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Preparation, Separation, Purification, Characterization and Human Cell Line Anti-Cancer Evaluation of Rice Bran Peptides

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PREPARATION, SEPARATION, PURIFICATION, CHARACTERIZATION AND HUMAN CELL LINE ANTI-CANCER EVALUATION OF RICE BRAN PEPTIDES

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

By

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Abstract

Bioactive compounds are revolutionizing the nutritional and medicinal world with their inherent disease-fighting properties. A wide range of functional groups fall under the category of imparting health benefits. Compounds from both animal and plant origins have been generated as bioactive agents that have opened up new vistas for alternative medicine and natural healing. For example, in a debilitating disease like cancer, these compounds can act to suppress or delay the underlying pathology over and above the conventional treatment strategies involving drugs or chemotherapy. In other words conventional and invasive therapy, although still considered appropriate at certain stages of cancer, their low success rates have necessitated and ushered in an era of nutraceuticals or naturally available bioactive compounds that can through diet reduce the progression of cancers thereby providing a replacement or supplement to pharmacologic treatments. As a result many, including Americans are supplementing traditional health care by turning to nutraceuticals and functional foods.

Animal and plant derived bioactive compounds have been shown to render positive health benefits. However with the outbreaks of a few meat-related illnesses, vegetarianism is on the rise. Compounds derived from plants including fruits and vegetables are being researched for the presence of bioactive compounds. Cereal grains are also being identified to be valuable sources of bioactive proteins and peptides that help arrest disease progression. The increasing knowledge of peptides with anti-disease properties like antihypertensive, antimutagenic, and anticancer has created commercial interest in using them as functional ingredients in food products. Bioactive peptide research had focused primarily on animal, fish, and dairy proteins but owing to disease

propensities and religious reasons many are turning to study and recommend plant derived products. Protein isolates and fractions from food sources have been shown to exert anti-mitogenic, anti-hypertensive, anti-cancerous, and anti-microbial effects, particularly peptides that are broken down from proteins available in foods have been documented to possess anti-disease characteristics. Hence exploring and testing for bioactivities, in compounds originating from plant sources has gained interest. Cereal proteins particularly have been utilized to obtain hydrolysates and peptides for testing against disease attributes like anti-hypertensive or anti-cancer etc.

The following research has been based on grounds of utilizing cereal grains' by-product and hopes to provide scope for reducing cancerous states with the use of natural, inexpensive and readily available value-added byproducts obtained during processing of certain cereal grains.

Rice bran is one such byproduct obtained during milling of rice. It contains 90% of the nutrients and nutraceuticals, and forms 8-10% of the total weight. The rice bran components include high quality protein, phenolics, dietary fiber, source of vitamins and minerals. Rice bran is primarily used as animal feed and to extract oil which is used in cooking. It has nearly 20% protein and could be a source of bioactive peptides and hence a functional food for health benefits. The State of Arkansas is ranked number one in the production of rice and, rice bran is a cheap co-product of rough rice milling. Rice bran production rate in the United States has reached 800,000 tons annually. Although rice bran production is so high, it is mostly under-utilized.

In spite of numerous potential health benefits little work has been done with rice bran to produce functional bioactive compounds. Rice bran protein has the potential for

creating anticancer peptides. The high quality protein can be broken down to generate peptides. *There are no systematic studies reported on the production of anti-cancer peptides from commercially available heat stabilized defatted rice bran by proteolytic enzyme hydrolysis and, particularly their resistance to gastrointestinal environment.* Therefore, a systematic study on the production of gastrointestinal resistant peptides from rice bran by enzymatic hydrolysis, and testing for anticancer activity was needed.

In this research rice bran was used for preparing peptides with an intention of providing benefits in controlling cancer cell growth. The protein was directly hydrolyzed using food grade enzyme to prepare peptides. These were treated with simulated gastro-intestinal juice before fractionation and collection according to their molecular sizes using ultra-filtration technique. The different sized peptides were tested for bio-activity by cell culture techniques to assess their ability to control cancer cell proliferation.

Identifying and characterizing rice bran peptides that can arrest human cancer cell proliferation formed the broad objective of this study. As a summary heat stabilized defatted rice bran was treated with endoprotease, alcalase after optimizing conditions for enzymatic hydrolysis using response surface design. Degree of hydrolysis (DH) was considered as the dictating response variable for enzymatic hydrolysis, which was set at nearly 25% to avoid both excessive hydrolysis (over 40% DH) and limited hydrolysis (under 15%). The resulting hydrolysates were treated with simulated gastric and intestinal juices to obtain gastrointestinal (GI) resistant peptides. The peptides were fractionated into molecular size ranges of >50, 10-50, 5-10, and <5 kDa using ultrafiltration. They were tested for their abilities to reduce cell viability and cause cytotoxicity to human cancer cells in vitro employing the trypan blue dye exclusion assay as well as a cell titer

assay that uses a tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; (MTS)] and the electron coupling reagent, phenazine methosulfate or [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT).

In order to provide a comprehensive picture for cancer cell proliferation inhibitory effects of the peptide fractions, and also due to the specificity of each cancer cell type, cancer cells derived from multiple sites representing colon, breast, lung and liver were chosen. The idea was to evaluate rice bran peptide(s) for multiple site activity against cancers. Two colon cancer cell lines including a tumorogenic cell line, two breast cancer cell lines including a tumorogenic cell line, a lung and a liver cancer cell line were selected for the study. The idea of including tumorogenic cell lines representing colon and breast was to decipher any probable anti-tumorigenic activity of the bran peptide fractions because colon and breast organs are prone to involve tumors as part of cancer pathology more commonly than other cancer types chosen for the study. Both GI and non-GI resistant fractions were initially screened with each cancer cell line for evaluating cell viability patterns of the fractions. Based on the findings the <5kDa fraction exhibited better anti-cancer activities followed by the 5-10 kDa fraction compared to other fractions, controls as well as non-GI resistant fractions. The <5 kDa fraction was selected for further confirmatory anti-cancer properties using relatively more specific assays. Dosage and anti-tumorigenic assays were also performed on the <5kDa fraction that initially showed better anti-cancer properties. This fraction was selected for characterization purposes.

The highlight of the research has been the demonstration of GI-resistant <5 and 5-10kDa bran peptide hydrolysates to inhibit growth of certain cancer cell types more effectively compared to controls, and nonresistant fractions. GI resistant fractions had better anti-cancer activities than non-GI resistant fractions of which the <5 and 5-10kDa fractions served as prime candidates for possessing anti-cancer activities based on initial screening. Specifically, the <5kDa fraction showed 70-75% cytotoxicity to colon (Caco-2, HCT-116), 70-80% cytotoxicity to breast (MCF-7, HTB-22) and 80% cytotoxicity to liver (HepG2) cells respectively. The 5-10kDa fraction caused 80% cytotoxicity to liver (HepG2) cancer cells alone. This implies that <5kDa fraction is able to arrest cancer cell proliferation and could also serve as anti-tumorogenic agent. The <5kDa fraction was characterized to obtain a pure peptide that when tested for cancer cell proliferation showed nearly 80% inhibition to colon, breast and liver cancer cells. Amino acid analysis of the peptide revealed the presence of Arginine, Proline and Glutamic acid residues. Full characterization of the peptide by proteomic tools coupled to mass spectrometry enabled determination of the amino acid sequence, Gln-Glu-Arg-Pro-Arg. Thus a penta-peptide from rice bran has been isolated and characterized for multiple-site activity against cancers. This could mean that a food-derived peptide could act as a cheaper natural alternative over synthetic anti-cancer drugs for treatment against cancers originating from multiple sites.

The significance of the findings observed in this study lies in the potential capacity by which a natural nutraceutical agent like rice bran could bear positive impact on human health through its protein components providing potentials for advancement in medicinal application from an agricultural standpoint, while it is considered as a co-

product of a larger process. Successful utilization of data from this research will revolutionize the concept of preparing peptides with bioactivity against human cancers and promote further study of value added products from the cereal grains like rice bran.

This dissertation is approved for
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Dedicated to my aunt Ambujam Anantharaman

Table of Contents

List of Figures

List of Tables

Introduction

Food proteins and biopeptides have biological functions in addition to their established nutritional value as a source of protein (Dziuba *et al.*, 1999). Such bioactive peptides are nutraceuticals and functional food ingredients possessing the ability to bring health benefits or reduce the risk of diseases like cancer, hypertension, obesity, diabetes etc. for human beings, and thus are in large demand by the global market. Numerous bioactive peptides have been obtained from enzymatic digestion of food proteins, although the types of biological activities of the peptides obtained are different with protein source, enzyme specificity, and processing conditions including pH, time and temperature (Wang and Gonzalez de Mejia, 2005). With vegetarianism on the rise, and high quality nutritional compounds utilized only as animal feed during several food/cereal grain processing stages, there is a need for evaluating such compounds for bioactivity for application into human health. The overall objective is thus to utilize protein components obtained from rice bran, an abundantly produced co-product during rice milling that is both underutilized and inexpensive, as a nutraceutical agent that can arrest human cancer cell type's proliferation. The need for evaluating rice bran for anti-cancer bioactive peptides is validated by limited information available of rice bran being used as a natural functional bioactive agent. Moreover there is an increasing commercial interest in the production of economical biopeptides from proteins, particularly from cereal grains where bioprocesses otherwise render certain nutritionally rich co-products only as feed for animal.

For obtaining protein or peptide hydrolysates from food sources both chemical and enzymatic approaches have been followed. However, it is evident from studies that

have attempted to obtain protein isolates from rice bran that, the proteins in bran are thought to be complexed with lipids and carbohydrates thereby providing difficulties in protein extraction (Tang et al, 2002). Use of both enzymatic and chemical methods has been explored wherein extraction of a substantial amount of protein from de-fatted rice bran was achieved. Heat stabilization and de-fatting processes with the aid of enzymes have been shown to weaken the protein-lipid and protein-carbohydrate interactions thereby improving protein extraction yields. Besides, to prepare peptides or protein hydrolysates it is preferred to directly access the bran protein instead of preparing protein isolates, where the purity and yield could be compromised. Hence direct hydrolysis of defatted rice bran would be preferred.

Since specificity and extent of hydrolysis are important factors that need to be controlled to not only obtain peptides on a consistent basis but also improve functional properties of food proteins and peptides (Nielson, 1997), the degree of hydrolysis is an essential parameter and the suitable response factor in any statistical design used for optimizing conditions for enzymatic hydrolysis. Degree of hydrolysis will determine the proportion of peptide bonds hydrolysed from rice bran protein.

For bioavailability from the food product consumed, the peptides need to be resistant to gastrointestinal environment. Thus the peptides need to be exposed to the gastric/intestinal pH, enzymes and microbial flora present in the gut in order to be resistant to the digestive processes. Soluble peptides that are resistant to gastrointestinal environment can only render bioactivity more effectively and, can thus extrapolate as bioavailable nutraceutical ingredients. The protein hydrolysates or peptides obtained from any source need to be exposed or evaluated for gastrointestinal resistance Very few

studies have involved gastric and intestinal treatments to food proteins/peptides that have implicated bioactivities because it has been implicated that the colonic microflora could have an influence to the fate of the peptides. Many of the known bioactive peptides have been produced *in vitro* using gastrointestinal enzymes, usually pepsin and trypsin to render them resistant to the gut environment. Angiotensin-1 converting enzyme (ACE)-inhibitory peptides and Caesino phosphopeptides (CPP), for example, are most commonly produced by trypsin. Other digestive enzymes and different enzyme combinations of proteinases—including alcalase, chymotrypsin, pancreatin, pepsin and thermolysin as well as enzymes from bacterial and fungal sources have also been utilized to generate bioactive peptides from various proteins (Haque et al, 2006).

Human cancers having varied etiologies (genetic and environmental) and high cancer mortality rates have surfaced as a top life-threatening condition. Most organs and cellular types have been reported to be affected by cancer when there is an imminent underlying pathology, with varying degrees. For treatment modalities it is essential to identify the cause for each type of cancer. Furthermore the agent in question needs to be evaluated for different cancer cell types because of specificity in the mode of action. Anti-cancer properties are generally evaluated by observing a reduction in the cancer cell viability and/or a possible metabolic arrest in the cancer cells upon addition of the compound in question and photometrically quantifying cell survival. While dye exclusion assays signify cell viability, dye binding/localization coupled to photometric detection can signify cell survival. Most studies have employed the MTT or MTS dye assay as a confirmatory assay for determining anti-cancer properties. Several others have used tumorogenic cell lines and have conducted clonogenic assays for possible anti-tumor

activities too. In this study dye exclusion, binding and clonogenic assays were performed to determine anti-cancer properties of rice bran peptide fractions/pure peptide on several human cancer cell lines including colon (Caco-2 and HCT-116 cell lines), lung (A-549), breast (MCF-7 and HTB-22 cell lines), and liver (HepG-2).

In order for the anti-cancer bioactive compound to impart bioactivity to its maximum potential, it needs to be free of potential interacting substances that may have a confounding effect on its activity. As an initial step in characterization of such bioactive peptides studies have used membrane fractionation procedures. Several membrane separation processes have been used depending upon the molecular size of the desired product. Ultrafiltration by far has been the most commonly employed fractionation procedure to be applied to food peptides ranging from 1-100kDa. It may be extremely useful to have definite molecular size fractions from the protein hydrolysate pool so that initial screening for bioactivity can be evaluated for in the fractions. Generating peptide fractions with definite molecular cut off membranes have been produced and also recommended. Fractionation, typically from higher (50-100 kDa) to lower (1-5 kDa) molecular cut off membranes have been used particularly for peptides obtained from food sources. In this study use of 50, 10 and 5 kDa will be done for generation of peptide fragments in the ranges <5, 5-10, 10-50, >50 kDa before testing for anticancer activities in human cancer cell lines thereby easing the characterization processes.

Purification of the peptide from the protein hydrolysate pool forms a very important task. Using powerful proteomic tools with high resolution, peptides can be purified from protein hydrolysates. Chromatographic separation methods are commonly used to achieve maximum purity. In purifying peptides from the lower molecular size

fractions, employing ion-exchange chromatography primarily followed by reversed phase chromatography can be the most efficient means of obtaining highly purified peptides than either ion exchange or reversed phase chromatographic separation alone. For example, a peptide, Lactoferricin was purified using cation-exchange chromatography (Dyonisius et al, 1997). while Luffacylin, another peptide was isolated from sponge guard seeds and characterized with the help of DEAE-cellulose ion exchange chromatography (Parkash et al, 2002). Analytical and preparative HPLC have been used in addition to ion exchange chromatography to improve purity and yield of the peptide. Specific c-18 peptide columns are designed especially for peptide purification. Multi-step processes involving repetitive chromatographic separation may be considered when the purity of the peptide is compromised at any stage of separation. The individual peptides or combination (two for synergistic activity) may then be evaluated for enhanced bioactivities and further characterization can be done to decipher the amino acid contents and sequencing. This information can be used to chemically synthesize the anticancer peptides for pharmaceutical use or other uses where purity is needed.

Objectives

Bioactive peptides are increasingly becoming popular candidates for traditional and alternative treatment strategies in disease states where conventional treatment has proven cumbersome or expensive. Although there are several food sources (animal and plant) to explore for bioactive compounds, cereal grains have gained much attention this decade for their sustained production, use and wider acceptance as compared to other food derived products with relatively poor sustenance and shelf life. Besides, bioactive

peptides incorporated food products are limited in the United States and there is a need for these products to reduce the risk of chronic diseases like cancer.

Heat-stabilized de-fatted rice bran (HDRB) can be a good starting material for creating new bioactive peptides, as non-defatted brans from cereals containing moderately higher concentrations of protein (oat, wheat, rice, and corn bran 17.0, 15.5, 13.0, and 8.0% approximate protein, respectively) have been neglected as a source of protein due to difficulties in extraction. Although soy protein hydrolysates and peptides for instance, have the potential to decrease cancer cell proliferation, availability and cost may be important factors for consideration. HDRB may thus qualify as a naturally abundant cereal source to prepare bioactive peptides from. The primary objective would be to prepare protein hydrolysates and peptides from rice bran by directly using protease at optimum degree of hydrolysis.

Even though bioactive peptides have been studied extensively, their resistances to enzymes and microflora in the gastrointestinal tract have not been evaluated. Therefore, a systematic study on the production of resistant peptides from HDRB by enzymatic hydrolysis is necessary before testing for anticancer bioactivity, which will comprise the core section of the study. Finally for enhanced bioactivity full characterization of the peptide(s) obtained from the pool of bran hydrolysates needs to be done. The individual pure peptides that demonstrate superior anti-cancer activities can be subjected to amino acid analysis and sequencing. This information can be used to chemically synthesize the anticancer peptides for pharmaceutical use or other uses where purity is needed.

This study will provide the basis for developing novel gastrointestinal environment resistant bioactive peptides active against different cancer cell types from rice bran. The following specific objectives are outlined in the context of this study.

Specific Objectives

a. Preparation of peptide hydrolysates from heat stabilized defatted rice bran by enzymatic hydrolysis using response surface design optimization.
b. Preparation of gastrointestinal juices resistant peptide hydrolysates and fractionation to generate peptide fractions of definite molecular sizes, >50, 10-50, 5-10 and <5kDa.
c. Determination of anti-proliferative and cytotoxicity activities of peptide fractions on human colon, breast, lung and liver cancer cells.
d. Purification and characterization from the fraction(s) having promising anti-cancer activity and test the purified peptide(s) for anti-cancer activity on cancer cells.
e. Amino acid analysis, mass spectrometry and sequencing of the purified peptide.

Dissertation Structure

Abstract

Introduction

Literature Review

CHAPTER 1
Human Colon cancer cell proliferation inhibition by peptide hydrolysates derived from heat stabilized de-fatted rice bran

CHAPTER 2
Human Breast anti-cancer properties of peptide hydrolysates derived from heat stabilized defatted rice bran.

CHAPTER 3
Human Lung and Liver anti-cancer properties of peptide hydrolysates derived from heat stabilized defatted rice bran.

CHAPTER 4
Purification and characterization from promising anti-cancer peptide fraction, amino acid analysis and sequencing of purified peptides.

Conclusion

Literature Review

Rice production in the United States and Arkansas

Rice is considered a staple food for a major part of the world. The global rough rice production was estimated to reach a new record level of 666 million tons in 2008 and an increase by manifold compared to previous years (2006: 8.8 million metric tons; 2007: 9.0 million metric tons). Asian countries like China and India top the overall rice production rate, followed by the United States. The United States is unique as one of the largest exporters of all rice types, supplying more than 13 percent of the rice that enters world trade. Approximately 88 percent of the rice consumed in the United States is grown in the United States. U.S. rice is in demand in high quality markets around the world (USA Rice federation, 2008).

Arkansas leads the nation in rice production. Arkansas farmers produce about 80 million hundredweight (cwt) of rice annually, which is 40 percent of the U.S. crop. The farm gate value of Arkansas-grown rice is about $700 million in 2008. The estimated economic impact of the rice industry in Arkansas is $2.5 billion. Despite rising prices for U.S. rough rice over the next 10 years, plantings are projected to increase 25,000 to 50,000 acres a year and to remain at 200,000-400,000 acres below the (record) 2005/06 level (USDA NASS (2008)–Arkansas agricultural Statistics Service).

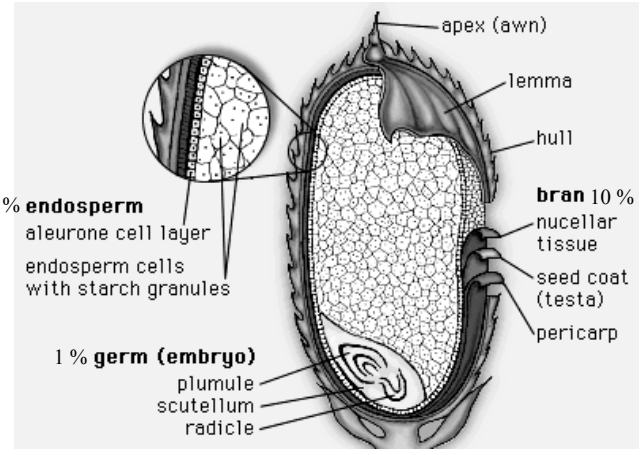
Rice bran production

Rice bran forms 8-10% of the total weight of rough rice and contains approximately 90% of its nutrients and nutraceuticals. Rice bran is considered the most valuable by-product of rough rice produced in abundant quantities in the Arkansas State.

However, it is mostly under-utilized. Rice bran production rate in the United States has reached 800,000 tons annually of which the State of Arkansas produces over 50% of the total production. ((USDA NASS (2007)–Arkansas data).

Rice components and co-products

Rice grain is composed of 3 basic components: the hull, a brown bran layer, and the kernel (Figure I). The kernel, bran, and hull represent approximately 70, 10, and 20% of the weight of rough rice. Milling of rough rice involves several stages (Figure II). Milling rough rice initially requires removing the hull, leaving brown rice. White rice is then produced by removing the bran layer. The bran layer normally contains 15% fat, and rice industries may subject the bran to further processing to extract the oil from the bran, producing residual de-fatted rice bran. Co-products of the rice milling industry - hulls, rice bran, de-oiled rice bran, and rice mill feeds (a mixture of hulls and bran) - may be an economical alternative to grains. Despite the large production of rice and rice co-products in the south-central United States, limited research is available on the nutritional value.



Adapted from Encyclopedia Brittanica. 1996

Figure I Structure of a rough rice grain

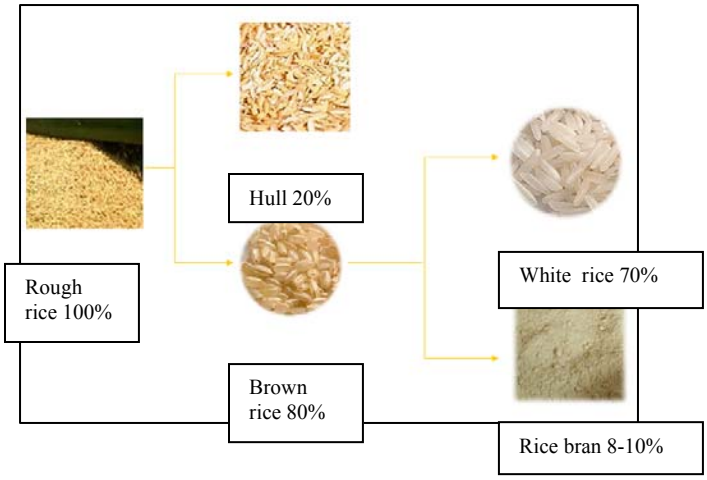


Figure II. Stages in Rice processing

Rice bran and composition

Bran is a component of the rice grain and is made up of the pericarp, seed-coat and nucleus (Juliano and Bechtel 1985, Juliano 1993) (Figure.I). Rice bran contains approximately 10.5- 12.0% water, 18.5-20.9% protein, 1.5-2.0% lipid, 15.3-16.8% starch, 25.2-25.6% total fiber, 2.0-2.5% total phenolics, 1.73-2.28% phytic acid, and 9.0-10.4% ash (Juliano, 1985; Hettiarachchy, 2003) in addition to B vitamins and minerals. Major anti-nutritional factors such as phytate, trypsin inhibitor, haemagglutinin-lectin s and oryzacystatin are also found within the bran (Juliano 1993).

Commercially, rice bran is stabilized by heat treatment to prevent deterioration resulting from hydrolysis and oxidation of oil. The commercial rice bran product after heat treatment and removal of oil (15-20%) is referred to as "heat-stabilized defatted rice bran" (HDRB) and is mainly used for animal feed and pet foods. Heat treatment results in protein denaturation and aggregation with other components, and lowers protein and phytochemical extractability. In addition, rice bran contains B-vitamins and minerals, and

trace elements. The positive nutritional and nutraceutical attributes of rice bran include hypoallergenicity, gluten-free, and a source of dietary fiber.

