Chapter 6

Isolation, Purification and LC-MS/MS Characterization of Peptides from Papain Digested Squid (*Uroteuthis duvauceli*) Protein Hydrolysate and their Antioxidant Activity

Contents

6.1 Introduction

Molecular weight and amino acids sequence of peptides obtained by enzyme hydrolysis determines the bioactivities (Kim and Mendis, 2006; Kim and Wijesekara, 2010). Hence, a detailed experiment for the separation and purification of protein hydrolysate based on the molecular weight and amino acid sequence were highly necessary to establish the antioxidant properties of peptides. Ultrafiltration, column chromatography, high performance liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry (LC–MS/MS) were the common methods for isolation, purification and sequencing of marine bioactive peptides (Je *et al.*, 2005; Jeon *et al.*, 1999; Hsu *et al.*, 2009). Several reports were available for the improvement of antioxidant properties of the purified protein hydrolysate (Je *et al.*, 2005). According to Picot *et al.*, (2010) low molecular weight protein fragments show better antioxidant properties. This was in well agreement with Wu *et al.*, (2003) and You *et al.*, (2010).

6.2 Objective of the study

This study was designed to isolate and purify antioxidant peptide from papain digested squid (*U. duvauceli*) protein hydrolysate based on the molecular weight and also to determine the antioxidant properties and amino acid sequence of the resultant peptides.

6.3 Materials and methods

6.3.1 Raw materials

Indian squid (*U. duvauceli*) was collected from the fish landing centre at Fort Cochin, India. Samples had an average length of 570±14 mm and weight 445±15 g. The samples were transported to the laboratory in iced condition. The squid mantle was dissected and used for the analysis.

6.3.2 Chemicals

Standards for papain, gallic acid 2,2-diphenyl-1-picryhydrazyl (DPPH) and linoleic acid were purchased from Sigma–Aldrich (Shanghai, China). All other reagents used for the study were analytical grade from standard sources.

6.3.3 Extraction of muscle protein

Extraction of muscle protein from Indian squid is described in Chapter 4, Section 4.3.3.

6.3.4 Preparation of Indian squid muscle hydrolysates

The squid muscle protein was subjected to papain hydrolysis at optimized conditions such as 1.5:100 (E/S ratio), 6.25 (pH), 5.15 hrs (time). Procedure of preparation of Indian squid muscle hydrolysates was described at Chapter-4, Section 4.3.5.

6.3.5 Separation of papaine digested squid muscle hydrolysates by ultrafiltration

A portion of squid protein hydrolysate obtained by papain digestion (Pap SPH) that showed better antioxidant potential was fractionated by ultrafiltration (UF, Amicon Model 8400, Millipore Corporation, Billerica, MA) with Millipore UF membranes having three different molecular weight cut-offs (MWCO) of 10 and 3 kDa were used respectively. A volume of 100 g L^{-1} Pap SPH was passed through 10 kDa MWCO membrane. The flow-through-1 was less than 10 kDa fraction and the retentate Pap SPH 1 was greater than 10 kDa fractions. Below 10 kDa fraction was passed through 3 kDa MWCO membrane. Flow-through-2, was less than 3 kDa and the retentate, Pap SPH -2 had MW range above 3kDa. All UF fractions of SPH-pap recovered were lyophilized and kept at ˉ80 °C until further use (Asha *et al*., 2016).

6.3.6 Determination of antioxidant activity

6.3.6.1 Estimation of DPPH radical scavenging activity

Methodology for the analysis of DPPH radical scavenging activity was described in Chapter 4, section 4.3.7.

6.3.6.2 Estimation of superoxide radical scavenging activity

Analysis of superoxide radical scavenging activity was elaborated in Chapter 5, Section 5.3.6.

6.3.6.3 Estimation of metal chelating activity

Procedure for analyzing metal-chelating activity is mentioned in Chapter 5, Section 5.3.7.

6.3.6.4 Estimation of reducing power

Reducing power was determined based on the methodology described in Chapter 5, Section 5.3.8.

6.3.7 Protective effect of the purified peptide against hydroxyl-radical-induced DNA damage

DNA protection effect of purified squid peptide was evaluated. DNA damage induced by hydroxyl radical and the reaction was carried out with a total volume of 12 µl containing 0.5 µl of pBR 322 DNA in µl of 50 mM phosphate buffer (pH 7.4), 3 µl of 2 mM FeSO4 and 2 µl of the purified peptide at different concentrations (Qian *et al*., 2008). After that, 4 µl of 30% H_2O_2 was added, and the mixture was incubated at 37°C for 30 min. The mixture was used for 0.8% agarose gel electrophoresis to identify DNA bands. After run DNA bands were stained with ethidium bromide and recorded.

6.3.8 Purification of the ultra filtered fraction with highest antioxidant capacity by RP-HPLC

The peptide fragment possessing highest antioxidant activity was purified by ultrafiltration technique by using Sep-Pak C-18 cartridges (Waters Associates) connected in series at a flow rate of 2 mL min^{-1} and followed by elution with 60% methanol. The eluate was collected, concentrated, freeze dried and stored at -80^{0}C for further analysis. For HPLC separation UF peptide fraction of below <3 kDa was reconstituted in 60% methanol and purified further on an analytical reverse-phase ShimadzuC-18 column equilibrated with 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 2 mL min^{-1}. The acetonitrile gradient in the eluting solvent was increased to 21% (v/v) over 10 min, held at this concentration for 40 min and then increased to 49% (v/v) over 60 min with linear gradients. Absorbance was measured at 214 and 280 nm.

Purified peptides were collected by repeating the steps for 15 times. Acetonitrile and TFA were removed by passing with nitrogen flush, pooled fractions were freeze-dried and reconstituted in ultra-pure water (Asha *et al*., 2016).

6.3.9 Characterization of RP-HPLC fraction peptides (<3 kDa) by LC-MS/MS

The RP-HPLC fraction below 3 kDa was collected and sequenced by liquid chromatography- positive electron spray ionization tandem mass spectrometry (LC-ESI-MS/MS). Total ion chromatogram was acquired in information dependent acquisition mode with a linear ion trap mass spectrometer (AB Sciex 4000 QTrap) hyphenated to a UPLC (Waters Acquity), equipped with a C18 column (BEH 130 peptide, 1.7 l, 2.1 9 100 mm). A gradient elution programme of 99% eluent A (water containing 0.1% TFA) and 1% eluent B (acetonitrile containing 0.1% TFA) at 0 min to 1% eluent A and 99% eluent B at 33 min was used. Enhanced mass spectra (EMS) and enhanced resolution spectra of the prominent peaks were acquired. Further enhanced product ion (EPI) spectra of the tentative molecular ion (m/z 320 to 430) was acquired and exported to Protein pilot software (ABSciex) to determine the amino acid sequence (Asha *et al*., 2016).

6.3.10 Statistical analysis

One way ANOVA was used to differentiate the antioxidant activities of Pap-SPH 1, Pap-SPH 2 and Pap SPH 3. SAS version 9.3 (SAS Institute, 2012) was used for statistical analysis. Once ANOVA was found to be significant at 5% level ($p<0.05$), Duncan and Tukey test was applied to differentiate the significance.

6.4 Result and discussion

6.4.1 Ultrafiltration of papain digested squid protein hydrolysate

Ultrafiltration is a vital technique applied to fractionate, purify, and concentrate the hydrolyzed peptides in laboratory and on commercial scale (Ghosh and Raja, 2003). It purifies and separates peptides based on their size and shape. Ultrafiltration technique employs selective membranes barriers. They can retard molecules such as peptides that are bigger to pass through the pores of the membrane while allow small fractions of peptides to cross the membrane (Baker *et al*., 1991). So this technique could be widely applied to separate target peptides by choosing appropriate cut off membrane (Korhonen and Pihlanto, 2003). This method is most effective to separate peptides of 2 kDa molecular weight or above and less effective for the separation of peptides below 1 kDa.

Ultrafiltrated peptide fragments obtained from papain digested squid protein hydrolysate are depicted in Table 6.1. Three fractions were collected and based on their molecular weight were named as Pap-SPH 1(>10 kDa), Pap-SPH 2 (10-3 kDa) and Pap-SPH 3 (<3 kDa), respectively. These isolated fractions were stored at $^{-}80\ ^{\circ}C$ for further analysis. Several reports are available for the successful separation of peptides by using ultrafiltration method (Wu *et al.,* 2003; You *et al.,* 2010; Kumar *et al.,* 2011a; Jai-Ganesh *et al.,* 2011; Ranathunga *et al.,* 2006; Kim *et al.,* 2007; Sumaya-Martinez, 2004). Isolation and separation of squid peptides using different molecular weight cutoff (MWCO) was reported by Asha *et al*., (2016). Similar experiment was conducted by Sudhakar and Nazeer (2015). Antioxidant peptides of jumbo squid (*Dosidicus gigas*) were fractioned by using ultrafilteration (Mendis *et al.,* 2005b). All these reports strengthen the authenticity of the methodology

used in the present experiment for the separation of squid peptides based on its molecular weights.

Table 6.1: Fractions obtained after ultrafiltration of papain digested squid protein hydrolysate (Pap-SPH) through molecular weight cut-off membranes filters

MWCO (kDa)	Peptide separated
>10	Pap-SPH 1
10-3	Pap-SPH 2
<3	Pap-SPH 3

6.4.2 Antioxidant properties of ultrafiltered papain digested squid protein hydrolysate

Incidence of increased oxidative stress and associated health problems are common in recent times. Since synthetic antioxidants make complications besides their positive effect exploring of natural compounds which can neutralize the oxidative stress are gaining much attention in health sector. This amplified the research to identify and separate natural antioxidants from marine sector for health improvements are evident. These natural antioxidants can be potential candidates for antioxidant ingredients for food and health products. Antioxidant peptides from marine sources especially, those isolated from mackerel (Wu *et al*., 2003), tilapia (Raghavan *et al* ., 2008) and Allaska Pollack (Je *et al*., 2005) have been reported.

Production of peptides by enzymatic digestion such as trypsin, pepsin, papain, alcalase digestion is the most pronounced and routinely employed method for the production of peptides. In the present study the squid protein was obtained by the digestion of papain at optimized hydrolysis condition. Papain breaks peptide chain at arginine or lysine residue that is subsequent to any of the hydrophobic amino acids. Papain enzyme cleaves the basic amino acids sites and exposes to various biomolecules for bioactivities. Hence,

degree of hydrolysis plays a vital role to determine the type and extent of bioactivities in protein hydrolysates.

In the radical scavenging assays, the radicals are stable by reacting with antioxidants, which produce decolourization and it can be measured by using spectrophotometer. The fractions *ie.,* Pap-SPH 1 (>10 kDa), Pap-SPH 2 (10-3 kDa) and Pap-SPH 3 (<3 kDa) isolated by using ultrafiltration technique were subjected to various antioxidant analyse such as DPPH (%) , superoxide (%), metal chelate (%) and reducing power (a-700nm) activities. All the samples showed remarkable antioxidant properties in all these assays (Table 6.2). There was a significant difference of DPPH, superoxide and metal chelate activities of Pap-SPH 1, Pap SPH 2 and Pap-SPH 3 ($p < 0.05$); whereas a homogenous values were noticed for reducing power ($p > 0.05$). Antioxidant potential of papain derived peptides from oyster protein hydrolysate was reported (Asha *et al*., 2016) and antioxidant potential of squid peptides produced by trypsin, α-chymotrypsin and pepsin were established (Sudhakar and Nazeer, 2015). Antioxidant activities are enhanced with the reduction of molecular weight of peptides which was observed in all the assays. Among the fractions, highest antioxidant properties were noticed in low molecular weight Pap-SPH 3 peptide which was justified by the observations in DPPH (40.33±1.32), superoxide (35.54±1.45), metal chelate (50.86±1.88) and reducing power (0.173±0.98) assays (Table 6.2). This is in well agreement with Picot *et al.,* (2010) and the authors suggested that lower molecular weight protein fragments possess better antioxidant properties. According to Kim *et al.,* (2010) molecular weight and amino acid sequence strongly influence the bioactivities of enzymatically hydrolyzed peptides.