Through further processing to extract and market the oil from rice bran, some rice milling industries offer de-oiled (defatted) rice bran as a co-product feed. One advantage of de-fatted rice bran over raw rice bran is a longer shelf life. Rice bran becomes rancid unless it has been stabilized in a manner such as extruding that will destroy the lipase enzyme. With the removal of fat, there is an expected reduction in degree of esterification. Extracting the oil concentrates the remaining nutrients. Raw rice bran contains 12-18% oil, whereas defatted bran contains about 1 to 3 percent oil only. Defatted bran contains higher percentage of protein (17-20%), vitamins (A and E) and minerals than full fatted bran obtained from raw and parboiled paddy.

Proteins and amino acids in rice bran

The bran component of rice grain contains 12-20% protein which includes proteins like albumins (37%), globulins (36%), glutelins (22%), and prolamins (5%). (Saunders, 1990, Betschart et al, 1977, Hettiarachchy, 2003,). Rice bran proteins are difficult to extract. The possible reason may be due to the tight bonding or interaction these proteins have with carbohydrate moieties within the rice bran (Hamada, 1995). Studies have attempted to extract proteins from rice bran (Wang, et al 1999). Some components that interact in such a way to hinder high extractability of proteins include fiber (12%), and phytic acid (1.7%) (Juliano, AACC 1994). Heat stabilization is generally done to maintain the quality of rice bran oil that results in interaction between proteins and carbohydrates, which in turn contributes toward less protein extraction efficiency (Tang et al, 2002; Tang et al, 2003).

Muksud et al (2000) had studied the amino acid contents of different varieties of rice bran. Eighteen amino acids were found in the total form, and thirteen amino acids in the free form. Aspartic acid (1.3-1.8%), glutamic acid (0.48-1.7%) and tyrosine (0.97-1.42%) as total form amino acids and aspartic acid (0.05-0.15%) and threonine (0.098-0.20%) as free form amino acids were reported to be present in appreciable quantities in all the varieties of rice bran. Wang et al (1999) have reported amino acid levels in rice bran protein isolate. They found higher amounts of acidic amino acids like glutamic acid (125mg/g protein) and aspartic acid (80mg/g protein), while arginine (89mg/g protein) and leucine (74mg/g protein) were also found to be predominant.

Several approaches have been considered to maximize extraction of protein and amino acids from rice bran. The amounts of protein and amino acids produced seem to be higher in cases where subcritical water hydrolysis method was followed compared to conventional alkali hydrolysis (Sereewatthanawu et al, 2007). In general acidic amino acids are abundant in rice bran protein prepared, and the manner of hydrolysis dictates the nature of peptides and amino acids that could be obtained.

Rice endosperm proteins: extraction and functionalities

Rice proteins are considered as competent ingredients because of their recognized nutritional, hypoallergenic and healthful qualities suited for human consumption (Eggum et al 1992, Fiocchi et al, 2003). Rice proteins are also ranked high in nutritional quality compared to other cereals like corn and wheat. Although rice is generally regarded as having the lowest protein content (7.3%) among the common cereal grains (wheat 10.6%, corn 9.8%, barley 11%) the net protein utilization of rice proteins (73.8%) is the highest among the cereal grains (wheat 53%, corn 58%, barley 62%,) (Bean and Nishita, 1985).

Much work has been done to assess the physicochemical properties of rice endosperm proteins following chemical and enzymatic processes. Physicochemical properties of rice endosperm proteins have been studied following chemical and enzymatic processes (Paraman et al, 2006). Since the major component of rice endosperm in starch studies have used chemical and enzymatic treatments that break down starch and release protein effectively. High-protein rice products can be obtained from rice flour by alkali extraction followed by precipitation at the isoelectric pH of the protein (Schafer et al, 2008). Starch-hydrolyzing enzymes such as alpha-amylase, glucoamylase, and pullulanase are often used to separate proteins in rice flour by solubilizing and removing starch. In addition to starch hydrolyzing enzymes, cellulase and hemicellulase enzymes have been used to further increase the protein content in rice protein concentrate. In a study by Paraman et al, (2006), alkali and salt treatments for protein extraction from rice endosperm resulted in 86.9 and 87.3% proteins while enzymatic methods with Termamyl and amylase S extracted 85.8 and 81.0% proteins respectively. Alkali- and salt-extracted proteins had higher solubility and emulsifying properties than proteins extracted from enzyme treatments. Comparatively, more favorable protein composition, lower surface hydrophobicity, higher solubility, and a lower degree of thermal denaturation of alkali- and salt-extracted proteins contributed to higher emulsifying and foaming properties than those of enzyme-extracted proteins.

It is relatively easier to extract proteins from rice endosperm with high yield than from rice bran because of the presence of a more complex interaction between components of protein in rice bran limiting extractability efficiencies. Moreover due to increased accessibility to proteins from rice endosperm much work has been focused on

preparing proteins from rice endosperm rather than from rice bran although rice bran has relatively more protein.

Protein extraction from rice bran

Rice proteins, either from rice flour or rice bran are generally extracted using physical and or chemical treatments. Several lipases, carbohydrases or proteases are used in the extraction process to achieve maximum purity of extracted proteins. Characterization and physicochemical properties of rice bran proteins have been studied by many scientists. (Yu et al, 2001, Hamada et al, 2000, Tang et al, 2002, Tang et al, 2003). These studies employed several lipid removing enzymes, endo- and exoproteases to extract maximum amount of proteins from rice bran. Functional properties of rice bran proteins have also been determined in several studies. Wang et al (1999) studied physical properties like hydrophobicity, emulsification, foaming capacity, solubility, and amino acid contents. These properties very much dictate the structure, and hence function of protein composites obtained from rice bran. Tang et al (2002) have extracted proteins from heat-stabilized defatted rice bran. They employed different processes including physical and enzymatic treatments to obtain maximum protein yield. Physical processes included freeze-thaw cycles, sonication, high-speed blending, and high pressure. Enzymatic treatments used amylase, as well as proteases separately or together. They were able to get a maximum of 54-57.8% protein following amylase and protease treatments with sonication. Their study observed that physical treatments in combination with enzymatic treatments improved the protein extractability efficiency. (Tang et al, 2002).

The alkali method for protein extraction from rice bran pioneered by many scientists (Cagampang 1966, Lynn 1969, Chen and Houston 1970, Betschart et al, 1977, Saunders 1990, Landers 1992, Prakash and Ramanathan 1994, Gnanasambandam and Hettiarachchy 1995, Zumbado et al, 1997, Chen 2000) enables high extractability especially from non-heat-stabilized rice bran (Betschart et al, 1977, Gnanasambandam and Hettiarachchy 1995). The alkaline method uses the principle that proteins can be made highly soluble at a very alkaline pH (pH 10 and above), and can then be iso-electrically precipitated out as a residue that can be solubilized eventually, and dried. This method though highly effective in getting most of the protein in a soluble state, causes some structural modifications that render the protein to lose their original function. (De Groot and Slump 1969). Functional properties like foaming, emulsification, and film formation are also affected during the alkali treatment method. (Kinsella, 1981).

Given the complexity in preparing protein isolates from rice bran in any form, scientists are considering direct utilization of the proteins instead of preparing isolates where the desired end products are either protein hydrolysates or peptides for functional ingredient benefits.

Protein hydrolysates and peptides from rice bran proteins

Research has focused on using hydrolysis as a means to obtain better efficiency in protein extraction from rice bran. Furthermore mild hydrolysis of the intact proteins present within rice bran could aid in not only releasing the proteins from bran but also in preparing protein hydrolysates and peptides for several functional properties. Bandyopadhyay et al (2008) prepared protein isolate from full fat rice bran by alkaline hydrolysis and isoelectric precipitation; obtained peptides from partial hydrolysis with

papain under controlled conditions and studied functional properties. From an industrial perspective utility to proteins or partially hydrolysed protein components from rice bran, hydrolyzed protein has been produced and exploited as vegetable protein (Jarunrattanasri et al, 2007).

Functional attributes can be destined by the nature of peptides obtained. For example, certain antioxidative peptide hydrolysates were generated following enzymatic hydrolysis and varied degree of hydrolysis, and it was noted that the hydrolysates that had lesser than 10% DH had exhibited more antioxidative properties (Abayomi et al, 2008). Such studies call for optimization to obtain peptides desirable for specific functionalities. With limited literature information about rice bran protein hydrolysates and peptides exhibiting functional properties, more research is needed particularly to evaluate potential nutraceutical attributes of rice bran peptides like its ability to arrest major disease progression states.

From these studies and also with the inherent complexity of proteins within rice bran it is thought that mild and controlled enzymatic hydrolysis is best suited over chemical hydrolysis (acid or alkaline) for generation of protein hydrolysates and peptides without compromising on functional properties. On the other hand for non-commercial purposes of using rice bran protein isolates, and where purity or yield is not important, protein isolates can be prepared following chemical means (alkaline extraction).

Bioactivity of rice bran

Rice bran obtained from certain pigmented rice seeds for instance has been proven to be anti-proliferative, anti-mutagenic as well as anti-oxidative (Nam et al, 2005). These characters are derived mainly from the phenolics, and other carbohydrate constituents. Such food products can readily be recommended to patients or subjects that

are predisposed to debilitating diseases like cancer. Rice bran's bioactivities have also been documented from a study that investigated whole rice bran extracts on chemical and cell assays. The investigators extracted rice bran from two blackish-purple pigmented (Sanhaehyanghyulla and Suwon 415) and one nonpigmented (Chuchung) brown rice cultivars. The peptides were able to scavenge superoxide anions, hydroxyl radicals, inhibit chemical induced mutagenesis, and also inhibit phorbol-ester induced tumor formation in mammalian cancer cell line (HL-60). The extracts from pigmented rice had a much higher activity than the non-pigmented rice. The authors recommend use of bran obtained from such pigmented rice (seeds) as a source of functional food bearing anti-disease characteristics.

Oryzatensin, an ileum-contracting bioactive peptide obtained from rice albumin was shown to have a sequence of amino acids Gly-Tyr-Pro-Met-Tyr-Pro-Leu-Pro-Arg. This peptide was shown to have immunostimulatory role that was mediated by histamine release. There has since been no other study to test rice or rice bran peptides for bioactivity. However peptides derived from other food proteins that have been evaluated for bioactivities serve as examples for evaluating cereal proteins and peptides as potential nutraceuticals in health and disease.

Gastrointestinal resistance

For bioavailability and for human consumption, peptides or hydrolysates derived from food sources and possessing bioactivities need to be resistant to physical (pH), proteolytic (enzymes) and microbial (microbial flora present in the gut) degradation. Soluble peptides that are resistant to gastrointestinal environment can thus render bioactivity more effectively than non-resistant peptides, and can thus extrapolate as

bioavailable nutraceutical ingredients. Bioactive peptides that impact bodily function are generally inactive within their parent protein, but exert specific function upon hydrolysis of the parent protein. Gastrointestinal (GI) enzymes help to release the peptides, and even a fraction of the compound is sufficient to exert the specific function for normal physiology.

There are three phases of assimilation for dietary proteins, *a)* initiation of proteolysis in the stomach by enzymes like pepsin and highly efficient endo- and carboxy-terminal cleavage in the upper small intestine cavity (duodenum) by secreted pancreatic proteases and carboxypeptidases; *b)* further processing of the resulting oligopeptide fragments by exo- and endopeptidases anchored in the brush-border surface membrane of the upper small intestinal epithelium (jejunum); and *c)* final facilitated transport of the resulting amino acids, di- and tripeptides, across the epithelial cells into the lamina propria. The nutrients from lamina propria enter capillaries for distribution throughout the body (Hausch et al, 2002). Enzymatic hydrolysis by most proteases and peptidases do not actively hydrolyze substituted and conformationally constrained amide bond of proline residues, and hence might lead to abundance of proline residues in gliadins and related proteins from wheat, rye, and barley (Schuppan et al, 2000). This, in turn, would lead to an increased concentration of relatively stable gluten-derived oligopeptides in the gut, some of which would be expected to cross the intestinal barrier to gain access to the subepithelial lymphocytes.

There have been relatively few studies examining bioactive potentials of peptides to evaluate the gastrointestinal resistances of the peptides. The complexity of the human gut has provided a predicament to enable selection of parameters (physical), proteolytic

(enzymes) and microbial (microbial flora present in the gut) for evaluation of gastrointestinal resistance. Ideally animal models closely representing human gut are most suited for determining gastric and intestinal digestive resistances of compounds. Hausch et al (2002) studied the ability of certain gliadin peptides to resist intestinal digestion using pancreatic protease digestion and determining the fate of the peptide. Such and several other studies have used in vitro simulatory proteolytic digestion as primary presumptive models for conducting gastrointestinal evaluation of compounds.

Generation of bioactive peptides from food

The methods for studying bioactive peptides are varied, although many rely on in vitro methods for demonstrating biological activity. Generation and identification of bioactive peptides have been performed in a number of ways. Bioactive peptides are inactive within the parent molecule until released by enzymatic hydrolysis, generally via the digestive process (either in vivo or in vitro) or by food processing effects. Once released, the bioactive peptides are capable of influencing a range of behavioral, gastrointestinal, hormonal, immunological, neurological, physiological, vasoregulatory, and nutritional responses.

Peptides derived from proteins by physical or chemical processing

Technologies involving food processing such as using heat, pH changes, or the ability of microbial enzymes to hydrolyze proteins, e.g., during fermentation can be utilized for generation of biopeptides. Addition of functional groups to peptides to promote functionality as chemical modification have also been in practice ever since the first kind of casein-derived peptides were phosphorylated, like the

caseinophosphopeptides (Mellander in 1950) for enhanced bone calcification in infants. During stages of food processing bioactive peptides have been liberated. For example during manufacture of milk products, hydrolysed milk proteins have been liberated. The plasmin present within milk can contribute to hydrolyze milk proteins to obtain bioactive peptides. Bacterial starter cultures that contain proteases help to break down protein to peptides as also seen during fermentation where long oligopeptides can be released upon breakdown of caesins. Cheese contains phosphopeptides as natural constituents and secondary proteolysis during cheese ripening leads to the formation of bioactive peptides that are angiotensin converting enzyme (ACE) –inhibitory (Pihlanto-Leppälä, 2001).

Peptides derived from proteins by enzymatic hydrolysis

Selection of proteases plays critical roles in liberation of bioactive peptide fragments. Pancreatic enzymes preferably pepsin, and trypsin and chymotrypsin have been popularly used to obtain peptides from food proteins. ACE-inhibitory peptides have most commonly been produced using trypsin. Tryptic, chymotryptic and peptic hydrolysis of casein protein has resulted in many short peptides possessing biological activities like immunomodulatory, ACE-inhibitory, and anti-oxidative properties. (Migliore-Samour et al, 1988, Suetsuna et al, 2000). One of the peptides (Ile-Ile-Ala-Glu-Lys) isolated by Nagaoka et al (2001) isolated from β-lactoglobulin by tryptic hydrolysis was found to have hypocholesterolemic action that suppressed absorption of cholesterol in Caco-2 cells in vitro. Another peptide (Trp-Leu-Ala-His-Lys) derived from β-lactoglobulin has been shown to possess anti-hypertensive activity resultant of ACE-inhibition (Pihlanto-Leppala et al, 2000).

Pepsin-liberated peptides from lactoferrin were found to disrupt the cell membrane for penetration into the cell to cause anti-microbial efffect (Schibli, D.J., and Vogel, H.J. (2000)). There is evidence to suggest that lactoferricin is generated by digestion of lactoferrin in the human stomach (Kuwata et al, 1998).

Other proteases like alcalase, alkaline protease and proteinases have also been used to obtain bioactive peptides mainly from cereal sources like soybean and wheat germ. Such proteases have widely been used to generate peptide hydrolysates on a commercial basis. Table I lists bioactive peptides obtained from various food proteins by enzymatic hydrolysis and their bioactivities.

Advantages of enzymatic hydrolysis over physical processing for generation of bioactive peptides

Generation of bioactive peptides during physical treatments involved in food processing stages can also cause structural and chemical changes with potential detrimental effects to the proteins and bioactive peptides. Such effects can prevent their release due to the formation of hydrolysis-resistant covalent bonds. Meisel et al (2003) has shown effects of dephosphorylation to be attributed to subsequent loss of bioactivity like mineral binding capacity.

Food processing can also damage proteins to such an extent as to render the bioactive peptides either inactive following digestion or prevent them from being released from the parent protein. Damaged proteins are frequently digested in a different manner, as resistant peptide bonds can be generated from heat and/or alkali treatments. Hence, peptides that would not normally occur naturally may be generated (Rutherfurd-

Markwick et al, 2005). The consequences of food processing on the generation of bioactive peptides, therefore, require careful attention. Owing to losses in function, potential damaging effects to proteins and generation of inadvertent peptides, treatments due to physical food processing is not preferred. On the other hand use of enzymatic hydrolysis using digestive enzymes that are specific is preferred over physical processing for the generation of bioactive peptides.

Table I Bioactive peptides and their activities

Protein	Protease	Peptide	Activity
Casein	Trypsin Trypsin-Chymotrypsin Pepsin	Phe-Phe-Val-Ala-Pro Val-Glu-Pro-Ile-Pro-Tyr-Gly-Leu-Phe Tyr-Phe-Tyr-Pro-Glu-Leu	ACE-inhibition Immunomodulation Antioxidative
β-lactoglobulin	Trypsin	Ile-Pro-Ala-Val-Pke-Lys-Trp-Leu-Ala-His-Lys-Ala-Leu-Pro-Met-His-Ile-Arg	Bactericidal ACE-inhibition
Rice albumin	Trypsin	Gly-Tyr-Pro-Met-Tyr-Pro-Leu-Pro-Arg	Ileum contraction Immunostimulation
Soybean	Proteinase S Alcalase	Leu-Leu-Pro-His-His Low molecular weight peptides	Antioxidative Antihypertensive
Wheat germ	Alkaline protease	Ile-Val-Tyr	ACE-inhibition Antihypertensive
Genetically modified soy protein	Trypsin and Chymotrypsin	Arg-Pro-Leu-Lys-Pro-Trp	Antihypertensive

Adapted from Korhonen et al, 2003.

Separation of food proteins/peptides

Proteins and hence, their constituent amino acids are present abundantly in many food sources. Isolation and identification of proteins or peptides of interest for bioactive

functional roles is thus a very important step. However characterization has proven to be a challenging task because many proteins/peptides can possess closely related physicochemical properties (Pouliot et al, 2006).

The separation of bioactive peptides from enzymatic hydrolysates provides scope to create functional foods that can slow disease progressions. Proper analytical separation and purification techniques can alone identify specific amino acid sequences, and hence peptides as important biological modulators. A number of separation techniques are available. Fractionation and purification techniques are normally performed based on where and for what purpose the final product/molecule is rendered applicable. Achieving purity generally requires the use of expensive methods such as liquid and gas chromatography particularly for industrial applications. As a first step in characterization, even before bioactivity can be determined, the generated peptide hydrolysates, say from a food source after extraction are normally subject to at least one of several membrane filtration procedures to fractionate the peptides into molecular sizes for ease of identification of bioactive fractions, and also for further characterization.

Membrane-separation processes

A range of filtration material is being used to fractionate molecules based on the membrane pore sizes. Choice of the appropriate filtration system depends on the type of product needed to be fractionated. For example, an ultrafiltration membrane typically consisting of pore sizes between 1-500 nm is used to fractionate proteins and peptides from a protein hydrolysate. Nanofiltration materials are used to separate smaller molecules like amino acids and salts based on size and charge. They usually operate with membranes of pore sizes between 0.1-1 nm. These filtration methods and some others

must operate under a consistent pressure ranging from 0.1 up to 50 bars. The following table (II) lists the comparative features of membrane filtration methods (Pouliot et al, 2006).

Table II. Membrane filtration methods

	Microfiltration (MF)	Ultrafiltration (UF)	Nanoflitration (NF)	Reverse osmosis (RO)
Pore size	> 0.1 μm	1-500 nm	0.1-1 nm	< 0.1 nm
Seiving mechanism	Size	Size, charge	Size, charge	Size
Separation products	Particles, globules (> 01μm)	Proteins/peptides (1-300Kda)	Amino acids, salts, solutes	Monovalent ions(K, Cl)
Operating pressure	0.1-2 bars	1-10 bars	15-30 bars	30-50 bars

Adapted from Pouliot et al, 2006.

The membranes are normally made up of polymers and bear configurations of hollow fibers, flat sheets, tubules or spiral windings. Owing to the subtle differences in physicochemical properties among mixture of peptides, a fractionation process such as UF or NF is usually preferred over other conventional separation methods. A scheme typical for production of peptides using fractionation methods is presented in figure III.

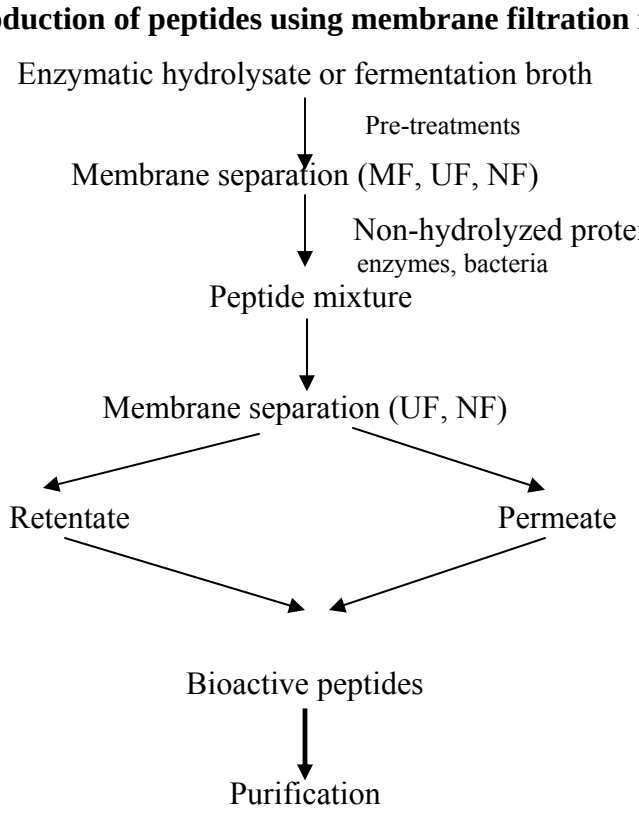


Figure III. Production of peptides using fractionation methods
(Adapted from Pouliot et al, 2006)

To cite examples of using membrane filtration methods, Turgeon and Gauthier used hollow fiber polysulfone ultrafiltration (UF) membranes having molecular weight cutoffs of 30kDa and 1kDa. They were able to obtain a pool of polypeptides that improved overall composition compared to the ratio of commercially available whey protein concentrate to higher protein content. They also obtained peptides lower than 5,000 Da, which could be used as emulsifiers in food industry (Turgeon and Gauthier, 1990). Nau et al (1995) used nanofiltration (NF) process to fractionate β-casein peptides. With 0.1 to 1nm pore size ranges Nau et al showed separation based on both electric

charges and size exclusion. Kimura and Tamano (1986) used charged ultrafiltration membranes to separate amino acids between 75 and 200 kDa. Separation of complex mixture of 15 amino acids using two membranes with molecular cut-offs 500 Da and 1,000 Da respectively was studied by Garem et al (1998). It was observed that separation with the high-molecular weight cut off (MWCO) membrane was pH dependent, and that maximum selectivity was obtained at pH 10 with a low ionic strength. Garem et al also used NF membranes to fractionate β-casein peptides into acidic and basic peptides at pH 8.0.

Soy protein hydrolysates were fractionated using ultrafiltration membranes where the effect of membrane pore sizes on the molecular size distribution as well as functional properties of the protein hydrolysate were studied (Deeslie and Cherian, 2006). Ultrafiltration has also been used to obtain bioactive peptides from soy for ACE-1 inhibitory activity (Cha and Park, 2005). β-lactoglobulin from whey was isolated using ultrafiltration membrane enzymic reactor where the authors were able to retain β-lactoglobulin in the reactor while peptides generated from hydrolysis of α-lactalbumin and serum albumin permeated through the membrane (Sannier et al, 2000).

Purification of food proteins/peptides

Liquid chromatography happens to be the most widely used method for analytic as well as preparative separations of food derived proteins and peptides. Coupling the chromatographic system to conventional UV and fluorescence detectors enables detection of proteins and peptides of interest. Furthermore, structural characterization of purified proteins and peptides can be achieved by methods such as amino acid analysis, mass

spectrometry, SDS–electrophoresis. The recent advent of ionization techniques, starting in 1981 with the introduction of fast atom bombardment (FAB), followed by electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) has made such analyses by mass spectrometry possible for proteins and peptides, with (0.01%) high accuracy. The latter two methods have become important tools for the detection and characterization of large biomolecules, because of their sensitivity, high mass range, and the capacity of isolating from complex mixtures. In addition, mass spectrometry (MS) analysis can be extended to include interfaces for MALDI source to enable precise structural information and characterize and even locate events such as post-translational modifications.

Several preparative chromatographic methods have been used to purify proteins and peptides from a complex mixture. Ion-exchange chromatography, hydrophobic interaction chromatography, affinity chromatography and size exclusion chromatography are the most commonly used chromatographic techniques. These are generally employed as initial separation steps where the peptides of interest are isolated out from a given pool that had exhibited bioactivity as a whole fraction. Following this, reverse phase HPLC has been widely employed with or without coupling to ion-exchange chromatography for efficient purification of the peptide of interest (Le´onil et al, 2000)

Ion-exchange chromatography

Cationic and anionic exchangers (resins) are used to separate molecules based on charge. The retention of the molecule in the matrix (column) depends on the intrinsic charge and also the induced electrostatic interactions. For proteins and peptides the pI (isoelectric point) is the important property for matrix retention. Several proteins and

peptides have been subjected to ion exchange chromatography for effective purification. Lactoferricin, a peptide from the protein Lactoferrin was separated with high purity using cation-exchange chromatography (Dyonisius et al, 1997). Another peptide, Luffacylin was isolated from sponge guard seeds and characterized with the help of DEAE-cellulose ion exchange chromatography (Parkash et al, 2002). First the seed extract was passed through DEAE-cellulose column, and then through a CM-sepharose column and finally purified to homogeneity with a FPLC column. The following profile depicts the elution profile of the guard peptide through a CM-sepharose column.

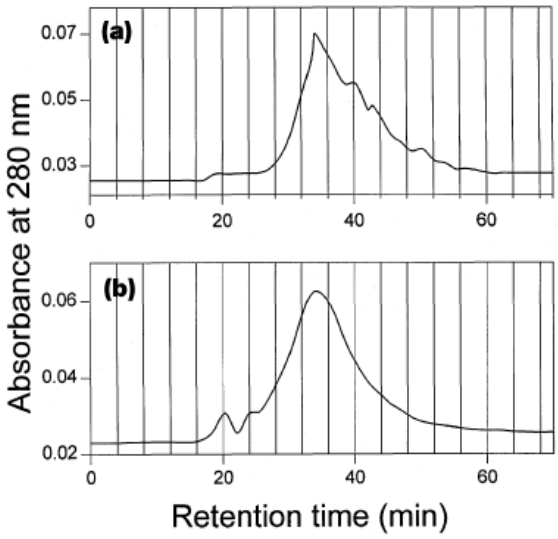


Figure IV. CM-sepharose elution profile of guard peptide with increasing NaCl concentrations.
(Adapted from Parkash et al, 2002).

Size exclusion chromatography (SEC)

SEC can be used to separate relatively large peptides, as the gel matrix is not suited for small peptides of <1kDa. The matrix consists of a gel of a specific pore size through which molecules may pass or get retained on the column owing to their

molecular sizes. In general, smaller molecules diffuse faster and better into pores than larger molecules, resulting in differential retention times. Prakash et al (2002) prepared protein hydrolysates prepared from rice bran employing size exclusion chromatography coupled with HPLC. The molecular fragments ranged between 1 and 150kDa after hydrolysis with alcalase and flavourzyme. Further, anion exchange chromatography of extracted rice bran hydrolysates was performed. Comparison between both alcalase and flavourzyme treatments for de-amidation showed on the chromatogram that the protein hydrolysates treated with flavourzyme had a longer retention time suggesting the occurrence of more negative charges due to de-amidation. The SEC as well as anion exchange chromatograms are presented (Figures IV and V).

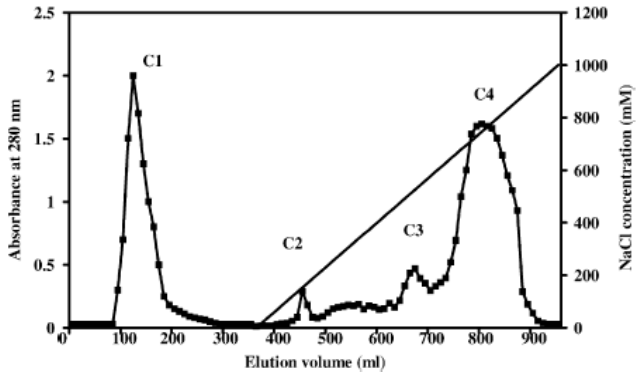


Figure V. SEC-HPLC elution profile of rice bran hydrolysates treated with enzymes.
Adapted from Parkash et al, 2002

For certain proteins and bacteriocins a combination of chromatographic techniques was used for efficient recovery. Stoffels et al (1993) had developed a two-step process of chromatographic separation, first based on adsorption onto hydrophobic interaction chromatography resin, and second on a cation-exchange resin. Stoffels et al used this two-way process for 100% recovery of nisin. In another similar study by Uteng

et al, (2002) bacteriocins were initially bound onto sepharose cation-exchanger resins and eluted with NaCl followed by a reverse phase HPLC process with application of low pressure. Thus they were able to obtain 90% pure fraction of bacteriocins.

Characterization of proteins and peptides can thus be performed using several chromatographic methods or in combination. Efficient purification generally requires manifold optimization trials and hence proves a challenging task. However sophisticated instrumentation and automation aids in faster and less cumbersome protein recoveries.

RP-HPLC for purification of bioactive peptides from food

HPLC/RP-HPLC has replaced classical methods as techniques for separation and purification of peptides. Recovery of biological activity is good with most low molecular weight peptides. Nowadays the most popular column material is reversed phase, in which separation is achieved through partition and through adsorption by unprotected silanol groups. In reversed-phase chromatography, the stationary phase is non-polar (or less polar than in the mobile phase) and the analytes are retained until eluted with a sufficiently polar solvent or solvent mixture (in the case of a mobile-phase gradient). Large peptides (>4,000 Da) are generally analyzed with reverse phase columns having particles with greater pore diameter (300A) so that the molecules will have greater access to the alkyl chains. On the other hand smaller molecular pore sizes (60-100A) are preferred for separation of smaller peptides. The physicochemical characteristics of peptides greatly influence the choice of stationary phase, for example C4 chains are suited for hydrophobic peptides while C8 chains are preferred for hydrophilic or relatively lesser hydrophobic peptides. The most widely used organic modifier for separation for peptides has been acetonitrile due to its high transparency in UV detection at around 200-220 nm. Ion-pairing reagents have been used to increase

efficiency in separation of peptides. Thus ion-pairing coupled solvents operating in reverse phase conditions are proven to yield highly pure peptides.

The formation of opioid peptides by in vitro proteolysis of bovine hemoglobin by pepsin was investigated by RP-HPLC and subsequent off-line identification of collected fractions by FAB-MS (Piot et al, 1992) The same technique was used to isolate opioid peptide from milk fermented with a strain of *Lactobacillus helveticus* and to identify some bioactive peptides released by pepsin and trypsin digestion of UHT milk fermented by *Lactobacillus casei*. Using RP-HPLC off-line with ESI-MS allowed Dionysius and Milne (1997) to identify peptides with antimicrobial activity in tryptic hydrolysate of bovine lactoferrin. Working with the same protein, Shimazaki et al (1998) identified a heparin binding peptide after pepsin hydrolysis, RP-HPLC separation and MALDI-TOF-MS measurements. RP-HPLC–ESI-MS was also reported to be useful to monitor the kinetics of a bioactive production during continuous hydrolysis of β-casein with chymosin in a membrane reactor. (Leonil et al, 2000).

The following table (III) illustrates the types of HPLC used for separation of food peptides, their separation mechanism and characteristics of separation.

Table III. HPLC methods involved with food peptides

HPLC type	Separation mechanism	Characteristics
RP-HPLC	Hydrophobic interaction of the peptides with the stationary phase	High resolution High speed Standard separation procedures Prediction of retention times Good detection and reproducibility Quantitative recovery using volatile buffers Moderate capacity in analytical columns
IE-HPLC	Ionic interaction of peptides with anion exchangers or cation exchangers as stationary phase	Moderate resolution High speed High capacity Prefractionation of peptides by charge Wide range of pH
SE-HPLC	Size exclusion of peptides on hydrophilic polymers as stationary phase	Moderate or low resolution High speed Very high capacity Desalting Good prefractionation by size Molecular weight determination Presence of nonspecific interactions

Adapted from Handbook of Food Analysis by Leo M. L. Nollet

Amino acid analysis

Amino acids are released and destroyed at different rates that depend upon the amino acid composition and characteristics of the proteins in the sample. In order to precisely determine the composition of amino acids present in a protein sample, it is most often necessary to hydrolyze the proteins to amino acids. Specifically acid hydrolysis followed by performic acid oxidation for cysteic acid and methionine sulphone, and an alkaline hydrolysis for tryptophan determination is carried out. A major problem of amino acid analysis in foodstuffs is the destruction of amino acids during acid hydrolysis. Unfortunately, this problem can be greatest with the essential amino acids likely to be limiting in practical diets:

methionine, cystine, lysine, threonine, and tryptophan. Proteins and protein foodstuffs differ so widely in their composition that specific hydrolytic procedures would need to be carried out for each material.

For detection of amino acids and quantification chromatographic techniques including HPLC and Gas-liquid chromatography are in practice. Pre-column derivatization is required for amino acid analysis. Dansyl chloride (5-dimethylamino-1-naphthalene sulphonyl chloride) is frequently used for this purpose, producing fluorescent dansyl derivatives that are separated by a reversed phase column chromatographic procedure. It has been demonstrated that separation particularly of peptides by ion exchange chromatography is more effective when followed with HPLC that uses various non-polar stationary phases, for separation of non-polar amino acid derivatives (Molnar and Horvath, 1977).

Characterization using proteomic tools

With the advent of modern tools that aid in precise and quick identification and detection of structural components within a complex as well as pure mixture, characterization protocols have been made simpler, quicker and reliable. Proteomic tools have been developed keeping in mind several factors involved in characterizing a compound for purity and to retain functionality. Chromatographic separations are being coupled to spectrometers in tandem to enhance separation as well as mass determining capabilities at the same time. Such features allow not only decreasing time but also provide increased sensitivities. Of the several tools available, the most popular and increasingly diverse especially with proteins is the mass spectrometer and its accessory coupling instruments available for characterization studies.

Mass spectrometry

Mass spectrometry (MS) is an analytical technique for the determination of the elemental composition of a sample or molecule. It is also used for elucidating the chemical structures of molecules, such as peptides and other chemical compounds. The MS principle consists of ionizing chemical compounds to generate charged molecules or molecule fragments and measurement of their mass-to-charge ratios (David, 2000). In a typical MS procedure, a sample is loaded onto the MS instrument, and its compounds are ionized by different methods (e.g., by impacting them with an electron beam), resulting in the formation of charged particles (ions). The mass-to-charge ratio of the particles is then calculated from the motion of the ions as they transit through electromagnetic fields.

The two primary methods for ionization of whole proteins are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). MALDI deals well with thermolabile, non-volatile organic compounds especially those of high molecular mass and is used successfully in biochemical areas for the analysis of proteins, peptides, glycoproteins, oligosaccharides, and oligonucleotides (Hillenkamp, 1991)**.** It is relatively straightforward to use and reasonably tolerant to buffers and other additives. The mass accuracy depends on the type and performance of the analyzer of the mass spectrometer, but most modern instruments should be capable of measuring masses to within 0.01% of the molecular mass of the sample, at least up to 40,000 Da. Two approaches are used for characterizing proteins. In the first approach, intact proteins are ionized by ESI or MALDI, and then introduced into a mass analyzer. In the second, proteins are enzymatically digested into smaller peptides using proteases such as trypsin or pepsin, either in solution or in gel after electrophoretic separation. Other proteolytic agents are also used. The collection of peptide products are then

introduced to the mass analyzer. When the characteristic pattern of peptides is used for the identification of the protein the method is called peptide mass fingerprinting (PMF), if the identification is performed using the sequence data determined in tandem MS-MS analysis it is called *de novo* sequencing.

MALDI is based on the bombardment of sample molecules with a laser light to bring about sample ionisation. The sample is pre-mixed with a highly absorbing matrix compound for the most consistent and reliable results and a low concentration of sample to matrix works best. The matrix transforms the laser energy into excitation energy for the sample, which leads to sputtering of analyte and matrix ions from the surface of the mixture. In this way energy transfer is efficient and also the analyte molecules are spared excessive direct energy that may otherwise cause decomposition.

Tandem mass spectrometry (MS-MS) is used to produce structural information about a compound by fragmenting specific sample ions inside the mass spectrometer and identifying the resulting fragment ions. This information can then be pieced together to generate structural information regarding the intact molecule. Tandem mass spectrometry also enables specific compounds to be detected in complex mixtures on account of their specific and characteristic fragmentation patterns.

Peptides fragment in a reasonably well-documented manner. The protonated molecules fragment along the peptide backbone and also show some side-chain fragmentation with certain instruments. There are three different types of bonds that can fragment along the amino acid backbone: the NH-CH**,** CH-CO, and CO-NH bonds. Each bond breakage gives rise to two species, one neutral and the other one charged, and only the charged species is monitored by the mass spectrometer. The charge can stay on either of the two fragments

depending on the chemistry and relative proton affinity of the two species. The most common cleavage sites are at the CO-NH bonds which give rise to the “b” and/or the “y" ions. The mass difference between two adjacent “b” ions, or “y" ions, is indicative of a particular amino acid residue.

Amino acid sequencing

N-terminal amino acid sequence of proteins and peptides helps in determining the correct sequence and number of amino acids that constitute proteins/peptides. Proteins can thus be probed for the occurrences of functional di- or tri-peptides within their structures to identify critical areas that impart significant functional characteristics to the protein. The findings can also provide exact amino acid composition of functional peptides to pharma chemists who could perform chemical *in vitro* synthesis of the same. Invariably N-terminal amino acid sequencing by the dideoxy method is the standard method of choice, and has been employed in several studies involving peptides. Low molecular weight peptides on alcalase treatment of soybean proteins have been isolated and identified to be effective against hypertension. Similarly a sequence of nine amino acids obtained from hydrolysis of rice albumin protein was found to promote immunostimulation. Several peptides ranging in amino acids of about 5-10 have been successfully isolated and proven beneficial against disease characteristics (Table I).

Caseinomacropeptide (CMP), the C-terminal moiety of κ-casein, i.e., residues 106–169, cleaved by chymosin during cheese making, is known to be a mixture of highly heterogeneous glycomacropeptides. CMP was reported to have diverse nutritional and biological significances due to its unique amino acid composition and sialic acid content. In particular, since CMP does not contain the aromatic amino acid residues, several reports

suggested the use of CMP as a protein source for the treatment of phenylketonurea, a hereditary disorder in which aromatic amino acids cannot be metabolized (Leonil et al, 2000).

Peptides derived from canola protein hydrolysate that inhibited ACE-1 activity when purified and analyzed for amino acids showed that the gel permeation chromatography fraction contained 45% content of aromatic amino acids in comparison to 8.5% of the hydrolysate. Two peptides, Val-Ser-Val and Phe-Leu were purified (Wu et al, 2008).

The activity of biofunctional peptides is based on their inherent amino acid composition and sequence. The size of active sequences may vary from two to twenty amino acid residues, and many peptides are known to have multifunctional properties (Meisel and FitzGerald, 2003) e.g., peptides from the sequence 60-70 of *ß*-casein show immunostimulatory, opioid and angiotensin I converting enzyme (ACE)-inhibitory activities. This sequence has been defined as a strategic zone (Migliore-Samour and Jolles, 1988; Meisel, 1998). The sequence is protected from proteolysis because of its high hydrophobicity and the presence of proline residues. Other examples of the multifunctionality of milk-derived peptides include the α_{s1}-casein fraction 194-199 showing immunomodulatory and ACE-inhibitory activity, the opioid peptides α- and *ß*-lactorphin also exhibiting ACE-inhibitory activity and the calcium-binding phosphopeptides (CPPs), which possess immunomodulatory properties (Korhonen and Pihlanto, 2003).

Precise sequence information can help peptides to be synthesized in vitro, and also in large scale where the finished product can be suitable for ingredient formulation functioning as a nutraceutical agent.

Determination of anti-cancer activities and mode of action

Typically, evaluation of growth inhibitory patterns, cell survival and proliferative indexes fall under the battery of screening tests for compounds predicted to have some bioactivity against cancerous states or cancer cell proliferation. Dye exclusion assays are indicators for cell survival while metabolic (cytotoxic) assays are more specific and prognostic in nature. Usually a combination of quantifiable (cell survival) and mechanistic (cytotoxicity) assays are recommended to arrive at a possible mode of action for the anti-cancer substances (Masters, 2000). On the other hand a few assays have been developed solely for the purpose of oncology research. One such is the clonogenic assay which relies also on the effectiveness of an agent on the cancer cell survival and proliferation indexes and differs in that clones of cells that are capable of forming tumors are used rather than non-tumorigenic cancer cells. It finds application especially in research laboratories to determine effects of drugs or radiation on proliferating tumor cells (Franken et al, 2006).

Human Cancers and natural anti-cancer compounds

Colon cancer

Colon cancer or colorectal cancer is the second leading cause of cancer-related mortality in the United States (American cancer society, 2008). Over the past decade, the incidence of colorectal cancer has not decreased, and hence no improvements in the mortality rates. Studies have shown that after the age of 50, men are more vulnerable to colorectal cancer than women (American cancer society, 2008).

Etiology of colon cancer

Most of the colon cancers develop as a polyp – a tissue growth into the center of the colon or the rectum. The type of polyp that normally tends to becomes cancerous is called the

adenoma. Ninety five percent of colorectal cancers are adenocarcinomas (American cancer society, 2008).

There is evidence that dietary components are considered as one of the most important environmental factors in the cause of the colorectal cancer. A number of studies, both clinical and epidemiological thus tend to provide clues that these food compounds may act as suitable markers or dietary determinants that when modified and prepared in a bio-functional form can serve as bioactive compounds in reducing the incidence of colorectal cancers. Food and food compounds that have been identified as those that promote high incidence of colorectal cancers include animal fat, red meats, chocolate, alcohol and refined cereals in Western societies whereas fruits, vegetables, fiber, whole grains and their compounds like folate and calcium have been identified as protective agents against not only colorectal but also other cancers (Levi et al, 1999). Although genetic factors are associated with the etiology of the disease and that they predispose individuals to colorectal cancer, much of this predisposition appears to be related to differences in dietary habits.

Bioactive agents against colon cancer

A number of *in vitro* studies suggest that proteins may be protective against colorectal and many other cancers. For example the whey protein has been proven to reduce the incidence of tumor formation in rats that were chemically induced to have colon cancer (Hakkak et al, 2001). The same study compared the effect of casein protein as diet to that of whey protein in decreasing the tumor formation, and found that casein did not prove as effective in reducing the tumor formation. Also whey proteins have been shown to protect more effectively than red meat against chemically induced formation of aberrant crypt foci

that are considered as markers for colon cancer, first detected in rodents (Bird RP, 1987) and later in Wistar rats (Belobrajdic et al, 2003).

Other proteins investigated for anti-carcinogenic properties of the colon include alpha-lactalbumin. This study demonstrated that at low concentrations of the protein alpha-lactalbumin (10-25 ug/mL) cell growth during the first 3 to 4 days was stimulated. After 4 days, cell proliferation had ceased with low viable cell counts suggesting probably a delayed apoptotic behavior (Sternhagen et al, 2001). In another study dietary intake of soy protein was shown to decrease tumor incidence in rat models of chemically induced colon cancer (Xiao et al, 2008), by inhibiting DNA damage and cell survival of colon epithelial cells through attenuated expression of fatty acid synthase. Animal models, usually for colon tumorigenesis, show that whey protein is superior to other dietary proteins for suppression of tumor development Whey protein components like β-lactoglobulin, α-lactalbumin and serum albumin have also been studied to effect anti-tumorigenic potential against colon cancer (Parodi, 2007).

Use of proteomics in colon cancer

Proteomics has greatly aided in the study of mechanisms of actions of several food components. Some of them include psychoactive compounds present in grape seed extract (Kim et al, 2006), and anti-microbial peptides from cheese extracts (Losito et al, 2006). Molecular targets that these dietary components act upon have been elucidated using the colon cancer cells as a model with the help of proteomic techniques. Employing techniques like the 2-dimensional gel electrophoresis, Mass spectrometry, and tryptic or proteolytic digests of proteins, biological actions of compounds like quercetin on the growth of cancer cells was studied to the level of the proteome within the cell (Davis and Hord, 2005). It was

also found from the same study that quercetin altered the expression of 20 proteins at least 2-fold, and the proteins included several heat shock proteins and annexins. These proteins play significant roles in the physiology of apoptosis, metabolism, and gene regulation (Wenzel et al, 2004).

Irrespective of the compound studied, a clear understanding of the mechanism of anti-cancerous or anti-proliferative effects can be visualized by studying the patterns of protein expression inside the cell that is subjected to study. That way proteomics proves useful.

Food and food components against colon cancer

Proteins and peptides have been shown to affect anti-proliferative effects on colon cancer cells, and these have been confirmed with animal studies. Predominantly flavones and isoflavones have been shown to possess anti-proliferative/anti-cancer effects on colon cancer cell lines. Cereals like rice or rice bran for example, has proteins, and other important components that inhibit cancerous cell proliferation. Tricin for example, present in rice bran is a flavone that is shown to cease colon cancer cell proliferation. Further it inhibits cycloxygenase activity, reduced prostaglandin production and thus interferes in intestinal carcinogenesis in mice (Cai et al, 2005). Certain chemopreventive phenols obtained from brown rice were shown to inhibit growth of both colon and breast cancer cells. Most of the functions that rice bran elicits are attributed to its non-protein components.

There is so far, no literature information on rice bran proteins/peptides that have anti-cancerous effects, or other bioactive functions. Such studies with rice and their components only seem to emphasize that the incidence of both colon and breast cancer are much lower in Asian countries where rice is a staple diet.