Table 6.2: Antioxidant activities of ultrafiltered papain digested squid protein hydrolysates

Assay	DPPH^{x}*(%)	Superoxide ^{y} *(%)	Metal chelate^{x}** (%)	Reducing power ^{z}* (A-700nm)
Pap-SPH 1	25.23±1.13^{a}	26.35±1.22^{a}	36.27±0.99^{a}	0.119±0.23^{a}
Pap-SPH 2	30.54±1.67^{b}	28.23±1.53^{b}	39.63±0.86^{b}	0.113±0.34^{a}
Pap-SPH 3	40.33±1.32^{c}	35.54±1.45^{c}	50.86±1.88^{c}	0.173±0.98^{a}

Hydrolysate concentration: x= 2mg mL^{-1}; y= 0.5mg mL^{-1} and z= 1.5mg mL^{-1}; STD = *Gallic acid and ** EDTA.

6.4.3 Protective effect of the purified peptide against hydroxyl-radical-induced DNA damage

Effectiveness of antioxidant potential of Pap-SPH 1, Pap-SPH 2 and Pap-SPH 3 was evaluated by protection against hydroxyl-radical-induced DNA damage. Protective action of isolated papain digested peptide fractions against hydroxyl radical-induced DNA damage to pBR322 is shown in Plate 6.1. pBR322 is a super coil form in DNA and when converted to open circular (OC) form indicates DNA damage due to hydroxyl radical action.

Free radicals cause cellular damage by denaturing proteins, lipid and nucleic acids. According to Riley (1994) nucleic acids are vulnerable to oxidative stress which leads to tissue damage. In the present study squid derived peptide fractions prevent DNA damage by protecting pBR322 from H_2O_2. Similarly no evidence of damage of DNA in negative control, might be due to the absence of H_2O_2 Whereas, H_2O_2 treated sample completely damaged pBR322 due to its strong free radical formation. Protection of DNA can be identified by the presence of DNA bands at respective lanes in the gel and vice versa (Plate 6.1). Among Pap-SPH 1, Pap-SPH 2 and Pap-SPH 3 treated samples Pap-SPH 3 revealed better DNA protection property, which is

confirmed by the retaining of DNA band at Pap-SPH 3 lane (*ie.,* lane 6). Several studies are reported for the DNA protection activities of peptides and protein hydrolysates obtained by enzymatic hydrolysis from Nile tilapia (Ngo *et al.,* 2010), yellow and northern whiting fish (Venkatesan and Nazeer, 2014) and stripe trevally (Klompong *et al*., 2009). Sheih *et al*., (2009) found antioxidant properties of peptides from algae protein waste hydrolysate in different oxidation systems. Findings of the present study clearly substantiate these reports and it can be concluded that purified peptide fractions of papain digested squid peptide effectively protected DNA from damage. Hence, these peptides could be a promising antioxidant food supplements to protect body from oxidative stress.

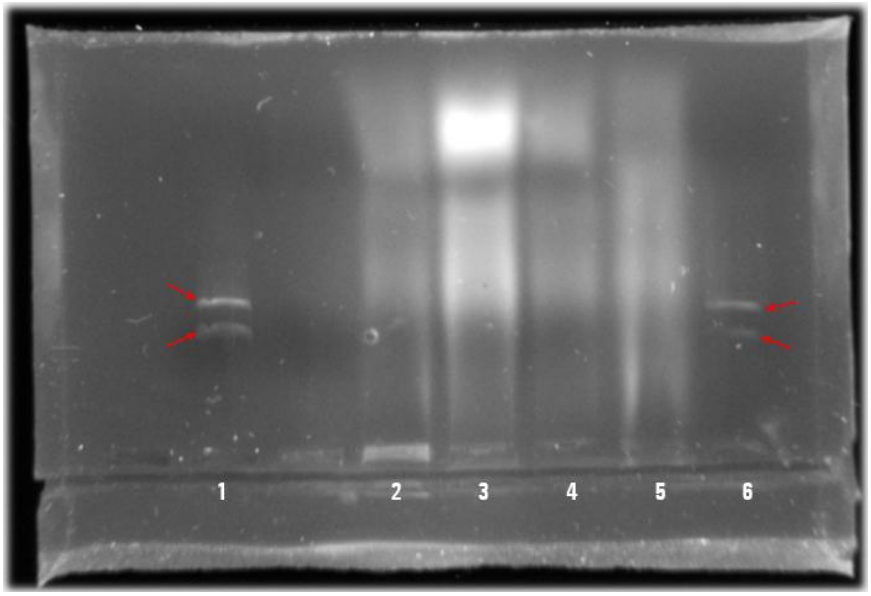

Plate 6.1: Protective effect of the purified peptide against hydroxyl-radical-induced DNA damage

Lane 1: Negative control plasmid pBR322; Lane 2: Positive control plasmid with H_2O_2 treatment; Lane 3: Squid protein hydrolysate treated; Lane 4: Pap-SPH 1; Lane 5: Pap-SPH 2; Lane 6: Pap-SPH 3.

6.4.4 Purification of ultra filtered fraction using RP-HPLC

HPLC method is widely used to separate and characterize peptides and the HPLC spectra of <3 kDa (Pap-SPH 3) fractions are depicted in Figure 6.1. Reverse phase HPLC is known for better separation and characterization of peptides of seafood. Reports are available for the separation of peptides from enzymatic hydrolysates of tuna dark muscle by-products by using reverse phase HPLC (Hsu, 2010). Antioxidant properties of peptides prepared from tuna cooking juice hydrolysates are reported and those peptides were isolated by using gradient solvent system in reverse phase HPLC (Hsu *et al*., 2009). In the present study typical peptide spectrum with several peaks in <3kDa fraction showed good accordance with previous reports and it was found that ultrafiltration methods are capable to generate peptides with low molecular weight. Asha *et al* ., (2016) suggested that low molecular weight peptides have more antioxidant potential due to the presence of exposed hydrophobic amino acids. This is in well agreement with the present experiment and the fractions of peptide mainly <3kDa possess strong antioxidant properties. Chromatogram indicates the efficacy of RP-HPLC for characterization of peptides (Figure 6.1). Peak 1 in the chromatogram is the major fraction which was denoted as Pap-SPH 3-F1 and this fraction was collected for further characterization.

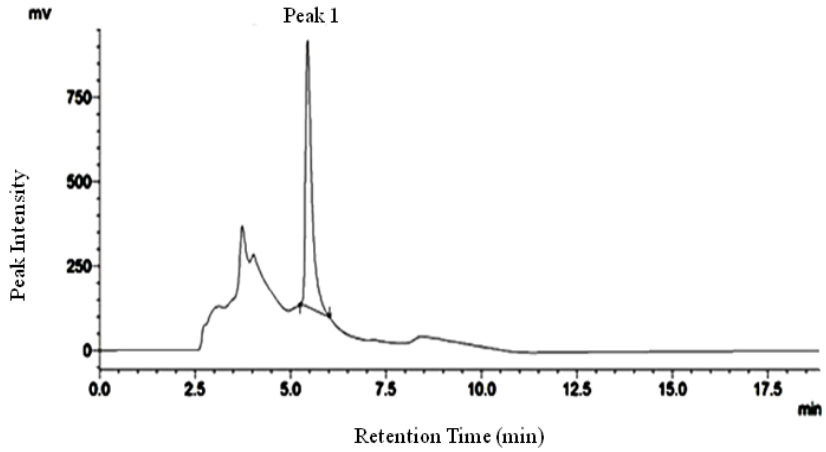

Fig. 6.1: Chromatographic separation of the ultrafiltration fraction 3 obtained from papain digested squid proteinhydrolysate (Pap-SPH 3)

6.4.5 LC-MS/MS analysis of Pap-SPH 3-F1

LC-MS/MS method is the most accurate and reliable method for characterization and sequencing of peptides and is widely employed to characterize and sequence the antioxidant peptides. LC-MS/MS spectrum of Pap-SPH 3-F1 is depicted in Figure 6.2. A rapid and simple LC-MS method using collagen marker peptides for identification of the animal source was reported by Kumazawa *et al*., (2016). They developed LC-MS methodology for characterization of peptides and successfully identified many peptide sequences. Marchis *et al*., (2017) identified muscle peptides in processed animal proteins by using LC-MS/MS and found unique amino acids during sequencing. Amino acid sequence of peptides from grass carp (Ren *et al.*, 2008) and sardine (*Sardinella aurita*) (Bougatef *et al.*, 2010) were characterized by using LC/MS/MS.