Breast cancer

Most breast cancers develop in the ducts (ductal carcinoma), some in the lobules (lobular carcinoma), and the rest in other tissues. Initially lumps form due to fibrocystic changes accompanied with scar-like tissue formation, swelling and pain. Gradually they become invasive. The most common form of breast cancer is the infiltrating (invasive) ductal carcinoma. This develops in the duct, penetrates the duct wall, and invades the fatty tissue of the breast. From there it can spread to any part of the body. This type of breast cancer accounts for about 80% of invasive breast cancers (American cancer society, 2006).

Breast cancer, the most common cancer among women is also the second leading cause of cancer death among women, next to lung cancer. Current statistics point out that there are slightly over 2 million women living in the United States who have been treated for breast cancer (American cancer society, 2008).

Chemoprevention by the use of drugs, vitamins, or other compounds to reduce the risk of developing cancer has gained considerable attention to block or suppress malignant transformations or disease progressions. Many food products are found to contain compounds and proteins that aid in alleviating the disease progression in many cancer states by preventing cancerous cell proliferation. If modified and administered properly these can replace expensive drugs or other invasive treatment modalities of cancer.

Food and food components against breast cancer

Fruits and vegetables are considered good sources of anti-cancerous/anti-proliferative agents (Hocman G, 1989). For instance, the genus Brassica that contains cruciferous vegetables like broccoli, and cauliflower are known to have certain carbohydrate moieties called the glucosinolates that when hydrolyzed cause reversal of chemically induced carcinogenesis (Verhoeven et al, 1997).

Several mechanisms have been hypothesized to understand the mechanism of action of these compounds in mitigating breast cancer. Some of them include apoptosis, arresting cell cycle progression, and also interfering with metablosim. Mammalian breast cancer cell lines investigated with the juices of Brassica family displayed anti-proliferative effects suggesting the presence of substances within the vegetables that are able to prevent cancer progression (Brandi et al, 2005).

Other than compounds having carbohydrate moieties, proteins are also considered good agents to reduce cancer progression. Most important and widely studied is the soy protein isolates. As such soy compounds render cancerous cell proliferation arrest, and added to this the protein isolates and hydrolysates are becoming effective against cancer progression too. In a study that tested the effect of soy protein isolates with breast cancer, colon cancer and also prostate cancer researchers found that the protein isolates reduced the incidences of chemically induced colon as well as mammary gland tumors in mice (Badger et al, 2005).

Cereals like rice, and rice bran contain compounds that can prevent cancerous cell proliferation. Tricin, a flavone extracted from rice bran has been shown to arrest breast cancer cell growth even at submicromolar concentrations. Although Tricin at this stage is not considered a chemopreventive agent for breast cancer, its accumulation in the gastrointestinal tract after consumption tends to reduce the risk of colorectal carcinogenesis propitious (Cai et al, 2004). Among cereals, rice is known to contain phenols that interfere with the progression of cancers particularly, the colon and breast cancer. Bran extracts constituting major phenolics when treated with mammalian breast and colon cancer cell lines reduced the cancer cell viability. Based on the results it has been suggested to consume bran rice rather than

milled white rice because of the presence of abundant phenolics in the former (Hudson et al, 2000).

Role of proteins and peptides in breast cancer

The RGD (Arg-Gly-Asp) peptide sequence is a common site for proteins in blood and the extracellular matrix to bind to and be able to exert biological functions. It has been shown to bind to tumor cells and interfere with cancer cell cycle progression. Peptides designed to possess this particular sequence when tested for their abilities to arrest cancerous cell growth exhibited positive characters. They inhibited tumor growth formation in a dose-dependent manner and also induced apoptosis and G1 phase cell cycle arrest in breast cancer cell lines (Yang et al, 2006).

Plant proteins are also found to interfere with breast cancer cell progression states. A novel protein, calcaelin isolated from the mushroom *Calvatia caelata* was found to reduce the cell viability of breast cancer cells (Ng et al, 2003).

Many proteins express differentially between healthy and cancerous cells. Studies have attempted to identify certain proteins that serve as markers for cancerous states, so that their expression levels can be monitored to study the disease progression (Roberts et al, 2004). Some of them include estrogen receptor-alpha, beta-caesin, bax, and cytokeratin 7.

There is so far, no literature information on rice bran proteins/peptides that have anti-breast cancerous effects, or other bioactive functions.

Lung cancer

Most lung cancers develop along the lining of the bronchi, and with time, cause pre-cancerous changes within the lung. When true cancer develops there is formation of

chemicals required for the development of new blood vessels which in turn allow tumor formation. Once lung cancer cells form, they break away and spread to other tissues (metastasis). Eighty five percent of all lung cancers are of the non-small cell type. Other classifications include squamous cell carcinoma, adenocarcinoma, and large-cell undifferentiated carcinoma (American cancer society, 2006).

Lung cancer is the number one cause of cancer deaths in the United States. In the year 2006, The American Cancer Society had predicted approximately 174,470 new cases of lung cancer (both small cell and non-small cell).

Food and lung cancer

Numerous studies and research have documented the positive links between food, food components and lung cancer. Fruits and vegetables are primary food sources that possess properties that fight against lung cancer. Since smoking is the chief cause of lung cancer the etiology of the formation of lung cancer, needs to be understood clearly in this context in order to provide appropriate treatment modalities. It has been shown that smoking causes oxidative stress, by generating many free radicals (Chow, 1993). This calls for researchers working in the field of nutrition and cancer to look for food sources or food components that possess anti-oxidant properties that can possibly counter the cancerous effect or slow down the disease progression.

Food and food components against lung cancer

Cumestrans are chemical compounds present in green vegetables like beans, peas, spinach, and sprouts. They have been shown to suppress lung cancer disease states. Studies

have also found association between consumption of large quantities of fish and a decreased rate of lung cancer (Takezaki et al, 2001).

Most isoflavones are common anticancer agents in reducing the rate of lung cancer. These isoflavones are present in a wide variety of food products including soybeans, yams and chickpeas.

Some plant seeds exhibit anti-proliferative properties, evident from a study that investigated the effects of methanolic extract of adlay seed on human lung cancer cell line. It was shown that the extract suppressed COX-2 expression via inhibition of gene transcription enabling anti-cancerous effect on human lung cancer cell line (Hung et al, 2003).

Role of proteins in lung cancer

Proteins are also found to exert similar anti-cancerous and apoptotic roles. In a study, milk treatment onto transformed cell lines resulted in an apoptotic picture. The protein responsible was identified as a multimeric form of alpha-lactalbumin. This effect was first observed in human lung cancer cell line (A549). Cell viability was almost completely reduced (98%), and the cells exhibited characteristic features of apoptosis. Other cell lines the same protein affected were a variety of epithelial cell lines (Caco-2, HT-29, NCI) kidney cell lines (Vero, GMK) and mouse cell lines (WEHI 164, B9). (Hakansson et al, 1995).

Animal studies conducted to determine the effects of feeding protein rich diets as against electrolyte or dextrose/amino acid diets observed that progression of pulmonary metastatic disease was reduced, and so did the tumor weight when fed with protein rich diets (casein) (Mahaffey et al, 1987).

There is so far, no literature information on rice bran proteins/peptides that have anti-lung cancerous effects, or other bioactive functions.

Several technological options in the literature available are on the production, preparation, gastrointestinal resistance evaluation, identification of specific bioactivities, and separation and purification strategies for food derived peptides. Depending upon the nature of the bioactivity and the target site it is critical to consider all the options available before conditions can be established for the production of bioactive peptides. With such an understanding peptides derived from rice bran have been prepared and evaluated for anti-cancer activity. In the following chapters the ways adopted for preparation and characterization of anti-cancer rice bran peptides have been documented.

Table IV List of proteins/peptides having anti-cancer effects

Type of cancer	Anti-cancer agent	Source	Reference
Prostate	Proteins	Soy	Bylund et al, 2000
Breast	Protein	Soy	Badger et al, 2005
	Peptides with RGD sequence		Yang et al
	Calcaelin protein	Mushroom Calvatia caelata	Ng et al, 2003
Lung	Alpha-lactalbumin	Milk	Hakansson et al, 1995
	Casein and other proteins	Animal	Mahaffey et al, 1985
Leukemia	Ribosomal inactivating proteins	Seeds of Abrus precatorius	Hideki et al, 2004
	Lfcin-B peptide	Lactoferrin-B protein	Yoo et al, 1997

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CHAPTER 1

Human Colon Cancer Cell Proliferation Inhibition By Peptide Hydrolysates Derived From Heat Stabilized De-fatted Rice Bran

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Abstract

Rice bran, an economical, underutilized co-product of rough rice milling, was used to produce peptide hydrolysates, which were investigated for anticancer activity against colon cancer cells. Protein hydrolysates prepared by Alcalase hydrolysis under response surface design-optimized conditions were treated further to obtain gastrointestinal (GI)-resistant peptide hydrolysates. They were fractionated into >50, 10−50, 5−10, and <5 kDa sizes and evaluated for inhibitory activity on proliferation of human colon (Caco-2 and HCT-116) cancer cell lines by Trypan blue dye exclusion assay and confirmed by the MTS cell titer and the clonogenic assays. GI-resistant <5 and 5−10 kDa sized peptide fractions inhibited growth of Caco-2 cells by 80% compared to controls and non-resistant fractions. Anti-proliferative effects were confirmed by the MTS assay wherein 70% cytotoxicity to Caco-2 cells and 75% toxicity to HCT-116 cells were observed. The 5-10 kDa fraction caused 50% cytotoxicity to Caco-2 cells and nearly 60% HCT-116 cells. Dosage and anti-tumorigenic inhibitory effects of <5kDa fraction on HCT-116 cells revealed that after 72h, 650 μg/mL caused growth inhibition by 80%, signifying high dosage and longer time needed for the <5kDa fraction to show anti-cancer effects. From the clonogenic assay IC_{50} dose of the <5 kDa fraction was calculated to be 770 μg/mL.

These results indicate that <5 kDa peptide fraction of the rice-bran has a potent anti-tumor activity for colon cancer cells.

Introduction

Cereal grains and their components are widely investigated for the presence of bioactive components. Bioactive components possess the ability to impart health benefits or reduce the risk of diseases (Dziuba,et al 1999).

In the United States cancer is the second leading cause of death. The Centers for Disease Control and Prevention (CDC) has estimated nearly 1.4 million new cases of cancer in 2008 and half a million deaths due to cancer occurred in 2007 (Cancer statistic, 2008, American Cancer Society). Colorectal cancer is the second leading cause of cancer-related death in the United States. Over the past decade, the incidence of colorectal cancer has not decreased, and hence no improvements in the mortality rates. Treatment and preventive options for colorectal cancer mainly focus on early detection. There is evidence that dietary components are considered as one of the most important environmental factors in the cause of the colorectal cancer and hence may act as suitable markers or dietary determinants that when modified and prepared in a bio-functional form can serve as bioactive compounds in reducing the incidence of colorectal cancers.

Bioactive peptides comprising 2 to 9 amino acids typically possess specific amino acid sequences comprising mainly hydrophobic groups in addition to proline, arginine or lysine (Kitts *et al*, 2003; Korhonen *et al,* 2003; Dzuiba *et al*, 1999). Several bioactive peptides (Dzuiba *et al*, 1999*,* Li *et al*, 2001; Parkash *et al*, 2002; Gauthier *et al* 2003; Algaron *et al*, 2004, Murakami *et al*, 2004, Quiros *et al* 2005) have demonstrated antioxidant, antiobesity, antiangiogenic, antihypertensive activities, hypocholesterolemic and immunomodulatory effects. Peptides and proteins from food sources have aided in cancer prevention and treatment. For example, whey proteins and alpha-lactalbumin have

been shown to inhibit colon cell proliferation (Sternhagen *et al,* 2001). Cereal grains including soybean, rice, and wheat and their components have been investigated for the presence of bioactive proteins and peptides (Yoshinori, 2006). For example, Oryzatensin, an ileum-contracting bioactive peptide obtained from rice albumin was shown to have an immunostimulatory role (Takahashi, et al 1996). Similarly, proteolytic hydrolysis of soybean protein using Alcalase and Proteinase S enzymes resulted in peptides that were anti-hypertensive and anti-oxidative respectively (Korhonen, 2003). Rice and its components have also been studied to exert specific anti-disease properties like antioxidative, anticarcinogenic and antimutagenic (Nam, 2005). However constituents like proteins and peptides from rice or co-products of rice milling have been studied to a limited extent to be able to confer anti-disease characteristics.

Rice bran is a cheap co-product of rough rice milling having nutrients including B vitamins, minerals and fiber (Juliano, 1985), including oil, which has health benefits (Sugano et al, 1997). It is being used as a low-cost animal feed and the State of Arkansas constitutes over 50% of the overall rice production in the country (NASS, USDA 2007). De-fatting the bran and directly hydrolyzing the high-quality protein using endoprotease can sustainably release peptides in a consistent manner explained earlier. The proteins in rice bran are complexed within carbohydrates and lipids and hence provide difficulties in protein extraction. Hence, direct hydrolysis of heat stabilized defatted rice bran (HDRB) was done to obtain high-quality and high-yield peptides for determining anti-cancer activities. This approach was not only unique but also can prove to be an economical way of producing anti-cancer peptides from rice bran on a large scale.

The objectives were to generate peptides from rice bran by specific food-grade enzymatic hydrolysis, generate gastrointestinal resistant peptides, fractionate them to obtain definite molecular sized peptide fractions, and evaluate for anti-cancer activities on human colon cancer cells.

Materials and Methods

Materials. Heat stabilized de-fatted rice bran was obtained from Riceland foods (Stuttgart, AR), Romicon ultrafiltration system used for fractionation (Koch membrane systems, USA), Food-grade Alcalase enzyme was purchased from a bacterial strain (Novozyme, USA). Human colon cancer cells including Caco-2 and HCT-116 cell lines were purchased from ATCC (Virginia, USA). Dulbecco's modified Eagles's medium, fetal bovine serum, gentamycin were obtained from Hyclone (USA), MTS kit from Promega (USA), MTT kit and McCoy's SA medium from ATCC (Virginia, USA). All other chemicals were purchased from Sigma (MO, USA).

Methods.

Response Surface optimization for enzymatic hydrolysis of HDRB.

A Response Surface Model (RSM) was designed to determine the optimum conditions for enzymatic hydrolysis of rice bran by food-grade Alcalase enzyme. A four by four factorial design was used to evaluate the effect of enzyme concentration, pH, temperature and time of incubation for digestion (Table 1.1). The concentration of the substrate, in this case being HDRB, was kept constant in the factorial design while other parameters like enzyme concentration, pH, temperature and time were varied. Degree of hydrolysis was treated as the response variable.

Sample preparation. HDRB was ground and passed through a 60 mesh sieve. It was stored at 4 ºC until further use. For extraction of protein hydrolysates, the sample was allowed to attain ambient temperature overnight. Then, the sample was mixed and homogenized with deionized water (1:8 w/v) and stirred for 30 min at ambient temperature (Wang et al, 1999). Samples from the mixture were used for RSM optimization of enzymatic hydrolysis to prepare protein hydrolysates.

Degree of hydrolysis. The amount of released alpha-amino groups can be measured by using reagents that react specifically with amino groups, yielding compounds that can be detected spectrophotometrically. Degree of hydrolysis was determined by the ortho-pthaldialdehyde (OPA) method (Nielson, 2001). Serine was used as the standard in the OPA method. The sample solution was prepared by dissolving 0.1g of freeze dried protein hydrolysate obtained after RSM optimization experiment in 10 mL deionized water. Serine standard/sample solution (400 µL) was added to the test tube (time 0) containing 3 mL OPA reagents, mixed for 5 s, let stand for exactly 2 min and the absorbance read at 340 nm in the spectrophotometer.

Degree of hydrolysis was calculated as follows: $DH = h / h_{tot} * 100\ \%$
Where, h is the number of cleaved peptide bonds, h_{tot} is the total number of peptide bonds per protein calculated as $h_{tot} = (\text{serine-NH}_2 - 0.4)$
Serine-NH_2 is the meqv serine NH_2 per g protein,

$$\text{Serine-NH}_2 = \frac{(\text{ODsample} - \text{ODblank}) * 0.9516\ \text{meqv/L} * (\text{sample volume in liter}) * 100 / (\text{sample wt in g} * \text{Protein \%})}{(\text{ODstandard} - \text{ODblank})}$$

Preparation of protein hydrolysates using optimized enzymatic hydrolysis parameters

After determining the degree of hydrolysis by the OPA method on the hydrolysate samples obtained during the RSM optimization experiment, parameters were selected corresponding to a median value of degree of hydrolysis. A median value was selected to ensure that the enzymatic hydrolysis was neither maximum nor minimum. Following the optimized conditions, protein hydrolysates were prepared from HDRB (prepared as described previously). In order to arrest proteolytic digestion after enzymatic hydrolysis the enzyme was inactivated by incubating the reaction mixture at 85 °C for 10 min (*Novo Indistri, 1988*). The mixture was then centrifuged at 3,000 g for 15 min to obtain soluble hydrolysates in the supernatant. Protein contents of the hydrolysates were analyzed before and after hydrolysis following inactivation of the enzyme to ensure no residual hydrolysis.

Treatment of HDRB protein hydrolysate with simulated gastric juice. Simulated gastric juice was prepared as follows: To deionized water (90 mL) in a 100 mL volumetric flask Sodium chloride (0.2 g) and concentrated hydrochloric acid (0.7 mL) were added and stirred for 30 min. The final volume was made up to 100 mL with deionized water and transferred into a beaker. The pH was adjusted to 2.0 and 0.32 g purified enzyme pepsin (Sigma-Aldrich Corp. St. Louis, MO) was added and stirred. The temperature of the solution was maintained at 37 °C. Five grams of freeze dried protein hydrolysate obtained from HDRB were dissolved in the simulated gastric juice and allowed to incubate at 37 °C with constant shaking. After 120 min, the time suitable for

evaluating digestive process in vitro (Takagi et al, 2003), pH was adjusted to 7.2 to inactivate the enzyme. The reaction mixture was centrifuged at 3,000 g for 20 min to obtain soluble peptide hydrolysates in the supernatant. The resistant supernatant hydrolysate was freeze dried and stored at 4°C.

Treatment of gastric juice resistant HDRB peptide hydrolysate to simulated intestinal juice.

Simulated intestinal juice was prepared as follows: To deionized water (90 mL) in a 100 mL volumetric flask potassium phosphate monobasic (0.68 g) and sodium hydroxide 0.2 N (7.7 mL) were added and stirred for 30 min. Final volume was made up to 100 mL and transferred into a beaker. The pH of the solution was adjusted to 8.0 and the mixture maintained at 37 °C. Pancreatin (Sigma-Aldrich Corp. St. Louis, MO) at a final concentration of 0.1% (Kiersten et al, 2005) was added and stirred. The simulated gastric juice treated hydrolysate (in freeze dried form) was dissolved in the simulated intestinal juice and allowed to incubate at 37 °C with constant shaking. After 120 min (Takagi et al, 2003), enzyme was inactivated by heating at 85 ^{o}C for 10 min (Kiersten et al, 2005). The reaction mixture was centrifuged at 3,000 g for 20 min to obtain soluble peptide hydrolysate in the supernatant. The Gastrointestinal (GI) resistant HDRB peptide hydrolysate was stored at 4°C.

Fractionation of GI resistant peptide hydrolysate by ultrafiltration. Fractionation is often needed to concentrate bioactivity, and also ease in characterization purposes. From the fraction that shows better bioactivity peptides can be characterized. Fractionation was

carried out with a Romicon ultrafiltration system (Koch membrane systems, USA) equipped with 1" diameter hollow-fiber polysulfone membrane cartridges. The filtered soluble gastrointestinal resistant peptide hydrolysate was run through sequential ultrafiltration columns with membrane-cartridges having nominal molecular weight cut offs (MWCO) of 50,000 Da, 10,000 Da, and 5,000 Da. In each MWCO cartridge, the peptide hydrolysate was ultra-filtered at a dilution factor of 5. Immediately after the first ultrafiltration, the retentate was dia-filtered twice with two volumes of de-ionized water. The permeates of the first (50,000 Da) ultrafiltration (UF) and the second, dia-filtration step (DF) were pooled and subjected to the second run through the 10,000 Da and then 5,000 Da MWCOs, respectively. The resulting retentates from each of the MWCO were freeze-dried and stored at 4 ^{0}C until use for anti-cancer bioactivity. Only GI-resistant peptide hydrolysates were subjected to fractionation.

Human colon cancer cell culture and anti-cancer activity testing.

Caco-2 was cultured at 37°C in DMEM (Dulbecco's modified eagles medium) in the presence of 10% fetal bovine serum, supplemented with 1mM L-glutamine, Sodium pyruvate, 1mM Sodium bicarbonate and 50 μg/mL gentamycin. HCT-116 cells were grown in McCoy's 5a medium supplemented with 10% fetal bovine serum (FBS; HyClone, Logan UT) and 100 μg/mL of penicillin and streptomycin (GIBCO-BRL).

After cell growth achieved 70%, cell viability was monitored employing the trypan blue dye exclusion assay after peptide treatments. The monolayer was allowed to grow for 2-3 days at 37°C on 24 well flat bottom plates. 100 μL of rice bran peptide fractions at 1 mg/mL protein content were added to the cultures. For positive (inhibitory) control

genistein at 200 and 400 μM in saline and for negative (viability) control saline alone was used. Genistein is a known anti-cancer agent able to arrest the cell proliferation of several cancer cells in vitro. After 24-48 h of treatments the media was removed, and the cells briefly dissociated with 0.1% trypsin-0.53mM ethylene diamine tetra acetic acid (EDTA) solution. Following this 0.5% trypan blue dye mixed in growth medium was added on to each well. Samples were then aspirated from each well and loaded onto chambers in haemocytometer cell and cell counts were taken. This assay reflected the number of viable cells that survived after treatment with peptide samples. This assay was done with GI and non-GI resistant fractions on Caco-2 cells.

Cell proliferation inhibition was determined using the phenazine methosulfate 3-(4,5-dimethyl thiazole-2-yl)]-2,5-diphenyl tetrazolium bromide (MTS) or MTT mix-based cell titer assay. After about 36 h of Caco-2 and HCT-116 cells growth respectively, cells were trypsinized, loaded onto a hemocytometer and counted. Approximately 1000 cells per well were used for growth in 96 well flat bottom plates. The cells were allowed to attach and grow for 36 h. After 36 h old media were replaced with fresh media, and samples of bran peptide fractions were treated with the cells. After 2-4 h exposure of the rice bran peptides to the monolayer, the phenazine methosulfate 3-(4,5-dimethyl thiazole-2-yl)]-2,5-diphenyl tetrazolium bromide (MTS) mix was added at a final volume of 20 μL/100 μL medium and then incubated for an additional 60 min under the same conditions. The reaction was terminated by adding 10% SDS, and the absorbance of formazan measured at 490 nm (*24*). Positive (inhibitory) and negative (viability) controls

were used similarly in the trypan blue dye exclusion assay. All assays were performed in triplicate and the results expressed as mean values ± standard error.

Dosage response of the best fraction that showed anti-cancer properties with both the exclusion assay and the titer based assay was done on HCT-116 cells starting from 50 to 650 μg/mL.

Clonogenic assay. Clonogenic assay is a frequently used cancer research assay that tests the efficacy of compounds on proliferating cancer cells [Hoffman et al, 1991]. When it is done using tumor cells, the ability of the bioactive compound to inhibit tumor progression can be evaluated. To determine the anti-tumorogenic activity of rice-bran peptides, the clonogenic cytotoxicity assay was performed. The clonogenic assay was performed using the HCT-116 cell line following a published procedure [Balusu et al, 2007]. Cells were trypsinized and a single cell suspension was prepared. Cells were plated at a density of 100 cells per 35 mm well. Cells were treated with different concentrations of the rice-bran peptide fraction. After 72 h, the medium was replaced with fresh medium. Cells were allowed to grow for another 8 days and then stained with 0.025% crystal violet. The excess crystal violet was removed with 30% methanol, plates were air dried at room temperature, and numbers of colonies were counted.