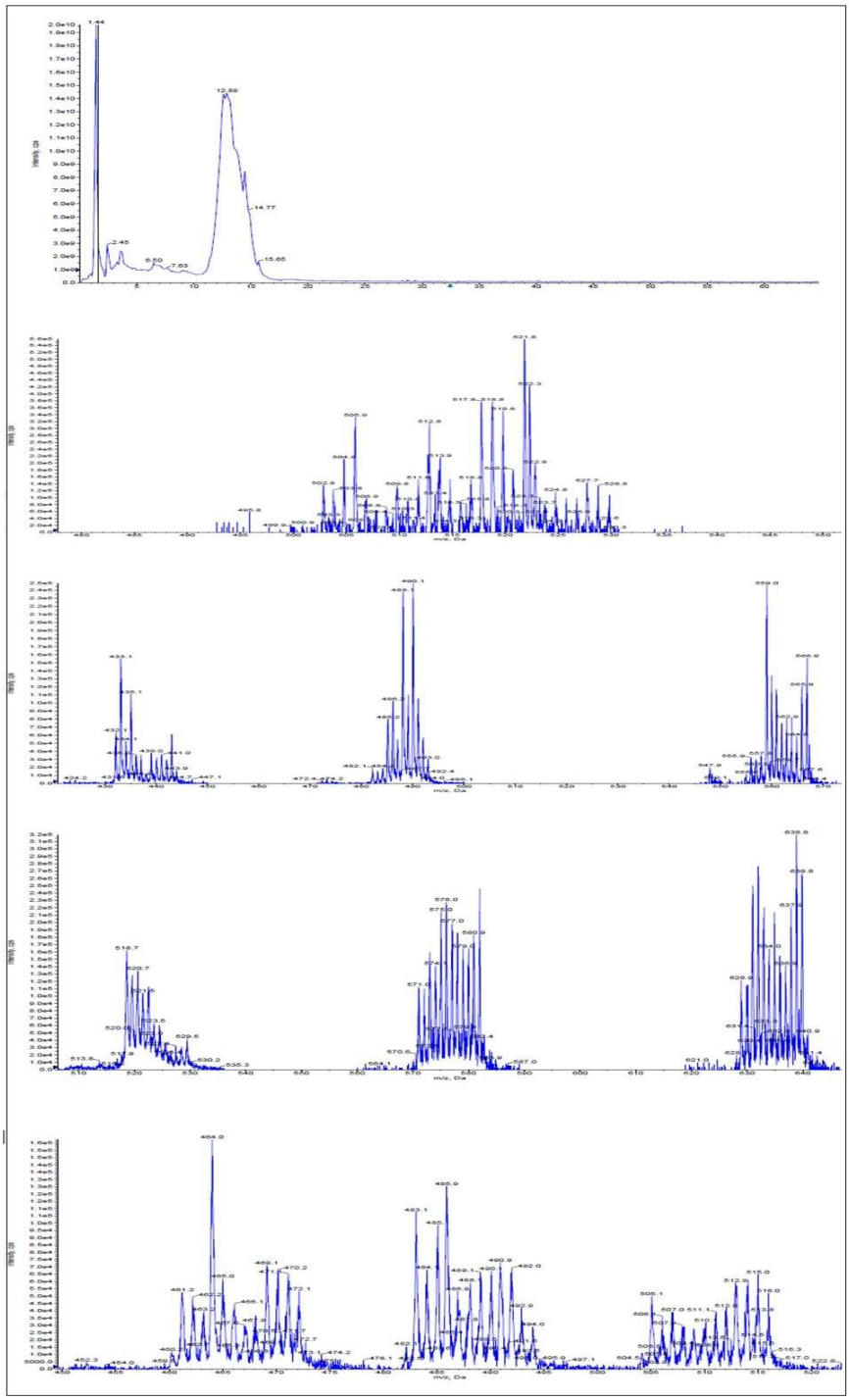

Fig. 6.2: LC-MS/MS analysis of Pap-SPH 3- F1

Several fractions of Pap-SPH 3-F1 peptide were sequenced with the help of LC/MS/MS and its amino acid sequence, theoretical molecular weight (MW), hydrophobicity, mass to charge ratio (m/z) and charge (z) are presented in Table 6.3. The presence of strong antioxidant potent hydrophobic amino acids such as alanine, proline, phenylalanine and valine were noticed in the Pap-SPH 3-F1 peptide. According to Tanaka *et al*., (2016) hydrophobic amino acids like isoleucine and valine and branched chain amino acids (BCAA) in the peptide expressed strong antioxidant property. Ichikawa *et al*., (2012) reported that BCAA activate DNA repair by reducing the surrounding oxidative stress. This substantiates the active role of hydrophobic amino acids to neutralize oxidative stress and thus cell protection. Threonine and serine found in the fraction acted as robust antioxidant molecules by scavenging reactive oxygen species and its antioxidant activity was due to the presence of hydroxyl group (Udenigwe and Aluko, 2011). Serine was cytoprotective and it elevated vital antioxidant mediators such as HO-1 and NO (Maralani *et al*., 2012). Antioxidant property of tyrosine was due to the action of phenol group and it had the capability of neutralizing ROS stress. Researchers demonstrated the relationship between the physicochemical properties of the C-terminal and N-terminal regions of the peptides and ROS neutralizing potential of peptides (Li and Li, 2013). Peptides containing amino acids like phenylalanine and glycine are found to exhibit strong antioxidant activity (Chen *et al*., 1996b; Qian *et al*., 2008a; Rajapakse *et al*., 2005). Aromatic amino acids like trypsin, histidine and phenylalanine residues can stabilize the reactive oxygen species by direct electron transfer. Whereas, amino acids like the glutamate, glycine, aspartic acid and tyrosine act as hydrogen donors thus quenching the unpaired electrons by providing protons (Young *et al*., 2007). Glycine play vital role in antioxidant mechanism as it can act as a direct proton donor which is

important for antioxidant scavenging (Qian *et al*., 2008b). Sulphur containing amino acids namely, methionine is an effective scavenger of H_2O_2, hydroxyl radicals, hypochlorous acid etc. under physiological conditions. Glutamine a basic amino acids was found to act as a strong antioxidant that protected the oxidative stress induced gastric mucosa damage in a rat model of portal hypertension (Marques *et al*., 2013). Arginine is essential for many physiological functions and it accelerated the antioxidant defense system in the experimental rats (Shan *et al*., 2013). It also has anti-atherogenic property (Wang *et al.*, 2008). Wallner *et al*., (2001) emphasized the role of proline, leucine, alanine, tyrosine and L-arginine as potential antioxidant nature. Similarly arginine, porline and hydroxylproline also exhibit potent ROS scavenging properties. Ahn *et al*., (2012) reported radical-scavenging properties of peptides of salmon. Hydrophobic amino acids like valinine and leucine accelerate the reaction between peptide and fatty acids and thus scavenge ROS generated at the lipid phase (Ranathunga *et al.*, 2006).

Table 6.3: Sequence of LC-MS/MS characterized Pap-SPH 3-F1

Peptide Sequence	Theoretical MW[1]	Hydrophobicity[2] (%)	m/z	z
KSAEPTVFVKT	1206.41	45.45	326.5221	3
KKENQDLNKLKI	1470.71	25.00	424.57	3
RAVISPFRR	1101.33	55.56	395.23	2
RIAVFPSRM	1076.34	66.67	395.23	2
RDLTDYLMKI	1267.52	40.00	499.74	2
RLDLAGRDLTDYLMKI	1893.25	43.75	541.95	3
KSYELPDGQVITIGNERF	2066.31	33.33	895.94	2
RIEELEEEIEAERA	1715.87	35.71	744.85	2
KAEGLTKEQQEDAKR	1730.91	20.00	744.88	2
KEITSLAPSTMKI	1418.73	46.15	589.30	2

[1]https://www.biopeptide.com/PepCalc/Calculate
[2]https://www.peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php

6.5 Conclusion

In the present experiment, papain digested squid protein hydrolysate was ultrafiltered based on the molecular weight and three fractions were obtained which were, Pap-SPH 1(>10 kDa), Pap-SPH 2 (10-3 kDa) and Pap-SPH 3 (<3 kDa), respectively. All these fractions revealed strong antioxidation reactions such as DPPH, superoxide, metal chelate and reducing power and also protected pBR322 of DNA from hydroxyl-radical; however, among the fractions, low molecular weight fraction *i.e.,* Pap-SPH 3 showed maximum activities. Pap-SPH 3 fraction was further separated by using RP-HPLC and it was found that Pap-SPH 3-F1 was the largest peak in the chromatogram, which was further characterized by using LC/MS/MS. Several antioxidant potent amino acids especially alanine, proline, phenylalanine and valine were observed in the sequence and excellent hydrophobicity of the fractions were also established. Hence, it could be utilized for antioxidant food supplements against oxidative stress.

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Chapter 7

In-Vivo Anti-Ulcer and Anti-Inflammation Studies of Papain Digested Squid Antioxidant Peptide in Male Albino Rats (*Wistar strain*)

Contents

7.1 Introduction

Peptic ulcer disease is the damage in the inner layer of stomach and beginning portion of the small intestine which causes severe distress to gastric intestinal (G.I) tract. Available treatments for curing peptic ulcer are the radiation therapy and chemotherapy for which the recovery rate is comparatively less. An effective therapy is yet to be discovered to treat peptic ulcer (Luck, 1998). One of the major reasons for the genesis of this disease is the involvement of reactive oxygen species (ROS). ROS is produced by the body to prevent and kill the invaded pathogens. The anti-oxidant system in the body comprises several antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and gluthathione peroxidase (GPX) (Fridovich, 1975). Abnormal accumulation of ROS due to imbalance in the activity of these enzymes leads to several complications to the body. These accumulated ROS causes cellular destruction due to lipid peroxidation, DNA damage, protein damage etc. (Fridovich, 1975). Moreover, excessive oxidative stress cause gastric ulceration by accelerating mucosal tissue damage, erosion and stomach

ulceration. Decreased enzyme activities were reported to trigger stomach lining damage and finally lead to peptic ulcer (Firton and Wiseman, 1996). Anti-oxidant compounds are known to reduce ROS damage by neutralizing their potential and protect cell. Compounds which can neutralize ROS by direct interaction or by indirect activation of anti-oxidant enzymes are gaining attention today to reduce ulcerative symptoms.

Inflammation is a kind of disease occurred in the body due to combination of physical, chemical and biological processes (Nathan, 2002). Major symptoms of inflammation include redness, swelling, heat, pain, and loss of function at affected area. Inflammation produces several physiological complications especially pain. So drugs such as analgesics are commonly used to reduce the pain developed during inflammation. Chemotherapy by using analgesic drugs creates many side effects apart from its beneficial action (Deciga-Campos *et al*., 2005). Hence, compounds which reduce inflammatory symptoms with no or fewer side effects are highly preferred. Natural compounds like peptides produced from food are gaining immense importance because of their beneficiary action without side effects (Erdmann *et al*., 2008: Sousa *et al*., 2012). Compounds having the ability to alleviate ROS are found to be effective to reduce inflammation (Mittal *et al.*, 2014). Many peptides of marine and terrestrial origin are found to exhibit anti-oxidant activity and seldom reports suggest the anti-inflammatory role of marine peptides especially from that of squid origin.

7.2 Objective of the study

Objective of the experiment was to investigate anti-inflammatory and anti-ulcer properties by using in-*vivo* male Albino rat model (*Wistar* strain) for the peptides synthesized from Indian squid

7.3 Materials and methods

7.3.1 Ultrafiltered Pap-SPH 3 fraction

Antiulcer and antiinflammation properties of Pap-SPH 3 fraction of Indian squid (*U. duvauceli*) was evaluated. Aliquot of Pap-SPH 3 was (procedure was detailed in Chapter 6, Section 6.3.5) orally fed to the animals (*Wistar* strain male Albino rats) after evaluating their lethal dosages.

7.3.2 Chemicals

All chemical reagents used in the experiments were purchased from Merck (Darmstadt, Germany). Ibuprofen and ranidine from Sigma-Aldrich Chemical Inc. (St. Louis, MO) were the standard reference drugs used for the animal experiments.

7.3.3 Experimental animals

Wistar strain male Albino rats (120–200 g) were used in the experiments (Plate 7.1). They were housed individually in polypropylene cages under hygienic conditions and were provided food and water ad libitum. The animals were maintained on a 12:12 hrs light: dark photoperiod under standard conditions of temperature and ventilation. The experiments were performed as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India, and with the approval of the Institutional Animal Ethics Committee.

Plate 7.1: *Wistar* strain male Albino rats

7.3.4 Experimental protocol

Anti-inflammatory and anti-ulcer activities of the squid peptides were assessed after checking their lethal dosages in rats animal models. Anti-inflammatory activity was determined using the formalin-induced rat paw oedema test.

Acute toxicity of the squid peptides was carried out on male *Wistar* Albino rats using Karber's arithmetical method for the determination of LD50 (lethal dose that causes mortality by 50%) (Turner, 1965). In this assay increasing doses of the test substance was administered orally to groups of four animals at different doses (0.5-5.0 kg kg^{-1}).

The animals were observed for one week, the number of survivors was counted and the optimum average dosage was determined.

$$LD50=\text{Maximum dose} - xy/n$$

Where, "maximal dose" is the dose at which 100% mortality occurred, "x" is the dose administered (mg kg^{-1} body weight) and "y" is the mean mortality rate.

7.3.5 Anti-inflammatory activity

The anti-inflammatory activity was determined by the modified method of Hunskaar and Hole (1987), using the formalin-induced rat paw oedema test. male *Wistar* Albino rats weighing between 120-180 g were divided into four groups of 6 animals each. The papain digested squid peptide (PAP-SPH3) was administered orally at a dose of 0.5 g kg^{-1} body weight prior to the induction of inflammation by the subcutaneous injection of 0.1 mL sterile saline solution of 3.5% formalin in the right hind paw. The control group received sterile saline solution (1 mL 0.9% NaCl solution) while the reference drug ibuprofen

at a dose of 100 mg kg^{-1} bodyweight was administered to the standard group intraperitoneally at least 30 min before the induction of oedema. Paw sizes were measured with a calibrated screw gauge before the administration of formalin, then thereafter at 1, 2, and 3 hrs after the injection of the inflammatory agent. The average size of the paw measured in millimeters was calculated from 3 measurements which did not differ from more than 1%. These individual measurements allowed us to determine the average paw size for each group (S_m) and then the percentage of oedema by comparison with the average size was obtained for each group before any treatment (S_o).

Percentage of inflammation-inhibition was obtained for each group using the following calculation:

$$\frac{[(S_m - S_0)\text{Control} - (S_m - S_0)\text{treated}]}{(S_m - S_0)\ \text{Control}} \times 100$$

Where, "S_m" is the mean paw size for each group after formalin treatment and "S_o" is the mean paw size obtained for each group before the treatment (Owolabi and Omogbai, 2007).

7.3.6 Anti-ulcer studies

Male and female *Wistar* rats (180-200 g) kept in standard laboratory conditions, were fasted overnight in single wire net floor cages with free access to tap water and were randomly assigned to four groups of six animals. Group I, served as the normal control, received oral administration of distilled water; group II served as positive control; group III as standard ranitidine (30 mg kg^{-1} of body weight group); papain digested squid peptide (PAP-SPH3) at a concentration of 0.5 g kg^{-1} body weight was administrated to group IV. Groups II to IV were induced with ulcer. All animals were fasted overnight before the induction of ulcer. Hydrochloric acid and ethanol (0.6% v/v) was

used as ulcerogenic agent which was administered intraperitoneally at a dose of 2.0 mL kg^{-1} body weight. After 4 hrs, all animal groups underwent surgery as per the procedure of Takeuchi *et al*., (1976) and gastric juice was collected. Rats were then sacrificed with overdose of chloroform and the stomach was removed after the esophagus had been clamped. The gastric juice was centrifuged and the volume was noted and samples were kept for further analysis. The stomach was inflated with normal saline and then incised through the greater curvature, examined for the number of lesions and stored for histopathological observation.