Data Analysis. Experimental data were analyzed using JMP 7.0 statistical software with the least significant differences between samples being $P < 0.05$. Response surface method was used as a model to optimize enzymatic hydrolysis of rice bran using Alcalase enzyme.

Results and discussion

Enzymatic hydrolysis of rice bran by Alcalase enzyme and protein contents.

Table 1.1 shows a four factorial Response Surface design optimization with optimum degree of hydrolysis as well as degree of hydrolysis as response values were determined. The four parameters, enzyme concentrations 1.5, 3.5 and 5.0 Alcalase units (AU)), pHs (6.0, 8.0, 10.0), temperatures (40°C, 50°C, 60°C) and incubation times for digestion (30min, 60min, 90min) were fitted to generate optimum concentrations of enzyme, pH, time and temperature for achieving optimum degree of hydrolysis. The three values for each parameter were selected based on the optimum condition for alcalase activity observed with a true substrate (Novozymes, 2004), and near optimum values were chosen. Box-Behenekehn surface response using the JMP 7.0 statistical software was used to evaluate the interactions between parameters to generate optimum values for enzymatic hydrolysis. Degree of hydrolysis at 23.4% was considered optimum.

Digested protein contents (in mg/mL) were obtained for each factorial combination. For example at 1.5AU enzyme concentration, at pH 8, at 40 ºC for 60 min of digestion the digested protein content was found to be 0.993 mg. The prediction profile from the response surface design shown in Figure 1.1 enabled selection of the optimum conditions needed for proteolytic digestion. The prediction profile designated 3.5AU enzyme concentration at pH 8, at 50 ºC, and a 60 min time of digestion for obtaining the optimum value for digested protein content, 1.025 ± 0.36 mg/mL with a DH of 23.4%. For consistent production of peptides from rice bran, optimized conditions were used for performing enzymatic hydrolysis.

Enzymatic hydrolysis has been the main approach to produce bioactive peptides from soybean, wheat, corn, rice, barley, buckwheat, and sunflower. Earlier studies from our laboratory had been directed towards optimizing the extraction of proteins from rice and rice bran using enzymatic and chemical approaches. Tang et al, (2002) had reported extraction of a substantial amount of protein from HDRB. The types of biological activities of the peptides are different with protein source, enzyme, and processing conditions including pH, time and temperature (Wang et al, 2005). In this study HDRB was digested with food-grade proteolytic enzyme to obtain peptide hydrolysates. Of the several food-grade proteases commercially available, cysteine and serine proteases are considered popular candidates for cancer research that not only serve as prognostic markers for certain cancers but also impart functions to certain tumor suppressor genes. Hence, the food-grade protease, Alcalase a commercially available serine protease that prefers uncharged residue's site for action was chosen. In this particular study an endoprotease was used instead of chemical treatments in order to attain specificity and also to maintain the bioactivity at its optimal level.

Colon cancer anti-cancer activity evaluation of GI resistant and non-resistant rice bran peptide hydrolysate fractions.

Trypan blue dye exclusion assay was conducted to determine cell viability after treatment of cells with peptide fractions. This assay evaluates the number of viable cells that remain after exposure of peptides on to Caco-2 cells. Both GI resistant peptides as well as non-GI resistant peptides were tested on the Caco-2 cells.

Figure 1.2 depicts the effect of GI and non-GI resistant peptide fractions on Caco-2 cells. On Caco-2 cells GI-resistant peptide fractions of sizes <5 kDa and 5-10 kDa were found to significantly inhibit the proliferation of viable cells compared to higher molecular weight fractions (>10 kDa and >50 kDa), non-GI resistant fractions and also negative control. There is approximately a three-fold reduction in viable cells between GI-resistant and non-GI resistant fractions that are below 10,000 Da. Similar patterns of inhibition between fractions that are above 10,000 Da were not observed. The positive control used was genistein at concentrations of 200 and 400 μM. At these concentrations genistein is known to inhibit proliferation of cancer cells effectively. It is an isoflavone that is a known anti-cancer agent (Steiner et al, 2008). At 200 μM concentration there were 20,000 viable cells/mL on Caco-2 cell line that was significantly less than the negative control (lacking genistein) that resulted in over 100,000 viable cells/mL. Proteins and peptides have been shown to possess anti-proliferative effects (Dzuiba et al, 1999) on cancer cells, and these have been confirmed with animal studies (data not shown). However there have been very few studies that have tested food proteins/peptides for their ability to suppress human cancer cell proliferation. Predominantly flavones and isoflavones have been shown to possess anti-proliferative/anti-cancer effects on colon cancer cell lines. A study reported inhibitory effects of a casein-derived peptide, β-Casomorphin-7 on human colonic lymphocytes (Elitsur et al, 1991), and in general casein-derived peptides could have the ability to arrest colonic epithelial cell proliferation and also induce apoptosis (Ganjam et al, 1997).

From experimental data it was found that the resistant fractions were more bioactive than the non-resistant fractions. When the peptide fractions were tested for gastrointestinal resistance, resistant peptides were generated, which not only meant that they were exposed to highly specific enzymatic cleavage rendering them to expose more side chains but also may imply their suitability in the digestive tract rendering absorptive and hence consumable properties. In our body peptides are usually generated when proteins pass through the intestine where gastrointestinal enzymes act and release the peptides before absorption. Depending upon the nature of the proteins, peptides and their amino acid sequences, these proteins/peptides may exert specific biological functions. Bioactive peptides when ingested should pass through the intestinal barrier and be transported to the target organs to impart antihypertensive or anticancer activities (Tang et al, 2002). Thus we support our experimental observations that gastrointestinal resistant peptide fractions (<5 kDa and 5-10 kDa) tend to be exposed imparting more bioactivity by effectively inhibiting proliferation of colon cancer cells more than the non-resistant fractions. Moreover soluble peptides were only generated in a process of eliminating organic or other constituents of rice bran that could possibly interfere with bioactivity. We believe although rice bran has organic bioactive components, soluble peptides derived from enzymatic hydrolysis of protein hydrolysates can aid in inhibitory action of human anti-colon cancer cell proliferation, and can have true biological activity in terms of bioavailability and delivery.

In order to confirm the peptide-bioactivity experimental findings, a more specific assay, the MTS based titer assay was conducted. Figures 1.3 depicts MTS based cell titer assay

results for confirming inhibitory actions of peptide fractions on Caco-2 and HCT-116 colon cancer cells respectively. This assay reflects cytotoxicity as an indication of early damage to cells thereby reducing metabolic (mitochondrial) activity. We find there is nearly 70% cytotoxicity to Caco-2 cells and 75% toxicity to HCT-116 cells. The 5-10 kDa fraction caused 50% cytotoxicity to Caco-2 cells and nearly 60% HCT-116 cells. The results of this test confirm that resistant peptide fractions <5 kDa and 5-10 kDa inhibit growth of colon cancer cells more effectively than the non-resistant fractions. For fractions that were >10 kDa pronounced bioactivity was not observed, probably because they are longer in length and hence may have needed more time of proteolytic exposure. Typically biopeptides studied so far have ranged in very short sizes comprising only 2-9 amino acids. Hence, it is not surprising to find less or no activity with peptides longer than 10 kDa. Since the trypan blue dye exclusion assay was enumerative of the cell viability after peptide treatments showing significant bioactivities with the GI resistant fractions, only GI resistant fractions were subjected to the MTS assay.

Dosage and anti-tumorigenic inhibitory effects of <5kDa fraction on HCT-116 cells

Treatment with rice-bran <5 kDa fraction at 24 and 48 h time-points revealed no inhibition in the growth of HCT-116 colon cancer cells compared to the untreated cells, whereas at the 72 h time-point there was a dose- and time-dependent inhibition in the growth of HCT-116 cells (Figure 1.4). The growth of HCT-116 cells was reduced by nearly 80% after the treatment with 650 µg/mL of the <5 kDa fraction at the 72 h time-point. The dose 650 µg/mL was considered the maximum dose that was preparable and

testable with the cancer cell lines because doses higher than 650 μg/mL had lower solubility thereby interfering with the anti-cancer activity assay.

The clonogenic assay also indicates the toxicity of the <5 kDa fraction to HCT-116 cells (Figure 1.5). The cytotoxicity of the <5 kDa fraction was pronounced after treating the cells with 500 μg/mL and that the IC_{50} dose of the <5 kDa fraction was 770 μg/mL. These results indicate that <5 kDa peptide fraction of the rice-bran has a potent anti-tumor activity for colon cancer cells.

The time- and concentration-dependent growth inhibition patterns observed with the <5kDa peptide fraction on HCT-116 cells reveal that the fraction could have a better and positive impact on reducing progression of human colon cancer. Further, the clonogenic assay, which also reflects cytotoxicity confirms that a higher dosage and longer time is needed for the <5kDa fraction to have strong inhibitory or cytotoxicity effect on the growth of especially the tumorigenic colon cancer cells. The dosage and time-dependent growth inhibition pattern may imply that the fractions may be slow-acting. Higher doses may reduce the time needed for killing the maximum number of cells.

Using a presumptive in vitro model like the cell culture to evaluate the bioactive nature of peptides obtained from rice bran by enzymatic hydrolysis, we have documented evidence that rice bran peptides has the potential as human anti-tumor bioactive agents. The findings reported from this study could form a basis for animal and human trials to determine the toxicity and confirmatory nature of these bioactive rice bran peptide fractions. Results from this study have also enabled us to perform characterization studies

on particularly the <5,000 Da sized peptide fraction, so that pure peptides can be obtained for *in vitro* synthesis and sequencing. Identification and characterization of bioactive peptides in the laboratory may generate the possibility of supplementing them into our diets for fighting against cancer or other diseases that have high mortality rates. Cereal grains are known to possess high quality protein which when consumed may be broken down by gastrointestinal enzymes in our body to release bioactive peptides. These could be our next generation natural anti-disease agents delivered at low cost and high efficacy. The use of bioactive peptides in foods, beverages and supplements is well established in Japan and is gaining interest in the US (Starling, 2005). PeptoPro, muscle cells repairing peptide, lactium, antistress bioactive peptide, and C12, blood pressure lowering peptide from casein are available in the US market. As a closing remark, so far peptides from plant proteins have not been commercialized in the US (Wang et al, 2005) and it would bear significance if peptides produced from rice bran can be commercialized to be incorporated into foods to fight diseases like cancer.

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	Enzyme	pH	Temp °C	Time (min)	Response
00+-	3.5	8	60	30	
+00+	5	8	50	90	
+0-0	5	8	40	60	
0000	3.5	8	50	60	
--00	1.5	6	50	60	
0+0-	3.5	10	50	30	
0--0	3.5	6	40	60	
00--	3.5	8	40	30	
0++0	3.5	10	60	60	
+0+0	5	8	60	60	
+00-	5	8	50	30	
0+-0	3.5	10	40	60	
0+0+	3.5	10	50	90	
+-00	5	6	50	60	
-+00	1.5	10	50	60	
-0+0	1.5	8	60	60	
-00+	1.5	8	50	90	
0-0+	3.5	6	50	90	
00-+	3.5	8	40	90	
0-+0	3.5	6	60	60	
++00	5	10	50	60	
0-0-	3.5	6	50	30	
0000	3.5	8	50	60	
00++	3.5	8	60	90	
-0-0	1.5	8	40	60	
-00-	1.5	8	50	30	
0000	3.5	8	50	60	

Table 1.1 Response surface optimization pattern for enzymatic hydrolysis of HDRB.

Parameters: Enzyme concentration (1.5, 3.5 and 5.0 AU), pH (6.0, 8.0 and 10.0), time of hydrolysis (40min, 50min, and 60min), temperature of hydrolysis (40°C, 50°C, and 60°C).

Response: Degree of hydrolysis

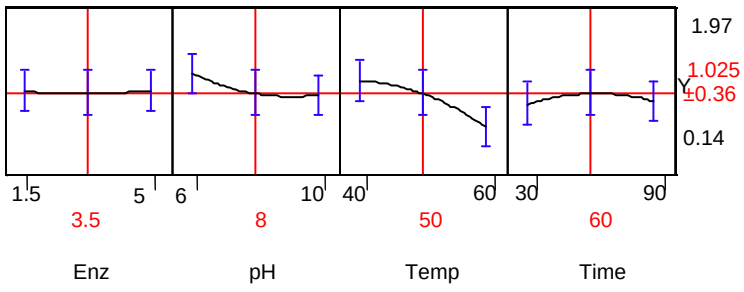

Figure 1.1 Box-Behnken RSM 3 points prediction profiler

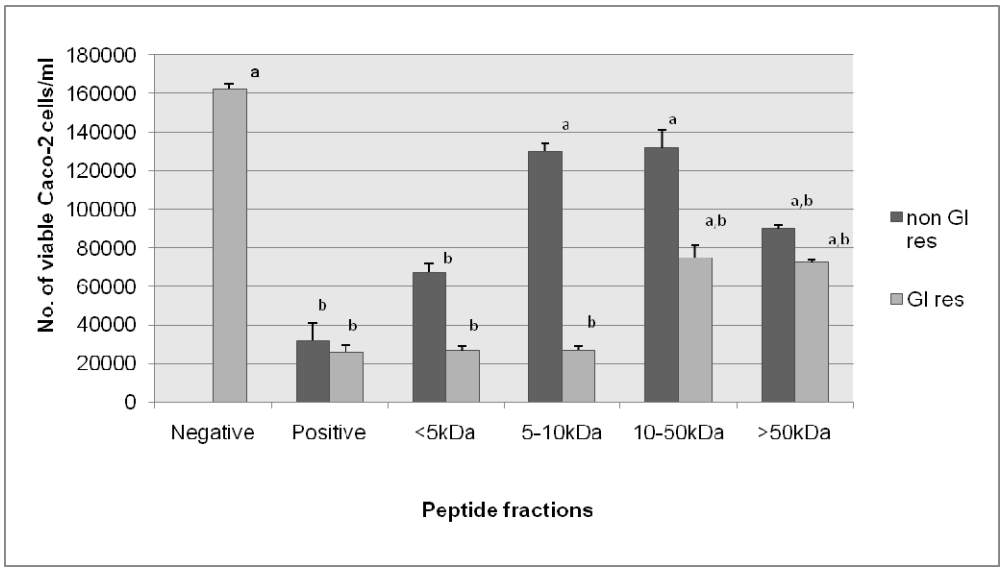

Figure 1.2 Viability of Caco-2 cells after exposure to rice bran peptide fractions measured by Trypan blue dye exclusion assay

Values are means trials ± SE. Values not connected by same letters are significantly different ($P<0.05$),
Negative (viability control): saline
Positive (inhibitory control)**:** Genistein (400µg/ml).
Peptide fractions 1mg/ml

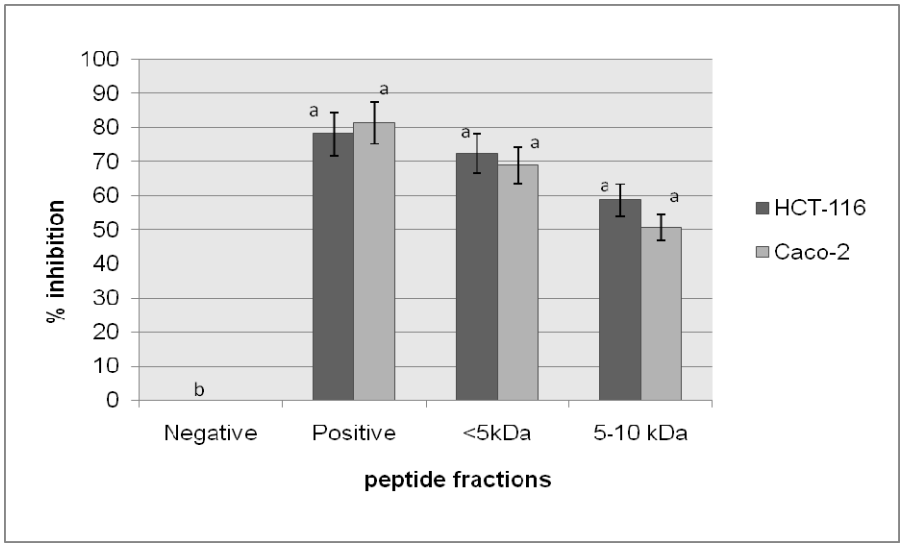

Figure 1.3. MTS confirmatory anti-cancer activities of rice bran peptide fractions (resistant GI fractions) on Caco-2 and HCT-116 cells.

Values are means of two trials ± SE. Values not connected by same letters are significantly different ($P<0.05$).

MTS - (3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

Negative (viability control): saline

Positive (inhibitory contro)l**:** Genistein (400µg/ml)

Peptide fractions 1mg/ml

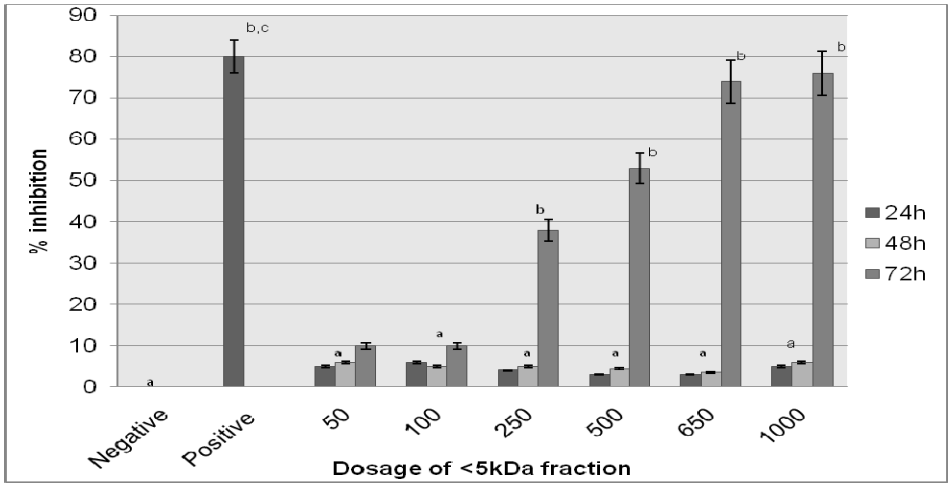

Figure 1.4. Dosage response for anti-proliferative activity of bran peptide <5 kDa fraction with HCT-116 cells.

Values are means of three trials ± SE. Values not connected by same letters are significantly different ($P<0.05$), MTS - (3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
Negative (viability control): saline
Positive (inhibitory control)**:** Genistein (400µg/ml)
Peptide fractions 1mg/ml

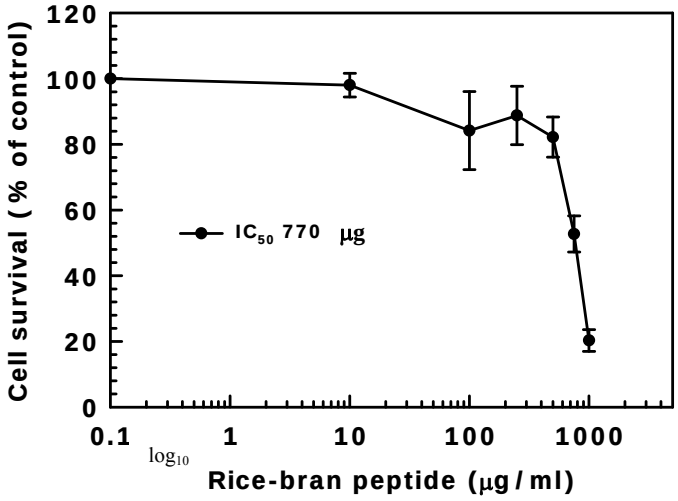

Figure 1.5. Clonogenic toxicity dose response of bran peptide <5 kDa fraction using HCT-116 cells.

Values plotted are means of trials with standard error bars. IC_{50} indicates 50% inhibitory concentration effective to inhibit cancer cell proliferation

CHAPTER 2

Human Breast anti-cancer properties of peptides obtained from heat stabilized defatted rice bran by enzymatic hydrolysis

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[Part of the chapter accepted for publication in The Open Bioactive compounds journal]

Abstract

Breast cancers are the leading causes of cancer related deaths and illnesses in the United States. There is evidence that modification of dietary factors can significantly have a positive effect on reduction in breast cancer progression states. Studies associating peptides and protein hydrolysates derived from cereal sources like soy to bear anti-carcinogenic effects have motivated research on proteins and peptides from rice or co-products of rice-milling such as the bran. These have not been evaluated for anti-disease properties particularly against breast cancer. From earlier study <5kDa and 5-10kDa fractions caused significant anti-cancer effects and so these fractions were selected for testing against breast cancer cells (MCF-7 and HTB-22). The fractions when tested against breast cancer cells caused reduction in breast cancer cell viability monitored by trypan blue dye exclusion assay. MTS cytotoxicity assay confirmed the anti-proliferative effects of the fractions by causing 70% inhibition to growth of MCF-7 cells and approximately 80% on HTB-22 cells. The 5-10 kDa fraction caused approximately 60% cytotoxicity to MCF-7 cells. Time- and dosage-dependent pattern of inhibition of <5kDa fraction shows maximum sensitivity to HTB-22 cells at 650 µg/mL at 72h time-point. This implies that the 5kDa fraction may be effective in inhibiting breast cancer cells with high dosage and long time of exposure.

Introduction

Breast cancers are the most leading causes of cancer related deaths and illnesses in the United States. According to the American Cancer Society, 40,480 estimated deaths for breast cancer (females) were reported in the year 2008 indicating not much reduction in the incidence or mortality rates compared to projected estimates in previous years (Cancer facts, American Cancer Society, 2008). With dietary and environmental factors playing critical roles in the cause and progression of these cancers (Anand et al, 2008), it is necessary to explore natural alternatives that are inexpensive and can be available in bulk.

Several food sources including fruits and vegetables, cereal grains and their components have been investigated for the presence of bioactive proteins and peptides (Mine et al, 2006). Mammalian breast cancer cell lines investigated with the juices of Brassica family displayed anti-proliferative effects suggesting the presence of substances within the vegetables that are able to prevent cancer progression (Brandi et al, 2005).

Of the cereal sources, soy and whey protein hydrolysates have been shown to inhibit proliferation of breast cancer cells in vitro. Particularly exposure to soy diets has proven to reduce the incidence of breast cancer states. Apart from isoflavones present in soy that are anti-cancer agents, protein concentrates, hydrolysates and peptides (lunasin) prepared from soy have also been shown to have anti-carcinogenic effects (Chiesa et al, 2008).

Cereals like rice and rice bran also contain compounds that can prevent cancerous cell proliferation. Tricin, a flavone extracted from rice bran has been shown to arrest breast cancer cell growth even at submicromolar concentrations. Although Tricin at this stage is not considered a chemopreventive agent for breast cancer, its accumulation in the gastrointestinal tract after consumption tends to reduce the risk of colorectal carcinogenesis propitious (Cai et al, 2004). Among cereals, rice is known to contain phenols that interfere with the progression of cancers particularly, the colon and breast cancer. Bran extracts constituting major phenolics when treated with mammalian breast and colon cancer cell lines reduced the cancer cell viability. Based on the results it has been suggested to consume bran rice rather than milled white rice because of the presence of abundant phenolics in the former (Hudson et al, 2000).

Proteins and peptides also are listed as bioactive compounds able to and already documented as imparting several physiological functions including antioxidant, anti-obesity, anti-angiogenic, antihypertensive, hypocholesterolemic and immunomodulatory (Li et al, 2001, Parkash et al, 2002; Gauthier et al, 2003; Algaron et al, 2004; Murakami et al, 2004; Quiros et al, 2005). Studies associating peptides and protein hydrolysates derived from cereal sources like soy to bear anti-carcinogenic effects have motivated research on proteins and peptides from rice or co-products of rice-milling such as the bran. These have not been evaluated for anti-disease properties particularly against breast cancer.

Since natural foods and their components may have the ability to serve as modulators of anti-tumorigenicity either as separate entities or act in synergism (Hemaiswarya et al, 2006), identification of such compounds within natural foods or co-products obtained during the processing of raw foods has promoted nutraceuticals as inexpensive alternatives to expensive therapeutic modalities, especially reducing the risks on those genetically predisposed. Furthermore, the combination of one or more compounds in drug design has proved clinically synergistic benefits toward the treatment strategies for cancer (Lao et al, 2004). These could serve to modify the dietary determinants and hence slow down the progression of cancers.

Bioactive compounds chiefly comprise the aromatic alcohols like phenols, acids, and several organic compounds and have been shown to contribute to mitigate disease pathology in cardiovascular disease and cancer (Kris-Etherton et al, 2002). These compounds need to be released or extracted with suitable solvents and procedures during particular stages of food processing or separately. Our aim was to utilize a naturally rich co-product obtained during the processing of rice. There have been relatively few studies that have focused on determining bioactive properties of rice and its components (Nam et al, 2005). To stress, constituents like proteins and peptides from rice or co-products of rice-milling such as the bran have not been evaluated for anti-disease properties even with evidences of peptides and protein hydrolysates from cereals tending to possess anti-disease characteristics. Hence, we aimed at investigating peptide hydrolysates derived from rice-bran, a co-product of rough rice-milling for a possible inhibitory activity against human breast cancer cells.

Rice-bran is a cheap co-product of rough rice-milling having nutrients including B vitamins, minerals and fiber (Juliano, 1985). In previous studies, it has been shown that whole rice-bran extracts possess anti-proliferative, anti-mutagenic as well as anti-oxidative properties (Nam et al, 2005). Such studies on identifying specific components that could possibly contribute to anti-disease characteristics within rice-bran protein isolates or peptides are very minimal. Thus, the objectives of our study were to prepare peptide hydrolysates from rice-bran using a food-grade proteolytic enzyme, conduct gastrointestinal (GI) treatment to the hydrolysates and fractionate the GI-resistant peptide hydrolysates into >50, 50-10, 5-10 kDa, and <5 kDa fractions and investigate bioactivity against breast cancer cells *in vitro*.

Materials and Methodology

Rice-bran. Heat stabilized de-fatted rice-bran (HDRB) was obtained from Riceland foods (Stuttgart, AR), the Romicon ultrafiltration system was purchased from Koch membrane systems (Massachusetts, USA), and the food-grade Alcalase enzyme derived from a bacterial strain purchased from Novozyme (North Carolina, USA).

Cell lines. Human breast cancer cells, MCF-7 and HTB-26 cancer cell lines were purchased from ATCC (Manassas, Virginia, USA), Dulbecco's modified Eagle's medium, fetal bovine serum, gentamycin, penicillin, and streptomycin were purchased from Hyclone (Logan, UT, USA). MTT kit was purchased from ATCC (Manassas,

Virginia, USA). All other chemicals were purchased from Sigma-Aldrich Chem. Co. (St. Louis, MO, USA).

Direct hydrolysis of HDRB to obtain protein hydrolysates. Based on a response surface method optimization design, HDRB (ground and sieved through 60 mesh) (1:8 w/v in de-ionized water) was digested with 3.5 AU Alcalase (Anson units of enzyme) at pH 8.0 and heated at 50ºC for 60 min. After this, the enzyme was inactivated by heating at 85ºC for 3 min (Novo Industri, 1988). The hydrolysis mixture was centrifuged at 3,000g for 15 min to obtain the hydrolysates in the supernatant. The supernatant was freeze-dried and stored at 4°C until needed.

Generating gastrointestinal (GI) juices resistant bran hydrolysates. It is essential that the peptides be resistant to the GI tract to impart uninhibited biological function and bio-availability. Hence the bran hydrolysates were passed through a simulated gastric and intestinal solution.

Treatment with simulated gastric juice. Five grams of freeze dried bran hydrolysate was dissolved in a simulated gastric juice and allowed to incubate at 37 °C with constant shaking. Simulated gastric juice contained Sodium chloride (0.2 g) and concentrated hydrochloric acid (0.7 mL) in 100mL of de-ionized water maintained at pH 2.0 and at 37 °C. Purified enzyme pepsin (Sigma-Aldrich Corp. St. Louis, MO) (0.32 g) was added and stirred. After 120 min of incubation of bran hydrolysates in he gastric juice, pH was adjusted to 7.2 to inactivate the enzyme. The reaction mixture was centrifuged at 3,000 g

for 20 min to obtain soluble peptide hydrolysates in the supernatant. In the i*n vitro* digestibility studies, the samples are typically examined up to 120 min. The resistant supernatant hydrolysate was freeze dried and stored at 4°C.

Treatment with simulated intestinal juice. The simulated gastric juice treated hydrolysate (in freeze dried form) was dissolved in the simulated intestinal juice and allowed to incubate at 37 °C with constant shaking for 120 min. Simulated intestinal juice contained Potassium phosphate monobasic (0.68 g) and sodium hydroxide 0.2 N (7.7 mL) in 100 mL de-ionized water maintained at pH 8.0 and at 37 °C. Pancreatin (Sigma-Aldrich Corp. St. Louis, MO) at a final concentration of 0.1% was added and stirred. After 120 min (Takagi et al, 2003), enzyme was inactivated by heating at 85 ^{o}C for 10 min. The reaction mixture was centrifuged at 3,000 g for 20 min to obtain soluble peptide hydrolysate in the supernatant. The GI resistant peptide hydrolysate was stored at 4°C.

Fractionation of gastrointestinal resistant peptide hydrolysate by ultrafiltration. Fractionation of the GI resistant peptide hydrolysate was done to separate the peptides into fractions before testing for anti-cancer activity. A Romicon ultrafiltration system (Koch membrane systems, USA) equipped with 1" diameter hollow-fiber polysulfone membrane cartridges was used for this purpose. Membrane-cartridges having nominal molecular weight cut offs (MWCO) of 50,000 Da, 10,000 Da, and 5,000 Da were chosen for a sequential ultrafiltration procedure. The permeate collected after passing the GI resistant hydrolysate through 50kDa membrane was collected and passed through 10kDa membrane resulting in 3 fractions: >50kDa (retentate of first ultrafiltration step), 10-

50kDa and <10kDa fractions (permeates). The <10kDa fraction was passed through 5kDa membrane to result in 2 fractions: 5-10kDa and <5kDa. The fractions were freeze-dried and stored at 4 ^{0}C until use for anti-cancer bioactivity. The GI-resistant bran fractions <5 kDa and 5-10kDa were tested for inhibitory activity against human breast cancer cells MCF-7 and HTB-26 cancer cell lines.

Human breast cancer cell culture and anti-cancer activity testing.

Human breast epithelial MCF-7 cancer cells were cultured in Minimum essential medium (Eagle). For growth the following supplements were added: 2mM L-glutamine, Earle's BSS adjusted to contain 1.5g/L sodium bicarbonate, 0.1mM non-essential amino acids, 1mM sodium pyruvate and 0.01mg/ml bovine insulin, 90%, fetal bovine serum, 10% and 50ug/ml gentamycin. The cells were cultured at 37°C in a humidified 5% CO_2 incubator. Human breast (HTB-26) cancer cells were cultured separately at DMEM (Dulbecco's Modified Eagles Medium) in the presence of 10% fetal bovine serum, supplemented with 1 mM L-glutamine, sodium pyruvate, 1 mM sodium bicarbonate and 50 μg/mL gentamycin. Cells were grown at 37°C in a humidified atmosphere of 5% carbon dioxide.

Breast cancer cell viability evaluation with peptide fractions: Monolayers of MCF-7 and HTB-22 cells were allowed to grow for 2-3 days at 37°C on 24 well flat bottom plates. The media were discarded; cells washed with saline and 100 μL of rice bran peptide fractions were added to the cultures. Separate wells for controls were allocated where for positive (inhibitor) control, genistein (anti-cancer compound) at 200 μM in saline and for negative (viability) control, saline alone were added. After 24-48 h of

treatments the media were discarded, followed by trypsinization of cells (0.1% trypsin-0.53mM ethylene diamine tetra acetic acid solution). The trypan blue dye (indicator of cell viability) in culture media were added to each well. The contents of each well were mixed and carefully aspirated to load onto haemocytometer chambers for counting the numbers of viable cells remaining after exposure of samples and controls.

MTT assay. The MTT [3-(4,5-dimethyl thiazole-2-yl)]-2,5-diphenyl tetrazolium bromide] cell titer assay was performed on the fraction that showed best anti-cancer activity based on the dye exclusion assay. This assay serves as a confirmatory test in determining the anti-cancer activity of rice-bran peptide fraction towards MCF-7 and HTB-22 cells. After 1-2 day culture of MCF-7 and HTB-22 cells respectively in growth media, approximately 1,000 cells/suspension were added on to flat-bottom 96-well plates. The cells were allowed to attach and grow for 36 h. Old medium was replaced with fresh medium and samples of peptide fraction were added to the wells for determining the anti-cancer effect. Concentration- and time-dependent pattern of inhibition was monitored. MTT dye was added after each time-point (12, 24, 36, 48h) followed by the termination of colored formazan product formed upon reaction of the MTT dye with the cells by a detergent solution. The readings were taken at 570 nm in a Tunable Versamax Microplate Reader (Molecular Devices, Sunnyvale, CA). For control, appropriate row or column of wells was left untreated at each time-point. As a separate experiment the dosage response of the best fraction that showed anti-cancer properties with both the exclusion assay and the titer based assay was done on HTB-22 cells starting from 50 to 650 μg/mL.

Data analysis. Experimental data were analyzed using SigmaPlot statistical software (Systat Software, Inc., San Jose, CA) with sample means and standard errors of approximately 10%.

Results and Discussion

Generation of peptide hydrolysates from rice bran

Response surface design was used to optimize conditions for enzymatic hydrolysis of rice bran to obtain peptide hydrolysates. The design selected 3.5 AU enzyme concentration, pH 8.0, 50ºC temperature, and a 60 min digestion time for enzymatic hydrolysis to obtain peptide hydrolysates from rice bran (refer figure 1.1, chapter 1). For obtaining peptides from cereal proteins, enzymatic approach is preferred over the chemical method where extraction by enzymatic hydrolysis yielded substantial amounts of protein when preparing protein isolates (Tang et al, 2002); however, combination of several parameters for a substrate contribute to different types of biological activities (Wang, 2005). In this study HDRB was digested with food-grade proteolytic enzyme (Alcalase) to obtain peptide hydrolysates.

MCF-7 and HTB-22 breast anti-cancer activity of GI resistant rice bran peptide hydrolysate fractions.

Figure 2.1 depicts the effect of GI and non-GI resistant peptide fractions on MCF-7 cells. On MCF-7 cells GI-resistant peptide fractions of sizes <5 kDa were found to significantly inhibit the proliferation of viable cells compared to negative control and non-GI resistant fractions. Negative control was left untreated and was overlaid with saline, supposedly

exhibiting normal pattern of cell growth. There is approximately a three-fold reduction in viable cells as seen with GI-resistant <5kDa fraction compared to the (inhibitory) control. The positive (inhibitory) control used was genistein (soy isoflavone) at concentration of 200 μM, an effective concentration for breast and other cancer cell inhibition. Anticarcinogenic soy protein components including Lunasin (peptide) (Galvez et al, 2001) and a tridecapeptide (MITLAIPVNKPGR) (Maruyama et al, 2003) has been shown to inhibit either breast cancer cell growth or mammary tumor formation in mice. Since then identification of specific protein components from soy or any cereal source is lacking. Demonstration of <5kDa rice bran peptide fraction to cause breast cancers (MCF-7 and HTB-22) cell proliferation inhibition could be indicative of the presence of peptides in cereals like rice and soy to directly inhibit cancerous cell growth as well as tumor formation.

In order to confirm the peptide-bioactivity experimental findings, a more specific assay, the MTS based titer assay was conducted. Figure 2.2 depicts MTS based cell titer assay results for confirming inhibitory actions of peptide fractions on breast cancer cells. This assay reflects cytotoxicity as an indication of early damage to cells thereby reducing metabolic (mitochondrial) activity. We find there is over 70% cytotoxicity to MCF-7 cells with the <5 kDa fraction very similar to the inhibition pattern observed with the positive (inhibitory) control, while on HTB-22 cells the same fraction showed nearly 80% inhibition. The 5-10 kDa fraction caused around nearly 60% cytotoxicity to MCF-7 cells and only about 45% inhibition to the HTB-22 cells. The results of this test confirm

that resistant peptide fractions <5 kDa inhibit growth of breast cancer cells similar to the positive (inhibitory) control, a known anti-cancer agent.

Figure 2.3 shows time- and dosage-dependent pattern of inhibition on HTB-22 cells by the <5kDa fraction. Results obtained from this experiment reveal that the fraction at 24 and 48h time-points showed no inhibition compared to the untreated cells. However, at the 72 h time-point there was a significant inhibition in the growth of HTB-22 cells in a dose- as well as time-dependent manner (Figure 2.3). The HTB-22 cells showed maximum sensitivity at 650 μg/mL of <5 kDa fraction treatment with a 65% inhibition in the growth at 72 h time-point.

The initial screening for determination of anti-cancer activity was done employing the trypan blue dye exclusion assay and confirmed using the MTS assay for anti-cancer properties of the fraction. The time- and concentration-dependent growth inhibition patterns observed with the <5kDa peptide fraction on HTB-26 suggests that the fraction could have a better and positive impact on reducing progression of human breast cancer. The <5kDa fraction showed a better inhibition on breast cancer cells. The dosage and time-dependent growth inhibition pattern may imply that the fractions may be a slow-acting. Higher doses may reduce the time needed for killing the maximum number of cells. Amino acid make-up and the purity of these fractions can determine the extent of inhibitory activity. Isolation and purification of <5 kDa fraction peptide(s) that show similar or enhanced extent of anti-cancer activity may form the basis for fully characterizing the anti-cancer bran peptide.

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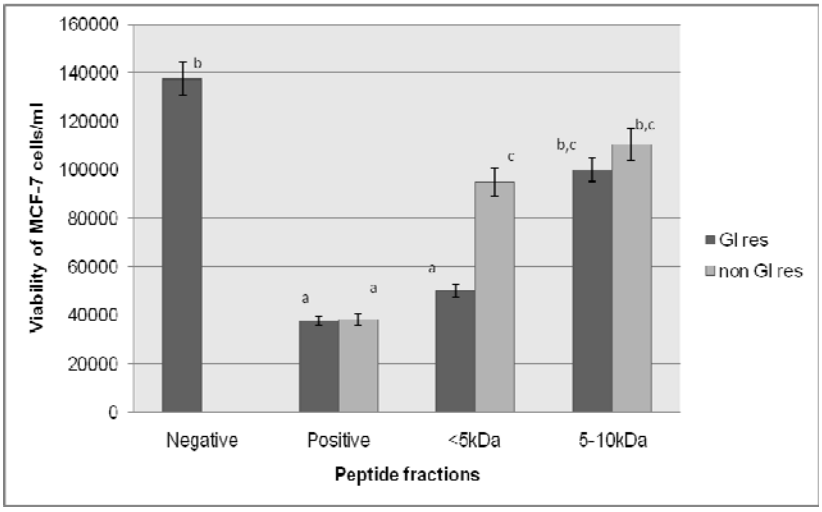

Figure 2.1 Viability of MCF-7 cells after exposure to rice bran peptide fractions measured by Trypan blue dye exclusion assay

Values are means trials ± SE. Values not connected by same letters are significantly different ($P<0.05$), Negative (viability control): saline; Positive (inhibitory control)**:** Genistein (400µg/ml) . Peptide fractions 1mg/ml

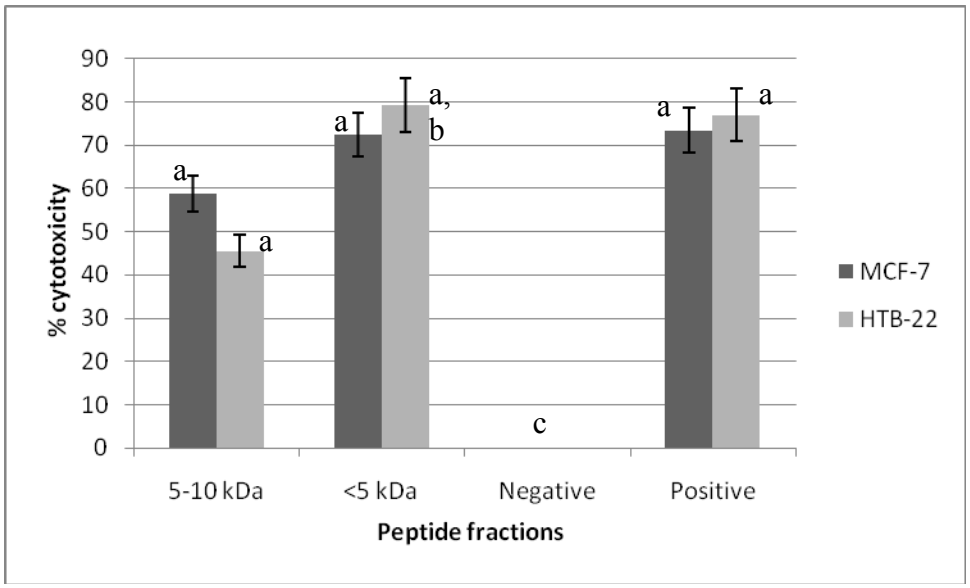

Figure 2.2. MTS confirmatory anti-cancer activities of rice bran peptide fractions on MCF-7 and HTB-22 cells.

Values are means of two trials ± SE. Values not connected by same letters are significantly different ($P<0.05$).

MTS - (3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

Negative (viability control): saline

Positive (inhibitory control): Genistein (200µM).

Peptide fractions 1mg/ml

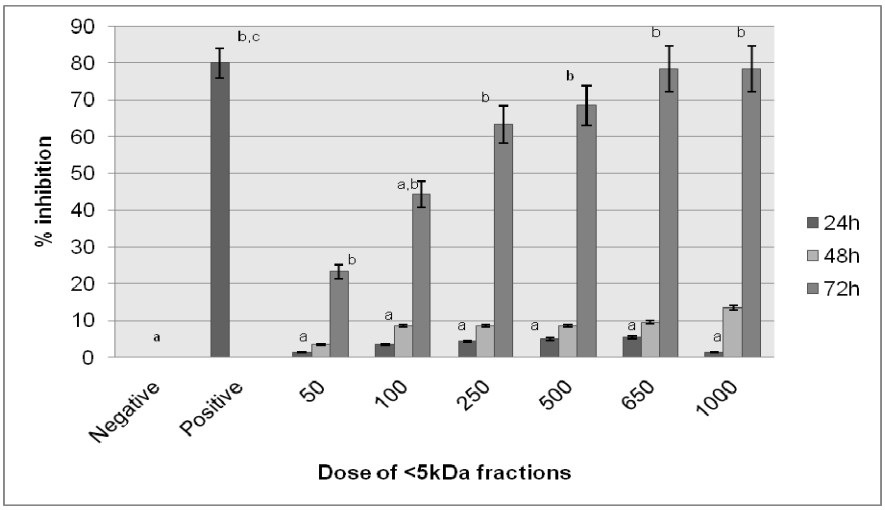

Figure 2.3. Dosage response for anti-proliferative activity of bran peptide <5 kDa fraction with HTB-26 cells.

Values are means of three trials ± SE. Values not connected by same letters are significantly different ($P<0.05$), Negative (viability control): saline; Positive (inhibitory control): Genistein (400µg/ml).

CHAPTER 3

Human Lung and Liver anti-cancer properties of peptides obtained from heat stabilized defatted rice bran by enzymatic hydrolysis.

Kannan A, Hettiarachchy N, Johnson MG, Nannapaneni R.

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Abstract

Lung cancer is the number one cause of cancer deaths in the United States, while Liver cancer is the most common cancer type in developing countries. As an alternative treatment, to reduce the risk of developing cancer the focus is on identifying compounds present in natural foods that could bear lung ang liver anti-cancer properties. Studies on identifying specific components that could possibly contribute to anti-disease characteristics within rice-bran protein isolates or peptides are very minimal. With evidence from earlier work that low molecular sized fractions inhibit growth of colon and breast cancer cells, the objective was to test comprehensively, the same fractions for anti-cancer activity on other cancer cell lines as well. Lung (A-549) and Liver (HepG2) cancer cells were tested using trypan blue dye exclusion and MTS cell titer assays. GI-resistant fractions were found to inhibit proliferation of viable cancer cells significantly compared to control and non-GI resistant fractions. MTS assay confirmed the anti-proliferative effects of the GI-resistant fractions showing 60% cytotoxicity to A-549 cells with the <5 kDa fraction and the same fractions caused more than 80% cytotoxicity to HepG2 cells. The 5-10 kDa fraction caused approximately but caused nearly 80% cytotoxicity to HepG2 cancer cells. The results show that the resistant peptide fractions <5 kDa and 5-10kDa inhibit the growth of liver and lung cancer cells with pronounced activity on liver cancer cells. Our earlier studies on characterizing the <5kDa fraction for

the presence of bioactive pure peptide has validated the anti-cancer effects seen in this study against lung and liver cancer cells. Identification and characterization of bioactive peptides in the laboratory may generate the possibility of supplementing them into our diets for fighting against cancer or other diseases that have high mortality rates.

Introduction

Lung cancer is the number one cause of cancer deaths in the United States. In the year 2006, The American Cancer Society had predicted approximately 174,470 new cases of lung cancer (both small cell and non-small cell) and since then we find a slow decline in new incidence rate. Liver cancer is the most common cancer type in developing countries, less common (2% of cancer deaths) in United States, affecting twice as many men as women. The American Cancer Society has estimated 21,370 new cases in 2008 with 18,410 liver-cancer related deaths in the United States in 2007 (American cancer society, 2008). Chemotherapy, radiotherapy and liver transplantation are the treatment options available for treating liver cancer. As alternative treatment, to reduce the risk of developing cancer focus is on identifying compounds present in natural foods that could bear anti-liver cancer properties.

Numerous studies and research have documented the positive links between foods and food components to lung cancers (Mine and Shahidi, 2006). In spite of intense research and significant progress made in the treatment of cancers like lung and liver over the past two decades, most cancers are still difficult to cure and long-term prevention remains essential, particularly knowing that the time between initial cellular degeneration and clinical detection of a malignancy is about 10–15 years.

Fruits and vegetables are primary food sources that possess properties that fight against lung cancer (Hocman G, 1989). Both alpha-carotene and beta-carotene are protective against liver cancer and lung cancer in cell culture and animal studies. Most isoflavones

are common anticancer agents in reducing the rate of lung cancer. These isoflavones are present in a wide variety of food products including soybeans, yams and chickpeas. Some plant seeds exhibit anti-proliferative properties, evident from a study that investigated the effects of methanolic extract of adlay seed on human lung cancer cell line (Hung et al, 2003). Soy, lycopene, green tea, flaxseed, melatonin, conjugated linoleic acid are some of the nutraceutical agents proposed to reduce the risk of lung cancer (Lockwood, 2007).