7.3.7 Histological observation

Histology of all the organs was conducted by the method of Galigher and Kozloff (1971). After sacrificing the rats by over dose of chloroform, tissues (stomach) were collected, washed in normal saline and fixed by using fixative (30% formaldehyde) for 24 hrs and dehydrated in a graded acetone series (50–70–90–100–100–100)% (v/v); 10 min $step^{-1}$. The samples were infused in with alcohol for complete dehydration. Then tissues were cleaned and embedded in paraffin (melting point 58–60 °C), were cut into ultra-thin sections (3–5 µm) by ultramicrotome and stained with the hematoxylin–eosin dye and finally, observed under a photomicroscope and morphological changes were observed.

7.3.8 Determination of pH

An aliquot of 1mL gastric juice was diluted with 1 mL of distilled water and pH of the solution was measured using pH meter.

7.3.9 Determination of total acidity

An aliquot of 1 mL gastric juice was diluted with 1 mLof distilled water into a 50 mL conical flask and added two drops of phenolphthalein indicator then titrated with 0.01N NaOH until a permanent pink colour was observed. The volume of 0.01N NaOH consumed was noted. The total acidity is expressed as mEq L^{-1} by the following equation.

$$\text{Acidity} = \frac{(\text{Vol. of NaOH} \times \text{N} \times 100)}{0.1\ \text{mEq/L}}$$

7.3.10 Macroscopic evaluation of stomach

The stomach were opened along the greater curvature, rinsed with saline to remove gastric contents and blood clots and examined by a 10 X magnifier lens to assess the formation of ulcers. The numbers of ulcers were counted. Scoring of ulcer was made as follows:

Normal colored stomach	0
Red coloration	0.5
Spot ulcer	1.0
Hemorrhagic streak	1.5
Deep Ulcers	2
Perforation	2.5

Mean ulcer score for each animal were expressed as ulcer index (UI). The percentage of ulcer protection was determined as follows:

Ulcer index was measured by using following formula:

$$\text{UI} = \text{UN} \times \text{US} + \text{UP} \times 10^{-1}$$

Where,

UI= Ulcer Index

UN = Average number of ulcers per animal

US = Average number of severity score

UP = Percentage of animals with ulcers

Percentage inhibition of ulceration was calculated as below:

$$\% \text{ Inhibition of Ulceration} = \frac{[(\text{Ulcer index of control} - \text{Ulcer index of test})}{\text{Ulcer index of control}} \times 100$$

7.3.11 Preparation of subcellular fractions of stomachs

Tissue samples from the stomach of *Wistar* Albino rats exposed to HCl and ethanol were taken for the biochemical assays. The ulceration or lesion part were weighed and homogenized with 200 mM potassium phosphate buffer (pH 6.5). Reduction of glutathione (GSH) levels in the homogenate was estimated and then centrifuged at 11,000 rpm for 20 min at 4 °C. Superoxide dismutase (SOD) and catalase (CAT) activity were established from the supernatant.

7.3.12 Determination of reduced glutathione (GSH) levels

GSH content in gastric mucosa was estimated by using the methodology described by Sedlak and Lindsay (1968). Tissue homogenate was mixed with trichloroacetic acid (12.5 %) and centrifuged at 4000 rpm for 15 min at 4 °C. Supernatant was mixed with Tris buffer (0.4 M, pH 8.9) and 5, 5′-dithiobis 2-nitrobenzoic acid (DTNB, 0.01 M) and the absorbance of the solution were measured at 420 nm. The GSH level was expressed as µg GSH g^{-1} of tissue.

7.3.13 Determination of superoxide dismutase (SOD) activity

The activity of SOD of tissue homogenate was estimated as per Marklund and Marklund (1974) method. Aliquots of tissue homogenate were mixed with Pyrogallol (1 mM) and buffer solution (Tris HCl 1 mM – EDTA 5 mM, pH 8.5) and kept for incubation for 20 min. The reaction was stopped by additing 1 N HCl and centrifuged at 14,000 rpm for 4 min. The absorbance of the supernatant was measured at 405 nm. The amount of SOD that inhibited the oxidation of pyrogallol by 50%, relative to the control, was defined as one unit of SOD activity. The enzymatic activity was expressed as U mg^{-1} of protein.

7.3.14 Determination of catalase (CAT) activity

Catalase activity was determined by using the procedure of Aebi (1984). Aliquots of supernatant samples were mixed with a solution containing 30% H_2O_2, milli-Q water and buffer (5 mM Tris EDTA, pH 8.0). The absorbance was measured at 240 nm for 60 sec by using spectrophotometer. The enzymatic activity was expressed as mmol min^{-1} mg^{-1} of protein.

7.3.15 Statistical analysis

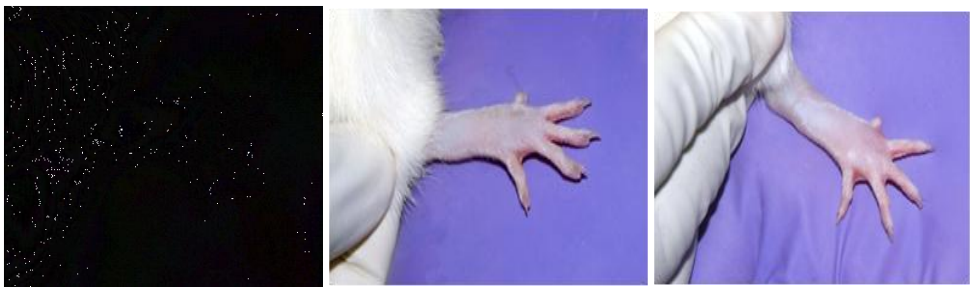
One way ANOVA was used to differentiate the antioxidant enzymes (ie., SOD, CAT and GSH) in Ethanol/HCl induced groups, control, ranitidine and PAP-SPH 3 treated samples. SAS version 9.3 (SAS Institute, 2012) was used for statistical analysis. Once ANOVA was found to be significant at 5% level ($p<0.05$), Duncan and Tukey test was applied to differentiate the significance.

7.4 Result and discussion

7.4.1 Anti-inflammatory properties of PAP-SPH 3

Formalin-induced paw edema is an ideal model to study the anti-inflammatory effect of compounds in rat model to assess the anti-inflammatory effect of peptides derived from Indian squid. Plate 7.2 and Figure 7.1 depicts the inflammation at hind paw of male *Wistar* Albino rats after a definite time interval *ie.,* 1 to 3 hrs. Paw edema was clearly decreased in PAP-SPH 3 treated sample compared to positive group and similar observation was noticed in the ibuprofen (standard drug) treated group. Oxidative stress play vital role in the inflammation process and usually the compounds which neutralize the ROS are found to reduce inflammatory symptoms like edema, redness and pain (Viitala and Newhouse, 2004). In the present study, significant anti-oxidant properties of peptides derived from Indian squid might be the reason for the reduction of inflammation. Percentage of inhibition of paw edema is depicted in Plate 7.2 and it was clearly evident that PAP-SPH3 significantly inhibited the paw edema compared to positive group. Ganesh *et al.,* (2008), Xu *et al.,* (2012) and Beck *et al.,* (2015) reported the anti-inflammatory effect of peptides, which is in well agreement with the present study. Hence, it can be substantiated that enzyme hydrolyzed Indian squid peptides are having the anti-inflammatory properties. Many marine derived peptides and hydrolysates are found to show anti-inflammatory activities. Anti-inflammatory properties were reported in the peptides synthesized from green sea urchin and protein hydrolysates from salmon (Björn *et al.,* 2012; Pilon *et al.,* 2011). Researchers reported the role of amino acids especially glycine and tryptophan for the reduction of inflammatory symptoms (Den-Hartog *et al.,* 2013; Zhang *et al.,* 2010; Kim *et al.,* 2010; Hasegawa *et al.,* 2011). However, Roberts *et al.,* (1999) found that those low molecular weight peptides were more effective to resist inflammatory

symptoms. This is in well agreement with the present experiment. Inflammation is closely associated with the risk of arthritis (Greenwald, 1991) and any compounds which reduce the inflammation also have the potential to reduce the risk of arthritis. Hence the peptides synthesized in the present study could be effectively utilized to produce anti-arthritic nutraceuticals. These findings point out the significance of marine derived peptides in nutraceutical field to fight against severe diseases.



POSITIVE **IBUPROFEN** **PAP-SPH 3**

Plate 7.2: Anti-inflammatory effect of squid peptide (PAP-SPH 3) in *Wistar* Albino rats against formalin induced paw edema

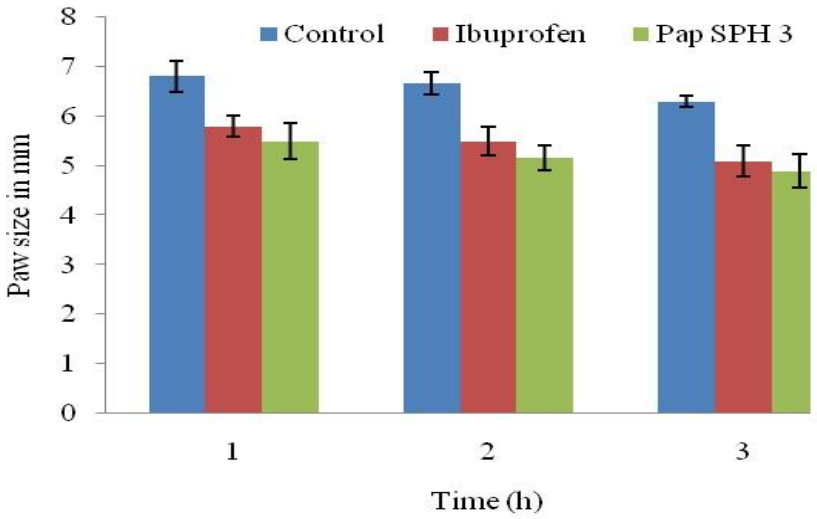


Fig. 7.1: Anti-inflammation effect of squid peptide (PAP-SPH 3)

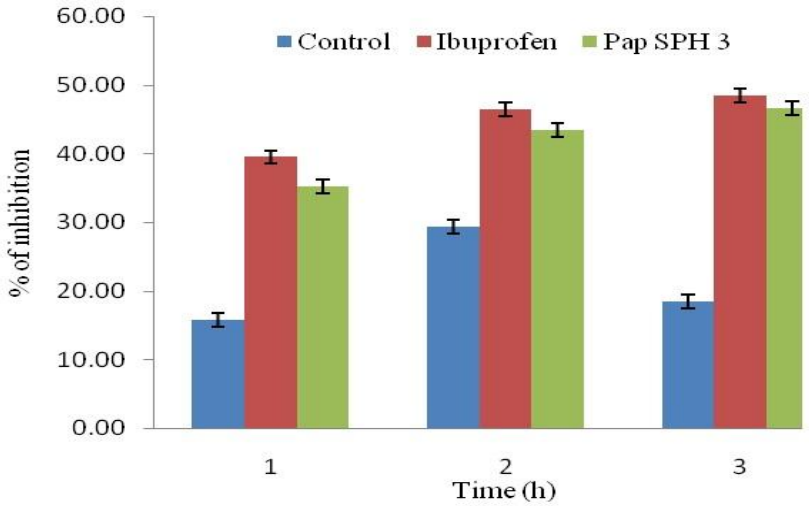


Fig. 7.2: Anti-inflammatory activity of Pap SPH 3 peptides from Indian squid on formalin induced paw edema in rat. Value represents the mean of six animals± SD

7.4.2 Anti-ulcer properties of PAP-SPH 3

Anti-ulcer activity of the squid peptide in male *Wistar* Albino rats is depicted in Table 7.1. After experimental period, animal groups namely, normal control, control (positive), ranitidine and squid peptide (PAP-SPH 3) were euthanized and stomach sections of each animal was separated and analyzed. Ulcer lesions of the stomach sections of the experimental animals of anti-ulcer activity of squid peptides in male *Wistar* Albino rats were presented in Plate 7.3. Ulcer lesions were not observed in normal control group; whereas, definite lesions and ulcer symptoms were noticed in positive group. Ulcer lesions were minimum in standard drug ranitidine treated group and reduction of ulcer lesions was clearly evident in PAP-SPH 3 treated group. Hence, the observations in PAP-SPH 3 treated group were comparable with ranitidine (standard drug) treated group. Ulcer protection indicators like percentage of ulcer inhibition, gastric volume (mL $100g^{-1}$ of body wt.), gastric pH and total acidity mEq L^{-1} were significantly reduced in PAP-SPH 3 treated

group which also exhibited striking similarities with ranitidine (standard drug) treated group. All these findings establish the anti-ulcer properties of PAP-SPH 3.