Proteins are also found to exert similar anti-cancerous and apoptotic roles. A multimeric form of alpha-lactalbumin was first observed to exhibit characteristic features of apoptosis in human lung cancer cell line (A-549). Other cell lines the same protein affected were a variety of epithelial cell lines (Caco-2, HT-29, NCI) kidney cell lines (Vero, GMK) and mouse cell lines (WEHI 164, B9). (Hakansson et al,1995).

Fruits and vegetables, their parts have been documented as anticarcinogenic to liver cancer cells in vitro. Apple peels have been found to exhibit significant antiproliferative characteristics on HepG2 cancer cells. Antiproliferative effects with other varieties of apples have also been reported by Liu et al (2001).

Although cereals like soy have been used to obtain protein hydrolysates to evaluate for anti-cancer activities, other cereal grains including rice and its components have not been investigated for bioactivities. In previous studies, it has been shown that whole rice-bran extracts possess anti-proliferative, anti-mutagenic as well as anti-oxidative properties (Nam *et al.*, 2005). Such studies on identifying specific components that could possibly

contribute to anti-disease characteristics within rice-bran protein isolates or peptides are very minimal and have motivated research on comprehensively testing bran-peptide fractions on various cancer cell lines in culture. Thus, the objective of our studies is to prepare peptide hydrolysates from rice-bran using a food-grade proteolytic enzyme, conduct gastrointestinal (GI) treatment to the hydrolysates and fractionate the GI-resistant peptide hydrolysates into >50, 50-10, 5-10 kDa, and <5 kDa fractions and investigate bioactivity against lung and liver cancer cells *in vitro*.

Materials and Methods

Rice-bran. Heat stabilized de-fatted rice-bran (HDRB) obtained from Riceland foods (Stuttgart, AR), the Romicon ultrafiltration system was purchased from Koch membrane systems (Massachusetts, USA), and the Food-grade Alcalase enzyme from a bacterial strain purchased from Novozyme (North Carolina, USA).

Cell lines. Human Lung (A-549) cancer and liver (HepG-2) epithelial cancer cell lines were purchased from ATCC, USA. Dulbecco's modified Eagle's medium, fetal bovine serum, gentamycin, penicillin, streptomycin were purchased from Hyclone, USA. MTT kit and McCoy's SA medium were purchased from ATCC (Manasssas, VA). All other chemicals were purchased from Sigma-Aldrich Chem. Co. (St. Louis, MO, USA).

Direct hydrolysis of HDRB to obtain protein hydrolysates.

HDRB (ground and sieved through 60 mesh) (1:8 w/v in de-ionized water) was digested with 3.5 AU Alcalase (Anson units of enzyme) at pH 8.0 and hydrolyzed at 50ºC for 60

min based on a response surface method optimization design. The enzyme was inactivated (Novo Industri, 1988) and the resulting hydrolysate mixture centrifuged at 3,000g for 15 min. The supernatant was freeze-dried and stored at 4°C until further used.

Generating gastrointestinal (GI) juices resistant bran hydrolysates.

It is essential that the peptides be resistant to the GI tract to impart uninhibited biological function and bio-availability. Hence the bran hydrolysates were passed through a simulated gastric and intestinal solution.

Simulated gastric juice was prepared as follows: To deionized water (90 mL) in a 100 mL volumetric flask Sodium chloride (0.2 g) and concentrated hydrochloric acid (0.7 mL) were added and stirred for 30 min. The final volume was made up to 100 mL with deionized water and transferred into a beaker. The pH was adjusted to 2.0. Purified enzyme pepsin from Sigma-Aldrich Corp. St. Louis, MO (0.32 g) was added and stirred. The temperature of the solution was maintained at 37 °C. Five grams of freeze dried hydrolysate were dissolved in the simulated gastric juice and allowed to incubate at 37 °C with constant shaking. After 120 min, pH was adjusted to 7.2 to inactivate the enzyme. The reaction mixture was centrifuged at 3,000 g for 20 min to obtain soluble peptide hydrolysates in the supernatant. In the i*n vitro* digestibility studies, the samples are typically examined up to 120 min. The resistant supernatant hydrolysate was freeze dried and stored at 4°C.

Treatment with simulated intestinal juice: Simulated intestinal juice was prepared as follows: To deionized water (90 mL) in a 100 mL volumetric flask Potassium phosphate monobasic (0.68 g) and sodium hydroxide 0.2 N (7.7 mL) were added and

stirred for 30 min. Final volume was made up to 100 mL and transferred into a beaker. The pH of the solution was adjusted to 8.0 and the mixture maintained at 37 °C. Pancreatin (Sigma-Aldrich Corp. St. Louis, MO) at a final concentration of 0.1% was added and stirred. The simulated gastric juice treated hydrolysate (in freeze dried form) was dissolved in the simulated intestinal juice and allowed to incubate at 37 °C with constant shaking. After 120 min, enzyme was inactivated by heating at 85 ^{o}C for 10 min. The reaction mixture was centrifuged at 3,000 g for 20 min to obtain soluble peptide hydrolysate in the supernatant. The hydrolysate was stored at 4°C.

Fractionation of gastrointestinal resistant peptide hydrolysate by ultrafiltration. Fractionation was carried out with a Romicon ultrafiltration system (Koch membrane systems, USA) equipped with 1" diameter hollow-fiber polysulfone membrane cartridges. The filtered soluble gastrointestinal resistant peptide hydrolysate was run through sequential ultrafiltration columns with membrane-cartridges having nominal molecular weight cut offs (MWCO) of 50,000 Da, 10,000 Da, and 5,000 Da. The permeates of the first (50,000 Da) ultrafiltration (UF) and the second, dia-filtration step (DF) were pooled and subjected to the second run through the 10,000 Da and then 5,000 Da MWCOs respectively. The resulting retentates from each of the MWCO were freeze-dried and stored at 4 ^{0}C until use for anti-cancer bioactivity. The GI-resistant bran fraction <5 kDa was tested for inhibitory activity against human lung (A-549) and liver (HepG2) cancer cell lines.

Cancer cell culture and anti-cancer activity

Human lung A-549 cancer cells were cultured in Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 90%; fetal bovine serum, 10% and 50ug/ml gentamycin at 37°C in a 5% CO_2 incubator. Human liver epithelial cancer cell line HepG2 was cultured at 37°C in DMEM (Dulbecco's modified eagles medium) in the presence of 10% fetal bovine serum, supplemented with 1mM L-glutamine, Sodium pyruvate, 1mM Sodium bicarbonate and 50 μg/mL gentamycin. After cell growth achieved 70%, cell viability was monitored employing the trypan blue dye exclusion assay after peptide treatments. Briefly, the monolayer was allowed to grow for 2-3 days at 37°C on 24 well flat bottom plates. 100 μL of rice bran peptide fractions at 1 mg/mL protein content were added on to the cultures. For positive control genistein at 200 and 400 μM in saline and for negative (viability) control saline alone was used. After 24-48 h of treatments the media were removed, and the cells briefly dissociated with 0.1% trypsin-0.53mM ethylene diamine tetra acetic acid (EDTA) solution. Following this 0.5% trypan blue dye mixed in growth medium was added on to each well. Samples were then aspirated from each well and loaded onto chambers in haemocytometer cell and cell counts were taken. This assay reflected the number of viable cells that survived after treatment with peptide samples.

Cell proliferation inhibition was confirmed using the phenazine methosulfate 3-(4,5-dimethyl thiazole-2-yl)]-2,5-diphenyl tetrazolium bromide (MTS) mix-based cell titer assay. After about 36 h of A-549 and HepG2 cell growth respectively, cells were trypsinized, loaded onto a hemocytometer and counted. Approximately 1000 cells per

well were used for growth onto 96 well flat bottom plates. The cells were allowed to attach and grow for 36 h. After 36 h old media were replaced with fresh media, and samples of bran peptide fractions were treated with the cells. After 2-4 h exposure of the rice bran peptides to the monolayer, the phenazine methosulfate 3-(4,5-dimethyl thiazole-2-yl)]-2,5-diphenyl tetrazolium bromide (MTS) mix was added at a final volume of 20 μL/100 μL medium and then incubated for an additional 60 min under the same conditions. The reaction was terminated by adding 10% SDS, and the absorbance of formazan measured at 490 nm. Positive (inhibitory) and negative (viability) controls were used similarly as used in the trypan blue dye exclusion assay. All assays were performed in duplicates and the results expressed as mean values ± standard error.

Data Analysis. Experimental data were analyzed using JMP 7.0 statistical software with the least significant differences between samples being $P < 0.05$.

Results and Discussion

Enzymatic hydrolysis of rice-bran by Alcalase enzyme The parameters for enzymatic hydrolysis [Alcalase concentrations (0.5, 3.5 and 10 Anson units), pHs (6, 8, 10), temperatures (40°C, 50°C, 60°C) and incubation times for digestion (30 min, 60 min, 90 min)] were previously used and optimized using RSM to obtain optimum peptide hydrolysates from HDRB (Kannan et al, 2008).

Lung and Liver anti-cancer activity evaluation of GI resistant peptide fractions

Trypan blue dye exclusion assay was conducted to determine cell viability after treatment of cells with peptide fractions. This assay evaluates the number of viable cells that remain after exposure of peptides on to HepG2 as well as A-549 cells.

Figure 3.1 depicts the effect of GI and non-GI resistant peptide fractions on A-549 cells. On A-549 cells GI-resistant peptide fractions <5 kDa and 5-10kDa were found to inhibit proliferation of viable cells significantly compared to negative control and their non-GI counterparts, although almost a two-fold inhibition was observed with 5-10kDa fraction. Figure 3.2 depicts the effect of GI and non-GI resistant peptide fractions on HepG-2 cells. On HepG-2 cells GI-resistant peptide fraction <5 kDa was alone shown to inhibit proliferation of viable cells significantly compared to non-GI resistant fraction and negative control. The positive (inhibitory) control used was genistein at concentration of 200 μM. It is an isoflavone that is a known anti-cancer agent, able to arrest the growth of both lung and liver cancer cells in vitro. Other cereal extracts and protein hydrolysates have shown positive effect on the inhibition of both lung and breast cancer cell proliferation on cancer cells. However there have been very few studies that have tested pure components for their ability to suppress human cancer cell proliferation specific to each organ and particularly none that have been made GI-resistant.

When the peptide fractions were tested for gastrointestinal resistance, resistant peptides were generated, which not only meant that they were exposed to highly specific enzymatic cleavage rendering them to expose more side chains but also may imply their

suitability in the digestive tract rendering absorptive and hence consumable properties. In our body peptides are usually generated when proteins pass through the intestine where gastrointestinal enzymes act and release the peptides before absorption. Depending upon the nature of the proteins, peptides and their amino acid sequences, these proteins/peptides may exert specific biological functions. Bioactive peptides when ingested should pass through the intestinal barrier and be transported to the target organs to impart antihypertensive or anticancer activities. Thus we support our experimental observations that gastrointestinal resistant peptide fractions (<5 kDa and 5-10 kDa) tend to open their chains up imparting more bioactivity by effectively inhibiting proliferation of both lung and liver cancer cells more than the non-resistant fractions. Moreover soluble peptides were only generated in a process of eliminating organic or other constituents of rice bran that could possibly interfere with bioactivity. We believe although rice bran has organic bioactive components, soluble peptides derived from enzymatic hydrolysis of protein hydrolysates can aid in inhibitory action of human anti-lung and liver cancer cell proliferation, and can have true biological activity in terms of bioavailability and delivery.

In order to confirm the peptide-bioactivity experimental findings, a more specific assay, the MTS based titer assay was conducted. Figure 3.3 depicts MTS based cell titer assay results for confirming inhibitory actions of peptide fractions on lung and liver cancer cells respectively. This assay reflects cytotoxicity as an indication of early damage to cells thereby reducing metabolic (mitochondrial) activity. We find there is over 60% cytotoxicity to A-549 cells with the <5 kDa fraction and the same fractions caused more

than 80% cytotoxicity to HepG2 cells. The 5-10 kDa fraction caused around 50% on A-549 but caused nearly 80% cytotoxicity to HepG2 cancer cells. The results of this test confirm that resistant peptide fractions <5 kDa and 5-10kDa inhibit growth of liver cancer cells more effectively than on lung cancer cells.

The findings reported from this study could form a basis for animal and human trials to determine the toxicity and confirmatory nature of these bioactive rice bran peptide fractions. Our earlier studies on characterizing the <5kDa fraction for the presence of bioactive pure peptide has validated the anti-cancer effects seen in this study against lung and liver cancer cells. Identification and characterization of bioactive peptides in the laboratory may generate the possibility of supplementing them into our diets for fighting against cancer or other diseases that have high mortality rates.

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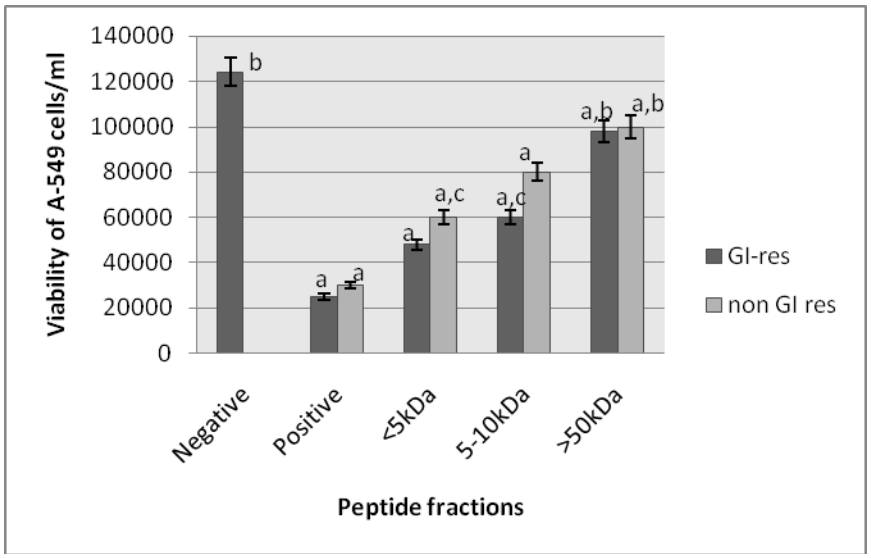

Figure 3.1. Viability of A-549 cells after exposure to rice bran peptide fractions measured by Trypan blue dye exclusion assay

Values are means of two trials ± SE. Values not connected by same letters are significantly different ($P<0.05$)

neg control: saline

pos control: Genistein (200μM)

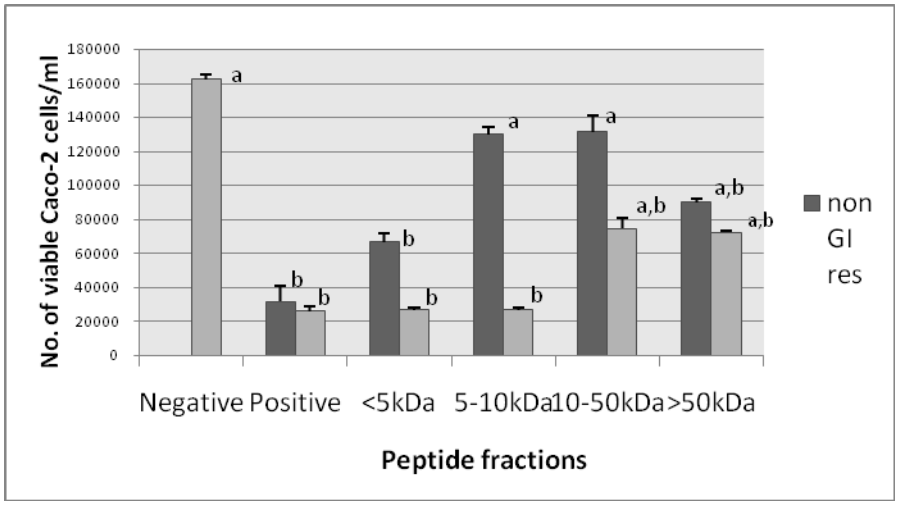

Figure 3.2. Viability of HepG2 cells after exposure to rice bran peptide fractions measured by Trypan blue dye exclusion assay

Values are means trials ± SE. Values not connected by same letters are significantly different ($P<0.05$), Negative (viability control): saline; Positive (inhibitory control)**:** Genistein (400µg/ml) . Peptide fractions 1mg/ml

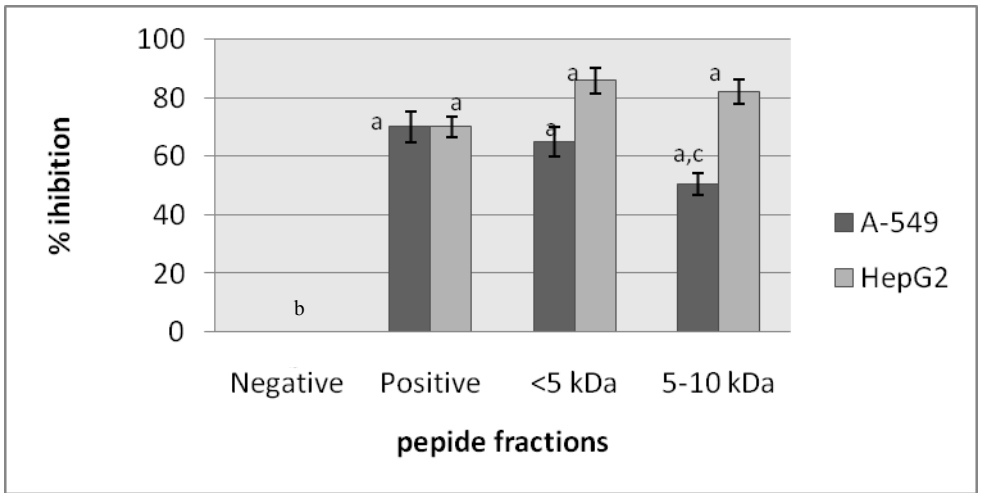

Figure 3.3. MTS confirmatory anti-cancer activities of rice bran peptide fractions on A-549 cells and HepG2 cells.

Values are means trials ± SE. Values not connected by same letters are significantly different (P<0.05),
MTS - (3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
Negative (viability control): saline;
Positive **(**inhibitory control**)**: Genistein (400µg/ml)
Peptide fractions 1mg/ml

CHAPTER 4

Purification and characterization from promising anti-cancer peptide fraction, amino acid analysis and sequencing.

Abstract

Specific peptide sequences with specific amino acids sequences are thought to be modulators of biological function. Characterization generally involves separation, purification, and detection of structural components of the peptide or protein under investigation. This will enable bioactive peptides to act as functional foods that can slow disease progressions. Based on earlier findings that rice bran peptide fractions have the ability to cause growth arrest in colon breast lung and liver cancer cell types in vitro, the <5kDa fraction originally separated by pressure-driven membrane based separation (ultrafiltration) that showed significant anti-cancer effects was subjected to further characterization to yield pure peptide(s) that show similar or enhanced anti-cancer properties. Purification by ion exchange followed by reverse phase HPLC was done to obtain single pure peptide(s) from the 5kDa fraction. 50mM Sodium Chloride elutae from ion exchange column caused appoximately 75% inhibition to colon and liver cancer cells growth and, 60% and 68% inhibitory activities on lung and breast cancer cells respectively. The eluate was purified using HPLC using peptide-specific column. It was observed that the 60-70 minute peak showed enhanced anti-cancer activity, namely. 84% inhibition on colon, 80% on breast and 84% on liver cancer cells. Accurate molecular mass of the pure peptide by MALDI-TOF revealed a mass of 685.378 Da. Amino acid analysis revealed the presence of Glutamic acid, Proline and Arginine. Tandem mass spectrometry for determining the amino acid sequence of the pure peptide(s) was done

using post-source decay fragmentation analysis. The sequence of the pure peptide read Glu-Gln-Arg-Pro-Arg (EQRPR).

Thus a pure multiple-site anti-cancer pentapeptide was isolated and characterized from rice bran.

Introduction

Specific peptide sequences with specific amino acids sequences are thought to be modulators of biological function. Several bioactive peptides have been characterized fully from food proteins using a systematic approach involving several steps and methods. Characterization generally involves separation, purification, and detection of structural components of the peptide or protein under investigation. Separation of bioactive peptides from enzymatic hydrolysates provides scope to create functional foods that can slow disease progressions. A number of food-derived bioactive (antioxidative) peptides, usually composed of 3 to 16 amino acid residues, have been isolated from these hydrolysates. Among these peptides, some contained hydrophobic amino acids (Val or Leu) at the N-terminus, Pro, His, or Tyr and in sequences containing mainly acidic acid residues (Glu, Asp) (Saiga et al., 2003).

Proper analytical separation and purification techniques can identify specific amino acid sequences, and hence peptides as important biological modulators. A number of separation techniques are available. There are pressure-driven membrane based separation techniques like microflitration, ultrafiltration, nanofiltration, and reverse osmosis. Soy protein hydrolysates were fractionated using ultrafiltration membranes where the effect of membrane pore sizes on the molecular size distribution as well as functional properties of the protein hydrolysate were studied (Deeslie and Cherian, 2006). Ultrafiltration has also been used to obtain bioactive peptides from soy for ACE-1 inhibitory activity (Cha and Park, 2005). β-lactoglobulin from whey was isolated using ultrafiltration membrane enzymic reactor where the authors were able to retain β-lactoglobulin in the reactor while peptides generated from hydrolysis of α-lactalbumin

and serum albumin permeated through the membrane (Sannier et al, 2000). Peptides identified in milk as a result of proteolysis after ultrafiltration ranged in sizes from 1023 to 2000 daltons (Wedholm et al, 2008).

As the subsequent stage in characterization, purity becomes an important criterion to concentrate all of the bioactivity within a pure compound. Purification of peptides ideally requires preparative mode of chromatography that relies on ion exchange, hydrophobic interaction, size exclusion, or affinity. These are generally employed as initial purification steps where the peptides of interest are isolated out from a given pool that had exhibited bioactivity as a whole fraction. Following this, reverse phase HPLC has been widely employed with or without coupling to ion-exchange chromatography for efficient purification of the peptide of interest (Le´onil et al, 2000). Prakash et al (2002) prepared protein hydrolysates prepared from rice bran employing size exclusion chromatography coupled with HPLC. The molecular fragments ranged between 1 and 150 kDa after hydrolysis with alcalase and flavourzyme. Adipogenesis inhibitory peptide was isolated and purified from black soybean (Rhynchosia volubilis Lour.) hydrolysate using systematic approach of separation by ultrafiltration with 3 and 10 kDa cutoffs MWCO, gel filtration chromatography (Superdex Peptide 10/300 GL column), and reverse-phase high-performance liquid chromatography (C18 column). The peptide was identified as Ile-Gln-Asn (Kim et al, 2007).

To assess purity and further characterize the structural components within the bioactive peptide, various proteomic tools can be used. Several food peptides have been characterized using Mass spectrometry (MS), a versatile tool not only for determining accurate molecular sizes but also for providing structural information of the pure

compound. MS is by far the most informative technique for determining the purity of protein/peptide samples. The mass spectrum provides the number of components present and an indication about the length of the proteins/peptides. It has been widely used as a modern tool to characterize peptides and proteins present in foods that can impart bioactive properties. MS in conjunction with database searching plays an increasingly important role in the characterization of peptides. Of the ionization modes, Matrix assisted laser desorption ionization (MALDI) has made it possible to not only determine the accurate mass of peptides obtained in a hydrolysate of food proteins but also is able to identify the purity of a pure protein or peptide, determine its fragmenting patterns and also determine the *de novo* sequence of the amino acids.