Table 7.1: Anti-ulcer activity results of the squid peptide in male *Wistar* Albino rats

Group treatment	Dosage (mg kg^{-1} rat)	Ulcer score	Ulcer index	% of Ulcer Inhibition	Gastric volume (mL $100g^{-1}$ of body wt.)	Gastric pH	Total acidity mEq L^{-1}
Normal Control	-	-	-	-	2.12±0.2	4.2	44.32±0.2
Ethanol/HCl induced (positive)	-	2.0	14.00±1.0	-	5.5±0.3	2.0	74.77±0.3
Ranitidine	20	0.5	2.00±1.0	81.54±1.0	1.5±0.1	5.0	30.25±0.5
PAP-SPH 3	500	0.5	4.00±0.1	72.73±1.5	3.2±0.2	4.3	48.03±0.8

Ethanol/HCl induced Ranitidine PAP-SPH 3

Plate 7.3: Ulcer lesions of the stomach section of the experimental animal of anti-ulcer activity of squid peptides in male *Wistar* Albino rats

Formation of reactive oxygen species is one of the major reasons behind the progression and severity of peptic ulcer. ROS facilitate the damage of mucosal membrane lipids and protein and leads to the erosion of mucosal membrane and facilitate the loss of membrane integrity (Brzozowski *et al.*, 1997; Mizui and Doteuchi, 1983). Anti-oxidant defense system plays crucial role to protect gastric mucosa to make it intact and functional by neutralizing free radicals. Loss of activity of anti-oxidant defense causes the progression of ulcer and compounds that can neutralize the free radicals are found to prevent the formation of ulcer (Bandyopadhyay and Chattopadhyay, 2006). SOD, GSH and CAT were most important anti-oxidant compounds which protect the

gastric mucosal membrane (Kwiecień *et al.,* 2002). Ethanol/HCl induced group showed a significantly different value for SOD, GSH and CAT compared to control, ranitidine and PAP-SPH 3 samples ($p < 0.05$) (Table 7.2). The main antioxidant present in the tissue is GSH, a tripeptide (L-γ-glutamyl-L-cysteinyl glycine), sulfhydryl group of the GSH tripeptide play vital role in the anti-oxidant activity by reduction and oxidation processes (Rozza and Pellizzon, 2013).

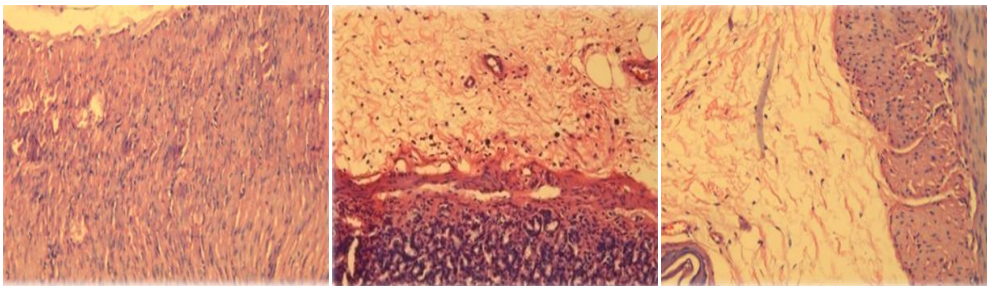
Table 7.2: Anti-oxidant defense system levels in experimental *Wistar* Albino rats

Group treatment	SOD (U mg^{-1} of protein)	CAT ($mmol^{-1}min^{-1}mg$) of protein	GSH (µg g^{-1} of tissue)
Normal Control	1784 ± 104^{a}	3118 ± 28^{a}	591 ± 34^{a}
Ethanol/HCl induced	295 ± 98^{b}	245 ± 82^{b}	299 ± 48^{b}
Ranitidine	1564 ± 52^{bc}	2984 ± 69^{bc}	575 ± 25^{bc}
PAP-SPH 3	1448 ± 112^{c}	2857 ± 55^{c}	484 ± 20^{c}

Histopathology (H & E staining) section of the stomach of anti-ulcer activity of PAP-SPH 3 treated male *Wistar* Albino rats is depicted in Plate 7.4 and it was clearly evident that there was less progression and severity of ulcer by regeneration of mucosal membrane, less blood vessel damage and inflammation by neutrophil infiltration in the PAP-SPH 3 treated group and standard drug ranitidine treated groups. During peptic ulcer, infiltration of neutrophils occurs at the site of mucosal membrane which induces severe inflammatory responses, mucosal lesion formation and blood vessel damage (Al-Wajeeh *et al.,* 2016). Enhancement of overall anti-oxidant defence system was noticed which will agree with the reduction in ulcer symptoms of the stomach section, reduction of neutrophil infiltartion and blood vessel damage in PAP SPH 3 treated samples and ranitidine treated samples and these findings indicates the effectiveness of squid peptide to prevent ulcer progression and better recovery.

Ethanol administration induces oxidative stress through a series of pathways resulting in the generation of reactive oxygen species (Manzo-Avalos and Saavedra-Molina, 2010). Superoxide dismutase (SOD) is a major anti-oxidant enzyme which converts the reactive superoxide radicals to H_2O_2, which destroy gastric mucosa and if not neutralized by catalase (CAT), it will cause lipid peroxidation (Lubrano and Balzan, 2015). CAT is mainly present in peroxisomes, catalyzes the conversion of harmful H_2O_2 into non-harmful water and molecular oxygen and thus protects gastric mucosal membrane (Kirkman *et al.*, 1999). Joint action of SOD, CAT and glutathione (GSH) contribute to the protection of gastric mucosal layer by diminishing the membrane damage caused by free radicals and thus protecting the stomach from peptic ulcer. Compared to ranitidine treated groups, PAP-SPH 3 also showed an increasing trend in the CAT, SOD and GSH levels, which indicates the protective action of squid peptide against ethanol induced peptic ulcer. Ethanol-induced gastric ulcer model is one of the ideal models to study the symptoms and changes that occur during gastric ulcer. Ethanol induces erosion of mucus membrane, impaire mucosal permeability and causes increased release of hydrogen ions which results in pH change (Kauffman, 1985). Peptic ulcer is characterized by the blood vessel damage of the mucosal membrane and produces several ulcer lesions and this ulcer symptom was accelerated by altered pH changes which ultimately lead to stomach discomfort and digestion issues (Brzozowski *et al.,* 1997). Decreased ulcer lesions and increased ulcer inhibition is widely used to assess the anti-ulcer activity of compounds of interest. Decreased ulcer lesions and recovered mucosal membrane were observed in the stomach section analysis. Protection of the mucosal membrane might be due to the activation of anti-oxidant defense system by the action of squid peptides. In the present study, increased ulcer inhibition, decreased

ulcer lesions, decreased gastic volume and increased pH was observed in PAP-SPH 3 treated groups which indicate better protection ability of squid peptides against ethanol induced gastric ulcer. There is lack of report for the anti-ulcer activity of peptides; however, anti-ulcer activity of orange (*Citrus sinensis* L.) peel aqueous extract and hesperidin on oxidative stress and peptic ulcer induced by alcohol in rat was studied by Selmi *et al.,* (2017) and the observations of that experiment was in well agreement with present study. Hence, squid peptides can be used for the development of health formulation for the peptic ulcer patients.



Positive control **Ranitidine** **PAP-SPH 3**

Plate 7.4: Histopathology section of the stomach of anti-ulcer activity of squid peptides in male *Wistar* Albino rats

7.5 Conclusion

Life style diseases such as ulcer and inflammation are increasingly reporting day by day. Nowadays, several synthetic drugs are available to treat such diseases but are not free from side effects. Major reason behind the formation of these diseases is associated with loss of balance of reactive oxygen species. This creates big risk in the body and leads to the formation of several other complications and leads to loss of health. Acute ulcer and inflammation gradually leads to the occurrence of more dangerous diseases such as cancer, arthritis etc and it makes a big concern all over the world. Main reason behind

the development of ulcer and inflammation is associated with food which lacks anti-oxidant molecules. Hence, nutraceuticals which act as both food and functional anti-oxidant molecules have better role to prevent these diseases. Observations of the present study revealed the anti-inflamation and anti-ulcer properties of the PAP-SPH 3 peptides synthesized from Indian squid and this property might be due to neutralizing the excess free radicals through the activation of anti-oxidant defense system. Findings of the experiment emphasize the role of squid peptides as an effective nutraceutical against ulcer and inflammation. Hence, peptides developed from Indian squid could be used as a nutraceutical agent against diseases like ulcer and inflammation.

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Chapter 8

Anticancer Properties of Indian Squid (*Uroteuthis duvauceli*) Peptide against Cervical Cancer Cell Line (HeLa)

Contents

8.1 Introduction

Apoptosis is the one of the most vital mechanisms for controlling cell death, it also termed as programmed cell death, which has a series of events namely, condensation of nuclear chromatin, plasma membrane breakdown, nucleases activation, DNA fragmentation and breakdown of the cells to form apoptotic bodies (Kidd, 1998). Apoptosis has been known to promote an important role in maintenance of tissue homeostasis through selective killing and removal of unwanted cells (Nayfield *et al*., 1991). Inducing apoptosis to kill cancer cells is an important concept of cancer prevention research.

Globally, cervical cancer is reported as second leading female tumor mortality (Chaturvedi *et al*., 2007). Surgery is the preferred method for removal of tumor in the beginning stage of cancer, whereas chemotherapy and radiotherapy are found to be much effective in advanced stages. However, nowadays these therapies have fewer acceptances because of many side effects such as cardiac and neurological toxicity (Florea and Büsselberg, 2011; Monsuez *et al*., 2010). In this scenario, development of effective anticancer

therapies with high specificity to cancer cells and less toxicity to normal cells has high relevance (Kang *et al*., 2012). Hence, investigation to find out effective anticancer therapies to restrict this fatal disease is highly indispensable.

Several peptides are proven to exhibit better immunogenicity and less toxicity (Lee *et al*., 2010) which enables them to be a good option as anticancer compounds. According to Hoskin and Ramamoorthy (2008) and Dennison *et al.,* (2006) numerous natural and synthetic peptides have been established to possess rapid cytotoxic activity against cancer cells in both wide range and specific manner. Hence, peptides have high specificity and efficacy against tumor tissue and have the capacity to protect normal cell. So it could be effectively utilized for the cancer treatment in order to minimize the consequences of chemotherapy and radiotherapy.

Squids are widely accepted seafood, which contains good source of nutritional components especially proteins (Torrinha *et al*., 2014; Bano *et al*., 1992). *U. duvaucelii* is recognized as a major squid species which possess significant nutritional and market acceptance (Sikorski and Kołodziejska, 1986).

8.2 Objective of the study

Objective of this research is to establish the anticancer properties of protein hydrolysate and peptides derived from Indian squid (*U. duvaucelii*) by enzymatic digestion. The cell line used for the experiment was HeLa (human epithelial cervix carcinoma) to investigate the anti-cancer effect of squid peptide and whether it is due to apoptosis or necrosis.

8.3 Materials and methods

8.3.1 Preparation of squid protein hydrolysate and ultrafiltered fractions

Anticancer properties of squid protein samples (*i.e.,* squid protein hydrolysate (SPH), Pap-SPH 1, Pap-SPH 2 and Pap-SPH 3) were analysed in HeLa cell lines . The procedure for the synthesis of squid protein samples was elaborated in Chapter 6, Section 6.3.4 and 6.3.5.

8.3.2 Apparatus and Chemicals

Equipments viz Confocal microscope, phase contrast microscope, BD FACS, multimode reader, agarose gel electrophoresis apparatus, refrigerated centrifuge, incubator, annexin V – apoptosis kit (cayman) and cell cycle analysis kit, Hoechst stain, MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], dulbbeco's modified eagle's medium (DMEM), trypsin, fetal bovine serum (FBS), ethidium bromide, agarose (Sigma- Aldrich), dimethyl sulphoxide (DMSO), hydrogen peroxide were the chemicals used for the experiments.

8.3.3 Cell culture and treatment

HeLa cells were obtained from National Centre for Cell Science (NCCS), Pune and were cultured in DMEM supplemented with 10% fetal bovine serum (GibcoBRL) and 2% antibiotic-antimycotic (GibcoBRL) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were seeded at appropriate density on plate for each analysis. After cells attachment, it was incubated at 37 °C with different concentrations of squid peptide hydrolysate. Initial experiment was to investigate the anticancer properties of squid protein hydrolysate (SPH), trypsin digested protein (TDP), Pap-SPH 1, Pap-SPH 2 and Pap-SPH 3 and based on the IC_{50} values of antiproliferative activity the most effective three samples were selected for further analysis. Untreated

cells served as negative control. The positive control of apoptosis was induced with 500µM H_2O_2 (Sigma) in the medium for 10 min.

8.3.4 Cell viability assay and cell morphology

Cell proliferation was determined by MTT assay (Mosmann, 1983). HeLa cells were trypsinized and seeded on 96 well plate. After 80% confluency, different concentration (*ie.*, 1 µg mL^{-1}, 10 µg mL^{-1}, 100 µg mL^{-1}, 250 µg mL, 500 µg mL^{-1}) of peptides (*ie.*, Pap-SPH 1, Pap-SPH 2, and Pap-SPH 3) were treated and incubated at 37 °C for 24 hrs. Sample free media was used for negative control. After 24 hrs incubation, 100µl MTT reagent (50µg $well^{-1}$) was added and incubated for 4 hrs in dark. After incubation, the reagents was removed and 200µl DMSO was added to all wells, covered with aluminium foil and agitated cells on shaker for 45 min. After shaking read absorbance at 570 nm using multimode reader. Each assay was carried out three times, and the results were expressed as mean value.

$$\text{Percentage viability} = \frac{\text{OD of test}}{\text{OD of control}} \times 100$$

8.3.5 Nuclear staining with Hoechst 33342(chromatin condensation)

The nuclear morphology of the cells was evaluated using the cell-permeable DNA dye, Hoechst 33342 presence of chromatin condensation or fragmentation was indicative of apoptosis. The HeLa cells were seeded in 24-well plates at a concentration of 1.0×10^5 cells mL^{-1}. After cells attachment, the cells were treated with various concentrations of the peptides samples (Pap-SPH 1, Pap-SPH 2, and Pap-SPH 3) and further incubated for 12 h at 37 °C in a humidified atmosphere. Then Hoechst 33342, a DNA-specific fluorescent dye, was added at a final concentration of 10 µg mL^{-1}, followed by 10 min of incubation at 37 °C. The stained cells were then observed under a confocal fluorescence microscope to examine the degree of nuclear condensation.

8.3.6 DNA fragmentation assay

Chromosomal DNA fragments released from nuclei were measured using DNA fragmentation assay. DNA extraction and electrophoresis were performed. Briefly, 2.5×10^6 HeLa cells were treated with different concentrations of squid peptides (*ie.*, Pap-SPH 1, Pap-SPH 2, and Pap-SPH 3) for 24 hrs at 37 °C. Cells were then pelleted and lysed in 0.5mL lysis buffer (10 mM Tris-HCl, pH 8.0, 20 mM EDTA, and 0.2% Triton X-100) and incubated at 37 °C for 60 min. After centrifugation to remove nuclei, chromosomal DNA in the supernatant (including both high molecular weight DNA and nucleosomal DNA fragments) were extracted with 200µl ethanol and 20 µl 4M NaCl precipitated and incubated overnight at ¯20 °C. After incubation DNA was pelleted by centrifugation and resuspended in 20 µl TE buffer, electrophoreses of DNA was carried out in 1.8 % agarose gel at 60 V, stained with ethidium bromide, and visualized under UV light.

8.3.7 Apoptosis assay

HeLa cells were seeded onto 6-well plates with a density of 2×10^5 cells per well. After 24 hrs incubation, two different concentrations (*ie.*,100 and 250 µg mL^{-1}) of Pap-SPH 1, Pap-SPH 2, and Pap-SPH 3 were added to the cells and incubated for 24 hrs. Cells were then trypsinized, aspirated and re-suspended in 1000 µL assay binding buffer. Mixed well and centrifuged at 400x g for 5 min discarded the supernatant and cells were further stained with fluorescein isothiocyanate (FITC) annexin-V (2 µL) and propidium iodide solution (2 µL) in binding buffer, from the annexin-V apoptosis detection kit (cayman) and then kept undisturbed for 10 min at room temperature in darkness, centrifuged at 400xg for 5 min and then re-suspended in 1 mL assay binding buffer, followed by flow cytometry analysis. Ratio of apoptotic cells was measured by flow cytometry as described by manufacturer's instructions.

8.3.8 Cell cycle analysis

Cell cycle distribution was detected using cell cycle analysis kit. HeLa cells were seeded onto 6-well plates for 24 hrs and rinsed twice with phosphate-buffered saline (PBS). Then two different concentrations (*ie.*,100 and 250 µg mL^{-1}) of samples Pap-SPH1, Pap-SPH 2, and Pap-SPH 3 were added to the cells and incubated for 24 hrs. After 24 hrs, the cells were washed twice with PBS and then trypsinized and fixed using 70% absolute ethanol, vortex gently and kept under ice for 30 min. Then centrifuged the samples at 2000 rpm for 5 min. Further, resuspend the cells in 0.25 mL PBS and 5µl RNase A incubated the samples at 37 ºC for 30 min then added 10µl propidium iodide (PI) and kept in dark at 4 ºC. The cells were further washed with PBS to remove the unbound PI and then centrifuged for 5 min at 1500 rpm. The supernatant was discarded and the cell pellet was further resuspended in 1 mL PBS.

8.4 Results and discussion

8.4.1 Cell viability and morphological observations

Cell viability assay by using MTT procedure is one of the well recognized methodologies to distinguish the anticancer properties of the compound. Anticancer properties of squid protein samples (*ie.,* squid protein hydrolysate (SPH), Pap-SPH 1, Pap-SPH 2 and Pap-SPH 3) were established based on the cell viability assay on HeLa cell line. Changes in the cell viability at different dosages of squid samples (*ie*., 1, 10, 100, 250 and 500 µg mL^{-1}) is depicted in Fig. 8.1. Results indicated that all the samples have the capacity to inhibit the growth of HeLa cell line compared to control group and it was proportional with the concentration. Similar observation was made by Priya *et al*., (2013) in squid peptide. Martinez-Cordova and Martinez-Porchas (2006) found antiproliferation property in the peptides synthesized from black clam (*Chione fluctifraga*). Similarly, peptides extracted from sponges such as carteritins A and B (cyclic

heptapeptides), lipodiscamides A-C, stylissamide X (proline-rich octapeptide) and callyaerin G from *Stylissa carteri*, *Discodermia kiensis*, *Stylissa* sp. and *Callyspongia aerizusa* also inhibited the growth of cervical cancer HeLa cells (Afifi *et al.*, 2016; Tan *et al.*, 2014; Ibrahim *et al.*, 2008).

Among the samples, the cells incubated with Pap-SPH 1, Pap-SPH 2 and Pap-SPH 3 showed maximum inhibition property against HeLa cell line and its IC_{50} values were 500.04 µg mL^{1}, 290.77 µg mL^{-1} and 180.73 µg mL^{-1}, respectively (Table 8.1). The efficacy of Pap-SPH 3 for antiproliferation property was well established based on the IC_{50} value. Cell cytotoxic assay indicated the sign of potential anticancer property of squid peptides; however, the involvement of apoptosis and cell cycle arrest will be evaluated. Apoptotic involvement is of great importance in cancer treatment since it kills especially mutated or damaged cancer cells by protecting normal cells. Among the treated squid samples, based on the cell viability assay and IC_{50} values Pap-SPH 1 (500.04 µg mL^{-1}), Pap-SPH 2 (290.77 µg mL^{-1}) and Pap-SPH 3 (180.73 µg mL^{-1}) were selected for further detailed investigations for the anticancer properties.

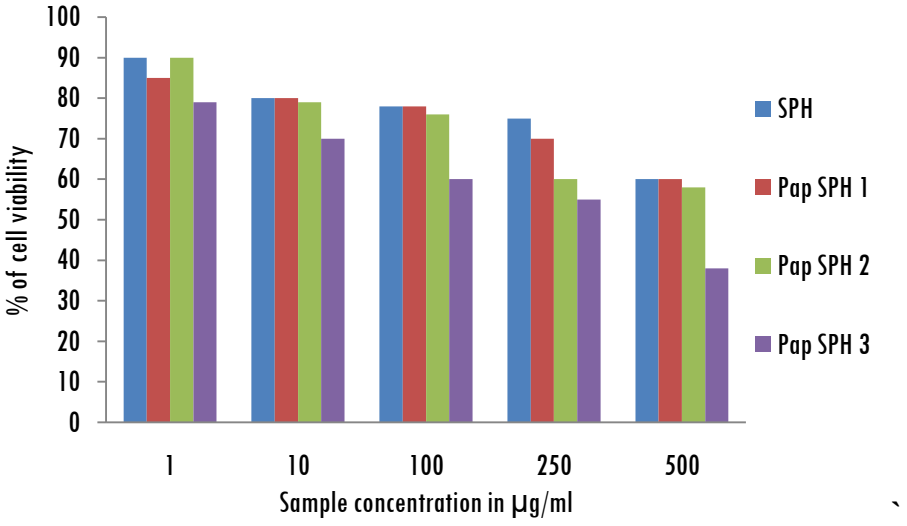

Fig. 8.1: Cell viability assay by MTT

Table 8.1: IC_{50} value (µg mL^{-1}) of samples

Peptide Samples	IC50 (µg mL^{-1})
Pap-SPH 1	500.04
Pap-SPH 2	290.77
Pap-SPH 3	180.73

Cell cytotoxic effects could be visualized by analyzing the colony formation assays, which reveals the mode of action of Pap-SPH 1, Pap-SPH 2 and Pap-SPH 3 for preventing the growth of HeLa cell lines. Significant colony reduction was noticed in all samples, which indicate the vital ability of peptides derived from Indian squid to prevent cervical cancer progression. The morphology of the HeLa cells was examined using a phase contrast microscope. In Pap-SPH 1, Pap-SPH 2 and Pap-SPH 3 treated HeLa cells showed round morphology with small shrinkage, swelling in some proportion of the cells, lysis of cell membranes and disintegration of organelles, which indicated the peptide induced toxicity to HeLa cells. These morphological changes could be comparable with H_2O_2 treated samples (**Plate. 8.1**). Reduction of total number of cells and accumulation of cells floating in the culture medium were noticed under phase contrast light microscope and these changes indicated peptide-induced cell death. In the control group, the cells exhibited an intact morphology of nucleus and cytoplasm.

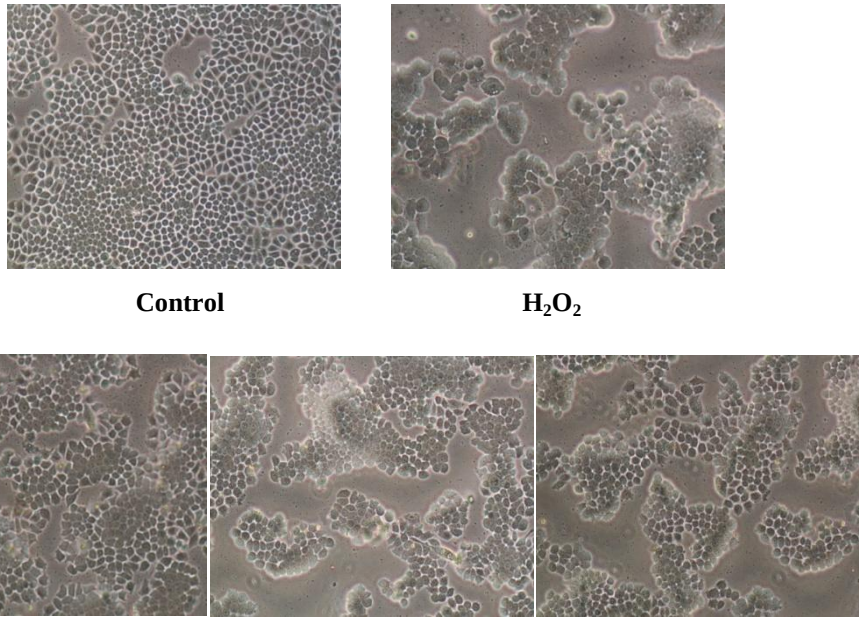

Plate 8.1: Morphological analysis: morphological changes of HeLa cells treated with Pap-SPH 3 (180 µg mL^{-1}), Pap-SPH 2 (290 µg mL^{-1}), Pap-SPH 1(500 µg mL^{-1}), H_2O_2 treated as positive control and untreated as negative control. Cells were observed using phase-contrast microscopy. Original magnification, 40X.

8.4.2 Changes in chromatin condensation (nuclear staining with Hoechst 33342)

Nuclear condensation assay with Hoechst staining identifies the cell that has undergone apoptotic changes. Cells treated with Pap-SPH 1 (500 µg mL^{-1}), Pap-SPH 2 (290 µg mL^{-1}) and Pap-SPH 3 (180 µg mL^{-1}) and its apoptosis changes are depicted in Plate. 8.2. Apoptotic HeLa cells (white arrows) displayed round and shrunken cell bodies with chromatin condensation inside the nucleus and these observations are comparable with H_2O_2 treated sample (highly condensed chromatin) which indicated peptides induced apoptosis. In control cell's nucleus were uniformly intact. Zhao *et al*., (2011) reported the nuclear condensation induced by a plant steroid isolated from *Russula*

cyanoxantha, towards HepG2 cells. According to Zhao *et al*., (2011) apoptotic nucleus undergoes characteristic condensation process and it was visualize by nuclear staining. In the present study, nuclear condensation in squid peptide treated sample signifies its role for apoptotic mediated cell death.

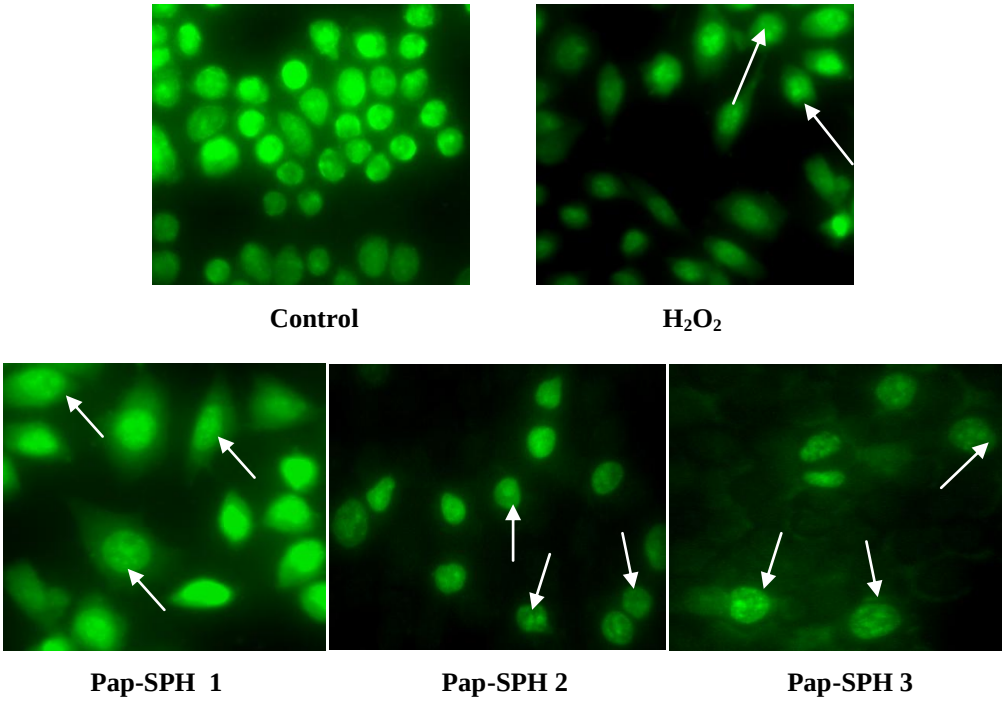

Plate 8.2: Cell apoptosis observed using Hoechst 33342 staining: HeLa cells were treated with Pap-SPH 3 (180 µg mL^{-1}), Pap-SPH 2 (290 µg mL^{-1}) and Pap-SPH 1 (500 µg mL^{-1}) for 24 h H_2O_2 treated as positive control and untreated as negative control. Photographs were taken under a fluorescence microscope (200×, original magnification). Arrows represent apoptotic cells with chromatin condensation inside the nucleus.

8.4.3 DNA fragmentation

DNA fragmentation was confirmed by staining with ethidium bromide in 2% agarose gel electrophoresis. Among treated samples Pap-SPH 3 showed an obvious DNA pattern of apoptosis slightly equal to positive control. Apoptosis characterized by the activation of endogenous enzymes with successive cleavage of chromatin DNA into inter nucleosomal fragments of around 180 base pairs,

which further justified by the presence of low molecular weight DNA fragments in the agarose gel (Plate. 8.3**)** DNA laddering is a characteristic event that occurs in apoptotic mediated cell death. Specific nucleases activated during apoptosis cleave the nuclear DNA into definite fragments of DNA with approximately 50bp and its multiplies; whereas, irregular or complete destruction happens in necrosis (Vaux and Silke, 2003). In the present study, definite DNA ladders observed in gel electrophoresis in peptide treated samples indicates the involvement of apoptosis and this was in good accordance with Pledgie-Tracy *et al.*, (2007). Authors have reported apoptotic specific DNA fragmentation by isothiocyanate present in cruciferous vegetables against breast cancer cell lines.

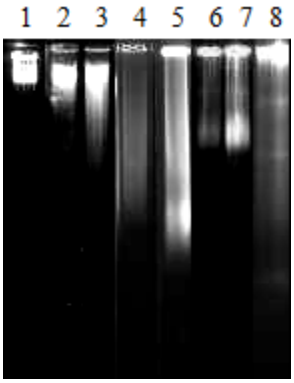

Plate 8.3: Typical DNA fragmentation was observed in peptides-treated HeLa cells, which is the one characteristic of apoptosis. LANE 1: Control, LANE 2 &3: Pap-SPH - 1007 & 500 µg mL^{-1}, LANE 4 &5: Pap-SPH 3-100 & 180 µg mL^{-1}, LANE 6 &7: Pap-SPH 2 100 & 290 µg mL^{-1}, LANE 8: H_2O_2

To further verify peptide-induced apoptosis of HeLa cells, flow cytometry was done with annex in V-FITC and PI staining. Flow cytometry is a sophisticated laser-based tool to identify the presence of apoptotic cells with the help of FTIV and propidium iodide stains. The observation in flow cytometry is depicted in Figure 8.2a. The lower right quadrant (Q4) depicts the percentage of early apoptotic cells (annexin V-FITC-stained cells) and the upper right quadrant (Q2) represents the percentage of late apoptotic cells (annexin V-FITC and PI-stained cells). The fully apoptotic cells were those in the lower right and upper

right quadrants. As shown in Figure 8.2b, no apoptotic cells were detected in the control group. Whereas, the ratio of apoptotic cells in Pap-SPH 1 (180 µg mL^{-1}), Pap-SPH 2 (290 µg mL^{-1}), Pap-SPH 3 (500 µg mL^{-1}) and H_2O_2 treated samples were 77.7%, 74,4%, 54.8% and 91.3%, respectively which confirms peptide induced HeLa cell apoptosis, but not necrosis. Increased late and early apoptotic cells observed in squid peptide treated samples was comparable with H_2O_2 treated samples. Zheng *et al*., (2012) reported quercetin induced apoptotic mediated anticancer activity against lung cancer cell lines. Result obtained in the present study have good accordance with Zheng *et al*., (2012) which substantiates the involvement of apoptosis for killing HeLa cells in squid peptide treated samples.

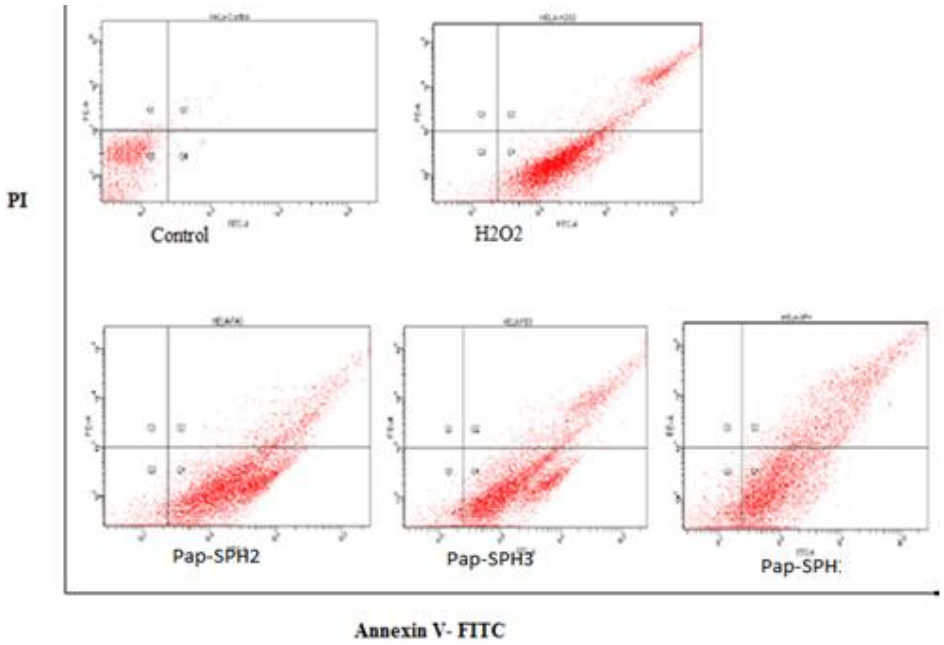

Fig. 8.2a: Squid peptides-induced apoptosis in HeLa cells was determined by flow cytometry using annexin V FITC-PI staining method. The cells were treated Pap-SPH 1 (180 µg mL^{-1}), Pap-SPH 2 (290 µg mL^{-1}), Pap- SPH 3 (500 µg mL^{-1}) for 24 h untreated as negative control and H_2O_2 treated as positive control for 24 h (a, b).

(a) peptides-induced apoptosis analyzed by flow cytometry. The lower right quadrant (Q4) indicates the percentage of early apoptotic cells (FITC-

stained cells) and the upper right quadrant (Q2) indicates the percentage of late apoptotic cells (FITC+PI-stained cells).

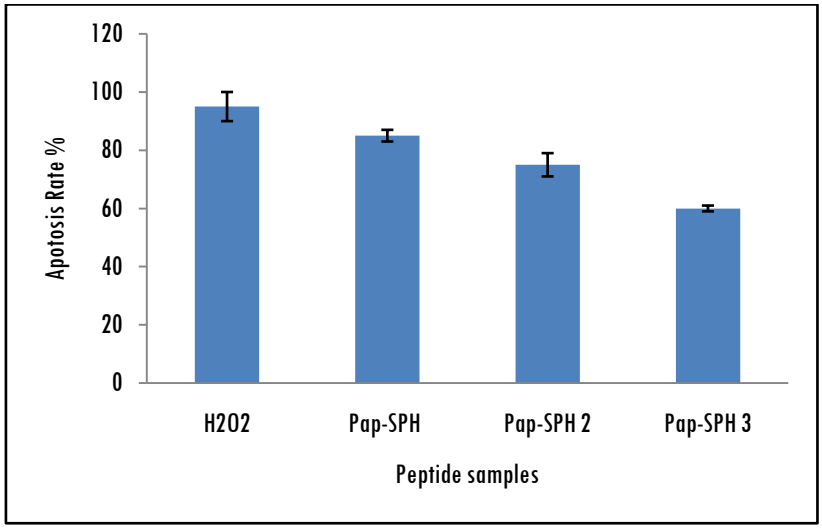

Fig. 8.2b: Peptide-induced apoptosis rate shown by bar graph.

8.4.5 Cell cycle distribution

Cell cycle events are the key stages in cell division and synthesis phase mainly account for the multiplication of cells in cancer. Compounds which can reduce or retard this phase in cell cycle have the advantage to resist the random cell division and as a result cell cytotoxicity can be achieved. At the same time reduction in G_2/M phase and increased G_0/G_1 favors the inhibition of cancer cells. Cell cycle distribution did not change dramatically, but a slight increase of sub-G_0/G_1 phase compared to control sample could be associated with apoptosis (Figure 8.3). Significant cell cycle arrest at G_0/G_1 phase was observed in Pap-SPH 1 (180 µg mL^{-1}), Pap-SPH 2 (290 µg mL^{-1}) and Pap-SPH 3 (500 µg mL^{-1}) treated samples. Among the treated samples Pap-SPH 3 showed better inactivation of cell cycle (37%) at G_0/G_1 phase. Researchers pointed the role of cell cycle control at G_1, S, G_2 and M phases of the cell

cycle to prevent the cancer development (Tuteja and Tuteja, 2000). Gu and Belury (2005) suggested the importance of cell cycle arrest and apoptosis activation to prevent cancer progression. Authors also reported the role of cell cycle arrest in cytotoxicity action of mushroom extract against leukemia cells. Activation and uncontrolled cell division is usual process in the progression of cancer and development of anticancer drugs which prevent cell division has high relevance. In this point of view the result obtained in the present study has great significance.

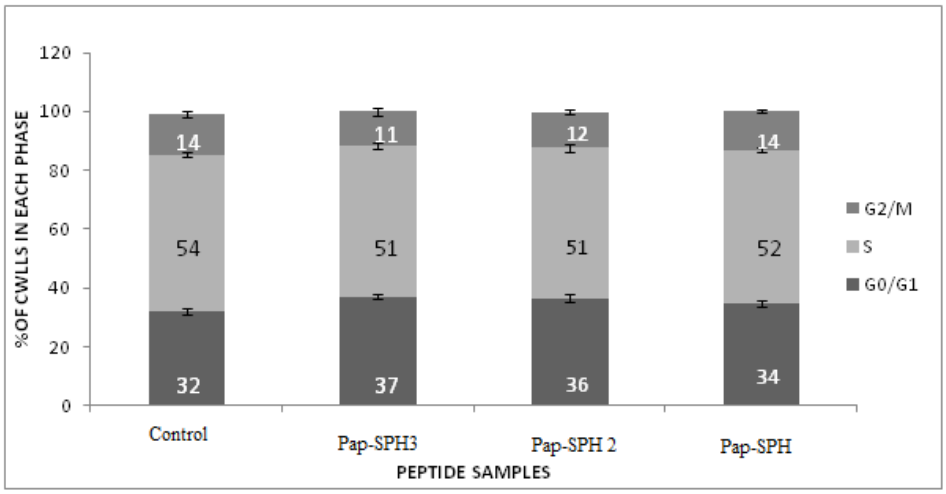

Fig. 8.3: Cell cycle distribution of Hela cells: The cell cycle analysis of untreated cells and treated cells were determined by flow cytometric analysis. The numbers indicate the average fraction of cells in a given phase of the cell cycle as determined from at least two independent experiments.

8.5 Conclusion

Relative oxygen species and free radicals generated during lipid oxidation mutates DNA and cause cancer by interrupting normal proliferation and apoptosis mechanism of the body. This causes uncontrollable cell growth or carcinogenesis in an organism. Hence, inactivating the free radicals prevent the formation of cancer. The compounds having strong antioxidant properties

and triggering apoptotics have great significance in current scenario; hence extensive research is going on to develop anticancer compounds with seldom side effects. Strong antioxidant properties of Indian squid peptides help to neutralize free radicals thus preventing the risk of cancer. Strong anticancer properties were observed in Indian squid peptides due to its antioxidant nature and ability to kill cancer cells by triggering apoptosis. This is well established by HeLa cell viability assay, morphological analysis of HeLa cells by phase contrast microscopy, chromatin condensation assay, DNA fragmentation, flow cytometric analysis and cell cycle distribution study. All squid samples showed potent anticancer properties, however the low molecular weight peptide *ie.,* Pap-SPH 3 (180.73 µg mL^{-1}) at low concentration exhibited maximum anticancer properties. Hence, the observations in the present experiment supported the involvement of apoptosis in squid peptide treated samples which open up the scope for development of squid peptide in the cervical cancer treatment.

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Chapter 9

Summery and Conclusions

Contents

9.1 Summary and future recommendations

Indian squid (*U. duvauceli*) composes 80.47% of moisture, 17.5% of protein, 0.52% of fat and 1.13% of ash and also possess commendable quantities of essential nutrients such as leucine, tryptophan (essential amino acids), DHA, EPA, arachidonic acid (essential fatty acids), sodium, calcium, magnesium and potassium (essential minerals). Essential amino acids, acidic amino acids, neutral amino acids, sulphur amino acids and aromatic amino acids were also found at significant levels in the squid. Biochemical profiling of Indian squid revealed its nutritional significance and capability to prevent malnutrition, hence exploration of this moderately exploited resource has high relevance.

Preparation of antioxidant squid protein hydrolysate using various proteases such as pepsin, trypsin and papain were carried out. Hydrolysis conditions of each protease were optimized based on the antioxidant properties of the resultant squid protein hydrolysate. Higher coefficient of determination (R^2) for the second order response surface regression model indicated its suitability to explain the total variability of antioxidant activity of squid protein hydrolysate in terms of linear, quadratic and interaction effect of

hydrolysis conditions. Based on the antioxidant activity, ridge and multiple response analysis, hydrolysis conditions such as E/S ratio (1.5:100), pH (6.25), time (5.15 hrs) for papain, E/S ratio (1:100), pH (2) and time (3.15 hrs) for pepsin and E/S ratio (1.4:100), pH (7.25) and time (6 hrs) for trypsin were optimized for the synthesis of squid protein hydrolysate. Similarities of predicted values and experimental values of the antioxidant activities of squid protein hydrolysates indicates appropriate and efficacy of generated model to predict the antioxidant activity and hydrolysis condition of each proteases. Among the proteases, papain digested squid protein hydrolysate revealed maximum antioxidant activity, which is further justified in another detailed study for the antioxidant properties of squid protein hydrolysate synthesized using each proteases at optimized hydrolysis conditions. DPPH radical scavenging activity, superoxide anion radical-scavenging activity, metal chelation ability, inhibition of lipid peroxidation in the linoleic acid model system, reducing power and degree of hydrolysis were analyzed in the resultant squid protein hydrolysate and the results indicated that papain digested squid protein hydrolysate exhibited excellent antioxidant properties, which justifies with the presence of significant quantities of hydrophobic amino acids. Hence, it can be concluded that papain was highly efficient for the synthesis of antioxidant hydrolysates from Indian squid muscle.

Papain digested squid protein hydrolysate was further ultrafiltered by using three molecular weight cut off of *ie.,* >10 kDa,10-3 kDa and <3 kDa and collected three fraction namely, Pap-SPH 1, Pap-SPH 2 and Pap-SPH 3, respectively. DPPH, superoxide, metal chelate and reducing power analysis evidently revealed strong antioxidant properties of these fractions and also showed strong ability to pBR322 of DNA from hydroxyl-radical. It was found that low molecular weight fraction *ie.,* Pap-SPH 3 showed maximum

activities. Hence, Pap-SPH 3 fraction was further purified by using RP-HPLC and the separated largest peak was named as Pap-SPH 3-F1. The amino acid sequence of the fraction was determined by using LC/MS/MS and several antioxidant potent amino acids showing excellent hydrophobicity were found. Hence, it could be an effective antioxidant against oxidative stress.

Anti-inflammatory and anti-ulcer properties of low molecular weight peptides purified from the papain digested squid protein hydrolysate (*ie.*, PAP-SPH 3) were investigated. PAP-SPH 3 possesses better efficiency to neutralize excess free radicals through the activation of anti-oxidant defense system. This might be the reason for the decreased paw edema in PAP-SPH 3 treated male Albino rats and which was comparable with the changes caused by ibuprofen (standard drug) treated male Albino rats. Generally, the anti-inflammatiory compounds are having the capabilities prevent arthritis and hence, PAP-SPH 3 might be possessing anti-arthritic properties. Further detailed research is recommended to establish anti-arthritic properties of peptides hydrolyzed from Indian squid. Compared to normal control group, significant reduction of definite lesions, percentage of ulcer inhibition, gastric volume (ml $100g^{-1}$ of body weight), gastric pH and total acidity mEq L^{-1} was noiced in PAP-SPH 3 treated group which was comparable with ranitidine (standard drug) treated group. In histopathology study, the stomach of male Wistar albino rats treated with PAP-SPH 3 clearly revealed an enhancement of anti-oxidant defense system and this well correlates with the reduction in ulcer symptoms of the stomach section, reduction of neutrophil infiltartion and blood vessel damage. Similar findings were noticed in standard drug (ranitidine) treated groups. In ethanol-induced gastric ulcer model experiment, the abilities of PAP-SPH 3 to enhance ulcer inhibition, decrease of ulcer lesions, reduction of gastic volume and increase of pH were well established which indicates the better protection

ability of squid peptides against ethanol induced gastric ulcer. Compared to ranitidine treated groups, PAP-SPH 3 also showed an increasing trend in the CAT, SOD and GSH levels, which reveals the protective action of squid peptide against ethanol induced peptic ulcer. Hence, antiinflammation, antiarthritis and antiulcer properties of squid peptides could be explored for the development of nutraceuticals for inflammatory, arthritic and peptic ulcer patients. Further research is recommended to establish the role of this peptide as antiulcer and antiinflammatory agents and also to explore the possibilities for the synthesis of nutraceuticals.

Anticancer properties of squid protein samples (*ie.*, squid protein hydrolysate (SPH), trypsin digested protein (TDP), Pap-SPH 1, Pap-SPH 2 and Pap-SPH 3) were established. All samples inhibited the growth of HeLa cell line and it was proportional with dosage. This might be due to the antioxidant properties of squid samples. In cell viability assay Pap-SPH 1, Pap-SPH 2 and Pap-SPH 3 exhibited maximum inhibition property and showed an IC_{50} values of 500.04 µg ml^{-1}, 290.77 µg ml^{-1} and 180.73 µg ml^{-1}, respectively. Morphological changes such as reduction of total number of cells and accumulation of cells floating in the culture medium, swelling in some proportion of the cells, lysis of cell membranes and disintegration of organelles, round and shrunken cell bodies with chromatin condensation inside the nucleus and cleavage of chromatin DNA into inter nucleosomal fragments indicated the peptide induced toxicity to HeLa cells. The ratio of apoptotic cells and reduction in G_2/M phase and increased G_0/G_1 phase favors the inhibition of cancer cells. Among the treated samples Pap-SPH 3 exhibited maximum involvement of apototics mechanism however; the involvement of Pap-SPH 3 for apoptosis and cell cycle arrest require further investigation. Hence, it can be concluded that Indian squid peptides possess strong

anticancer properties and could be explored for the synthesis of drugs for the treatment of cancer.

9.2 Overall conclusion

Indian squid (*Uroteuthis duvauceli*) contains essential nutrients and its peptides have strong antioxidant properties due to the enrichment of strong antioxidant potent amino acids; moreover its anticancer, anti-ulcer and anti-inflamation properties pave the way to explore this under exploited resource to develop new arena of drugs or nurtraceuticals for curing cancer, ulcer and inflammation.

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