The shorter the peptides the more accurate will be the prediction by MS-MALDI of the sequence, nevertheless the score for predicting the best hit for sequences present in longer peptides can enable identification of C-terminal amino acids to a reasonable accuracy. Although MS was applied more popularly to biologically important peptides, its use is growing in food peptide characterization as well. Characterization of dairy products, proteins and peptides in fish, legumes and cereals, cheese, and milk proteins have become possible using MS. Lunasin, an anti-cancer peptide purified from soybean seeds was characterized using MALDI peptide mass mapping (Jeong et al, 2003). The identity of lunasin was established by comparison of the peptide mass map of the putative peptide obtained from in-gel tryptic digest with predicted masses of lunasin cleaved by trypsin (Shevchenko et al, 1996).

Precise characterization of peptides hence becomes a valuable tool in ascribing each peptide to its bio-functional role. Because peptides fragment in a sequence specific

way, the original amino acid sequence can be derived from the fragment ion signals. Post source decay (PSD) has been used successfully to obtain amino acid sequence information for many peptides (Spengler et al, 1997).

In this context and based on the findings that rice bran peptide fractions have the ability to cause growth arrest in certain cancer cell type proliferation, the <5 kDa fraction originally separated by pressure-driven membrane based separation (ultrafiltration) that showed significant anti-cancer effects was subjected to further characterization to yield pure peptide(s) that show similar or enhanced anti-cancer properties. Hence the objectives were to subject the <5 kDa peptide fraction to ion exchange and HPLC purification to obtain single pure peptide(s). This was followed by evaluation of single pure peptides for similar or enhanced anti-cancer activity to that of the <5 kDa fraction. The pure peptide that showed anti-cancer activity was subjected to mass spectrometry analysis for determining the accurate molecular mass, amino acid analysis and tandem mass spectrometry for determining the amino acid sequence of the pure peptide(s).

Materials and Methods

Hp 1090 series, HPLC system, reverse-phase C-18 peptide column, analytical grade, sephadex G-75 resin from Pharmacia biotech AB, Uppsala Sweden, biopore C18 preparative HPLC column, amino acid analyzer from Beckman Coulter, Bruker Reflex III (Bruker Daltonics GMBH, Bremen, Germany) and Bruker Ultraflex II time-of-flight mass spectrometers at the Statewide mass spectrometry facility, University of Arkansas. All other chemicals purchased were of HPLC grade and purchased from Sigma, MO, USA.

Methods

Ion exchange chromatography

A sephadex G-75 ion exchange resin was packed into a glass column and equilibrated with 10mM phosphate buffer, pH 8.0. 10mL of < 5kDa peptide hydrolysate (~1mg/mL protein concentration) was loaded onto the column at 1mL/min flow rate. The elution was started by washing the unbound hydrolysate eluted with 10mM phosphate buffer till about 5 bed volumes. After washing, the hydrolysate was eluted using 10mM phosphate buffer containing 50mM NaCl followed by elution with 10mM phosphate buffer containing 100mM NaCl for a total 5 bed volumes. The eluates were collected, concentrated in an Amicon concentrator with buffer exchange and stored at 4°C until used for anti-cancer activity determination. The anti-cancer activity of the eluates obtained after ion-exchange was determined using the MTS as described in earlier chapters.

Preparative HPLC purification of IEC eluate showing anti-cancer activity

Preparative scale peptide-specific column (Biopore Prep ID 22 x L 250 mm part # 34955) was used to separate peptides from the IEC eluates that showed better anti-cancer activity and the absorbance of the eluate monitored at 215nm. The gradient from solvent A (1.2mlTFA/1000ml deionized water) to solvent B (0.1%TFA in Acetonitrile:water 50:50) was varied from 100% solvent A to 100% solvent B over 80 minutes at 2ml/min flow rate monitored at 215nm. The peaks were collected and tested for anti-cancer activity, and the peak that showed anti-cancer activity was fully characterized using mass spectrometry, amino acid analysis and sequencing.

Amino acid analysis

A modified method of AOAC 982.30a (1990) was used for hydrolyzing purified peptides. 10mg of peptide samples were hydrolyzed in 10mL of 6.0 N HCl under vacuum at 150°C for 12 h and evaporated under nitrogen at 60°C. Sodium diluent buffer pH 2.2 (1 mL) was added to the dried peptide, filtered and the filtrate was analyzed for amino acids. The peptides were pretreated with performic acid prior to hydrolysis to preserve cysteine and methionine, while alkali hydrolysis was conducted to determine tryptophan (AOAC 982.15, 2000). Amino acid analysis of the filtrate was conducted on an automated amino acid analyzer (Beckman 6300, Beckman Instruments, Inc. Palo Alto, CA) at a flow rate of 0.67mL/min (0.44 mL/min for buffer solutions and 0.23 mL/min for ninhydrin solution). Sodium citrate buffers (pH 3.3, 4.3 and 6.3) were used as eluents. The amino acid contents (in g/100g sample) were quantified by comparing them with amino acid profiles from external amino acid standards as follows ($Peak_{sample}/Peak_{standard}$) x $Concentration_{standard}$ x $MW_{standard}$

Mass spectrometry characterization of pure peptide

For preliminary intact mass determination, 1.0 µl of the HPLC purified peptide was mixed with 1.0 µl saturated HCCA and spotted on Bruker MTP 384 ground stainless steel MALDI target. MALDI-TOF (time of flight) mass spectra were acquired on a Bruker Reflex III (Bruker Daltonics GMBH, Bremen, Germany) and Bruker Ultraflex II time-of-flight mass spectrometers operated in the positive-ion reflectron mode. High resolution exact mass of the same peptide was obtained by using MALDI Ionspec 9.4 T ion cyclotron resonance (ICR) mass spectrometer. For the exact mass measurements 2, 5

dihydrobenzoic acid (DHB) was used as the MALDI matrix and followed exactly the same spotting technique.

MALDI fragmentation

Fragmentation of intact peptide ions were performed to obtain sequence information using MALDI post source decay studies. MALDI-PSD fragmentation of these ions were analyzed using the "Lift" mode in Bruker Ultraflex II (Bruker Daltonics GMBH, Bremen, Germany) time-of-flight/ time-of-flight mass spectrometer (TOF/TOF MS).

Data analysis and *de novo* sequencing

Fragmentation pattern obtained by MALDI-TOF-TOF was interpreted using Bruker Biotools software, which uses *de novo* sequencing algorithm to determine the best sequence for the observed fragmentation pattern.

RESULTS AND DISCUSSION

Ion exchange chromatography and anti-cancer activity of eluates: The <5 kDa fraction was subjected first to ion exchange chromatography and the peaks were eluted using 50mM and 100mM NaCl. On colon and liver cancer cells the 50mM NaCl eluate showed around 75% inhibition by the MTS dye assay. On breast cancer cells there was 68% inhibition while on lung cancer cells there was 60% inhibition (Figure 4.1). While the 50mM eluate showed cancer cell inhibitions better than the 100mM eluates, the 50mM eluate possibly had the pool of <5kDa peptides that contribute towards the anti-cancer effect. Ion exchange chromatography was the most popular tool used especially to separate peptides from protein hydrolysates in the past decade. With the advent of sophisticated purification techniques involving reverse phase HPLC, LC-MS and specific columns, the need for fully

characterizing a pure peptide has become imminent following just ion exchange purification. Moreover bioactive peptides separated out in ion exchange columns have been subjected to an additional reverse phase HPLC step to get pure peptides amenable for mass spectrometry and sequence studies. Hence the 50mM IEC eluate was subjected to HPLC purification using a peptide-specific column.

Reverse phase HPLC purification

The 50mM IEC eluate was subjected to HPLC in a preparative HPLC with a peptide-specific wide bore column (ID 22 x L 250 mm). The gradient from solvent A (1.2mlTFA/1000ml deionized water) to solvent B (0.1%TFA in Acetonitrile:water 50:50) was varied from 100% solvent A to 100% solvent B over 80 minutes at 2ml/min flow rate monitored at 215nm.Figure 4.2 shows the HPLC profile of 50mM IEC eluates. The peaks were collected at 10 min intervals and tested for anti-cancer activity. It was found that the 60-70min fraction showed anti-cancer activity and this fraction was further purified in HPLC. Figure 4.3 shows the purification of a single peptide obtained from 60-70min fraction isolated from ion exchange chromatography. The peak eluted at 62 min was evaluated for anti-cancer activity.

Reverse phase HPLC has been the most preferred tool for purification of the peptide of interest from the extracts or from the ion exchange eluates. Several peptides from food sources have been isolated to purity using this technique where a mixture of solvents, usually acetonitrile and water with ion pairing reagents like trifluoroacetic acid aids in formation of a polar to non-polar gradient. The extracts when injected into a specific column get separated out with the programmed gradient thereby separating and identifying polar, nonpolar, hydrophobic peptides. The 62 min eluate of the peptide suggests the presence of both polar and non-polar amino acids that make up the peptide.

Anti-cancer activity of pure peptide

The pure peptide showed 84% inhibition on colon cancer cells, 80% on breast cancer cells and 84% on liver cancer cells, very similar to the positive (inhibitory) control (genistein) at 400μg/ml. On breast and liver cancer cells the pure peptide showed 80% and 85% inhibitions respectively, while on lung cancer cells there was 69% inhibition (Figure 4.4). The results show that the peptide bears strong anti-cancer activities better than the <5kDa peptide fraction from which it was purified. It is conclusive to observe that purification of a single peptide from the <5kDa rice bran hydrolysate resulted in identifying the anti-cancer component within rice bran. When its activity is tested and compared against all cancer types, we do not find enhanced activity of the peptide towards lung cancer cells. More specific lung cancer types like small cell, squamous cell, and non-small cell lung cancer carcinomas may need to be tested with the peptide to look for better inhibition of proliferation.

Amino acid analysis Amino acid analysis revealed the predominance of 4 amino acids suggesting the anti-cancer peptide could be a short peptide. The amino acids identified were arginine, proline, glutamic acid, and glutamine (Table 4.1). The activity of biofunctional peptides is based on their inherent amino acid composition and sequence. The size of active sequences may vary from two to twenty amino acid residues, and many peptides are known to have multifunctional properties (Meisel and FitzGerald, 2003). Here we find the possibility of a tetra to penta peptide bearing anti-cancer properties against several types of cancer cell lines. Other studies have also produced short peptides possessing biological activities like immunomodulatory, ACE-inhibitory, and anti-oxidative properties (Migliore-Samour et al, 1988, Suetsuna et al, 2000). The peptides

range from di-, tri- to penta-peptides like Ile-Ile-Ala-Glu-Lys (Nagaoka et al 2001) isolated from β-lactoglobulin by tryptic hydrolysis. The shorter the peptide chains the more accessibility would exist for solvents and exposure of functional groups to impart functional properties. Moreover presence of proline signifies more accessibility to solvent owing to its predominance in turns of a polypeptide chain. It also has a rigid conformation than most other amino acids. Occurrences of proline residue in bioactive peptides are not uncommon and are also thought to be a major player to contribute to bioactivity of the peptide

Dose-response pattern of the pure peptide (Figure 4.5) revealed that from 600µg/mL to 1000 µg/mL maximum inhibition was achieved on most cancer cells even at 24 hour period. There is an increase in inhibition pattern as the dosage increases and seems to plateau off beyond 600µg/mL. The dosage for the pure peptide is similar to what was observed with the 5kDa fraction on most cancer cell lines as per the earlier studies except there is a 24 h response for significant anti-cancer activity.

MALDI characterization of peptide

Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) analysis was performed on the HPLC purified peptide. Figure 4.6 shows the MALDI-TOF-MS spectrum of the purified fraction and confirms the purity of the fraction. The accuracy of the mass was confirmed by MALDI-ICR (ion cyclotron resonance) -MS where the mass of the ion was measured to 10 ppm accuracy. By means of MALDI–TOF MS, single protonated molecular ions ($M+H^+$) of the intact peptide were located at *m/z* 685.378 suggesting the possibility of a penta-peptide.

Further tandem (MS-MS) was performed on the predominant peak by isolating the ion inside the mass spectrometer to enable post source decay (PSD) fragmentation to obtain amino acid sequence (Figure 4.7). Three amino acids were identified from the C-terminus end as Arg-Pro-Arg. The N-terminal amino acids when matched against the database showed a high prediction of Glu-Gln amino acids in the N-terminus end (Table 4.2). The *de novo* sequencing thus revealed the peptide to have the amino acid sequence as Glu-Gln-Arg-Pro-Arg (EQRPR). The presence of charged (glutamic acid) and heterocyclic amino acid (proline) in the sequence could have attributed anti-cancer properties to the peptide. Low molecular sized bioactive peptides as tri- and penta-peptides have been isolated and purified from soy protein and wheat germ respectively (Korhonen et al, 2003). The sequences for these peptides were Ile-Val-Tyr, and Leu-Leu-Pro-His-His.

All MS/MS spectra of peptides contain fragment ions, which result either from single cleavages of peptide backbone bonds and multiple cleavages. The extraction of sequence information from MS/MS spectra or fragment ion spectra without any prior knowledge about the sequence, i.e., *de novo* sequencing, allows elucidating the structure and sequence of a particular peptide. Such peptide sequences can then be used to identify a protein solely by homology searches such as BLAST Biotools that uses a scoring method to sort the most probable sequence.

Fully characterizing a pure peptide, thereby identifying its structural components confirms the peptide to its identity, and enhanced bioactivity. This suggests and confirms the finding that an anti-cancer peptide has been isolated and purified from rice bran after enzymatic hydrolysis.

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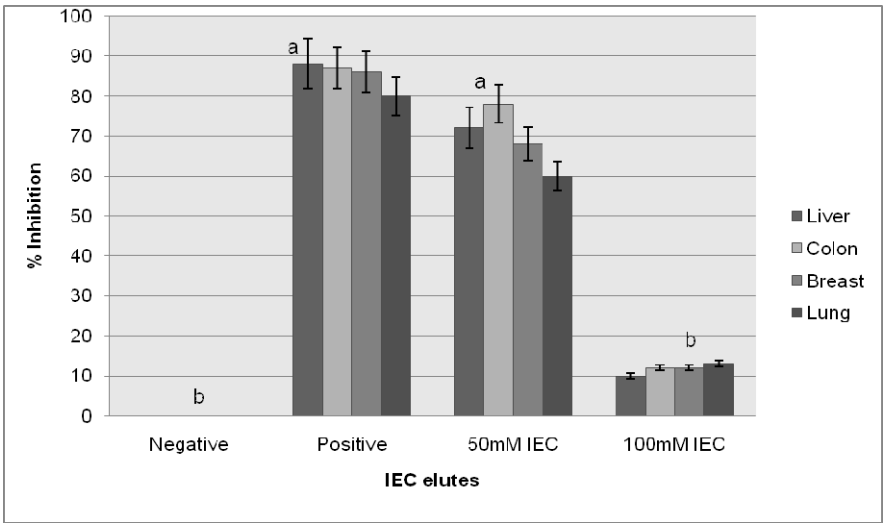

Figure 4.1 MTS anti-cancer activities on IEC eluted fractions.

100mM: 100mM NaCl eluate; 50 mM: 50mM NaCl eluate
Values are means trials ± SE. Values not connected by same letters are significantly different ($P<0.05$), Negative (viability control): saline; Positive (inhibitory control)**:** Genistein (400µg/ml) .
MTS - (3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

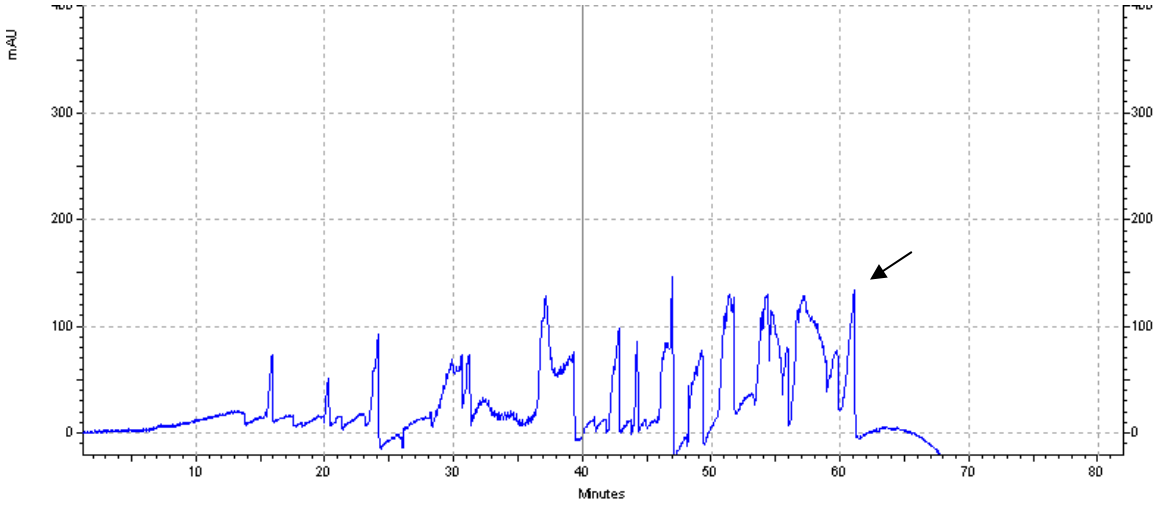

Figure 4.2 HPLC profile of 50mM IEC eluate.

50mM NaCl eluate from ion exchange chromatography (IEC) subjected to HPLC. Peaks were collected every 10 min of the run and analyzed for anti-cancer activity. 60-70min peak (arrowhead) showed anti-cancer activity, which was further purified.

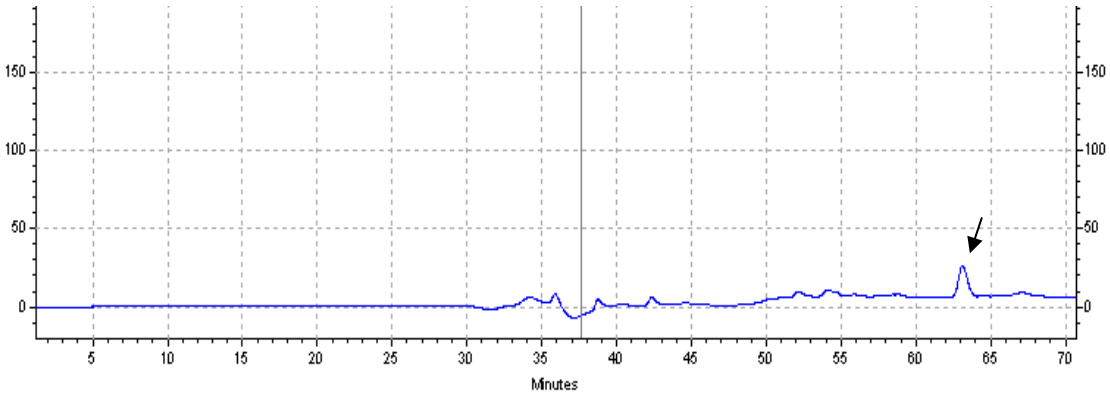

Figure 4.3 HPLC purification of single peptide from 60-70min fraction from IEC.

62min peak (arrowhead) was collected for evaluation of anti-cancer effect.

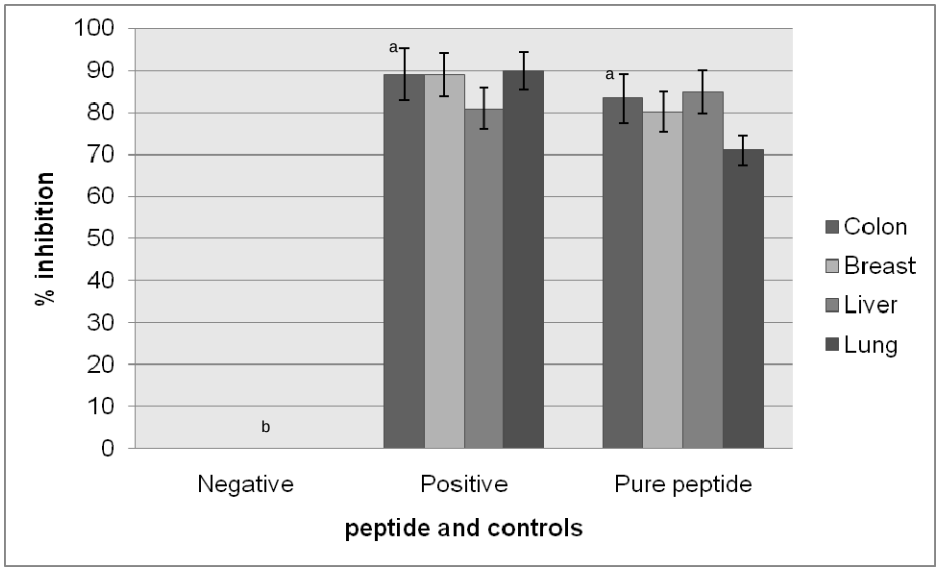

Figure 4.4 MTS anti-cancer activities of pure peptide.

Values are means trials ± SE. Values not connected by same letters are significantly different ($P<0.05$),
MTS - (3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
Negative (viability control): saline
Positive (inhibitory control)**:** Genistein (400µg/ml)
Peptide fractions 1mg/ml

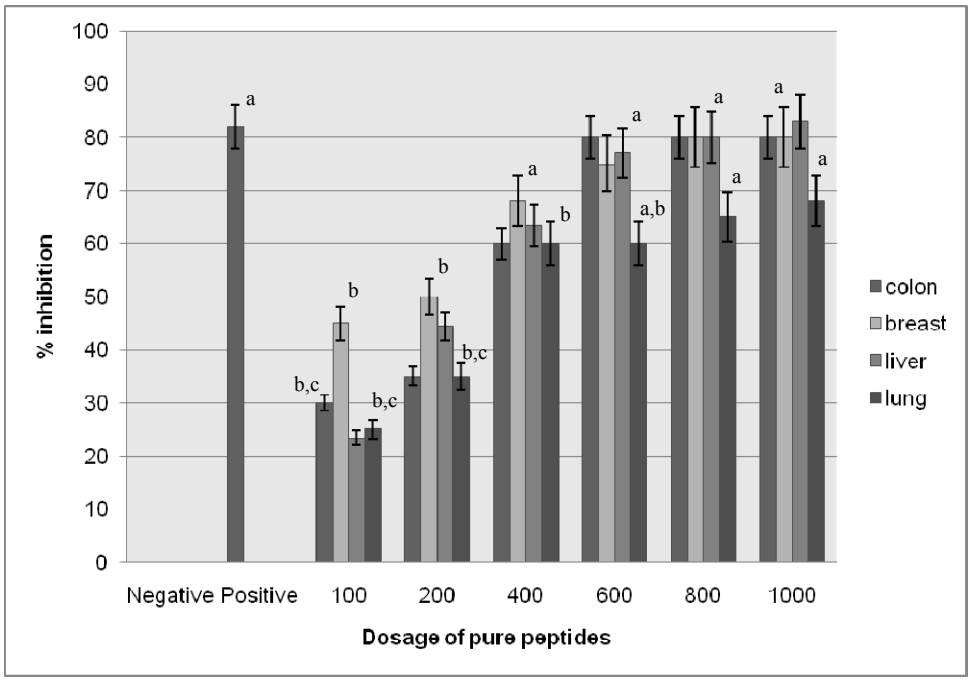

Figure 4.5: Dose response at 24h of pure peptide on cancer cells

Values are means trials ± SE. Values not connected by same letters are significantly different ($P<0.05$),
MTS - (3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
Negative (viability control): saline
Positive (inhibitory control)**:** Genistein (400µg/ml)

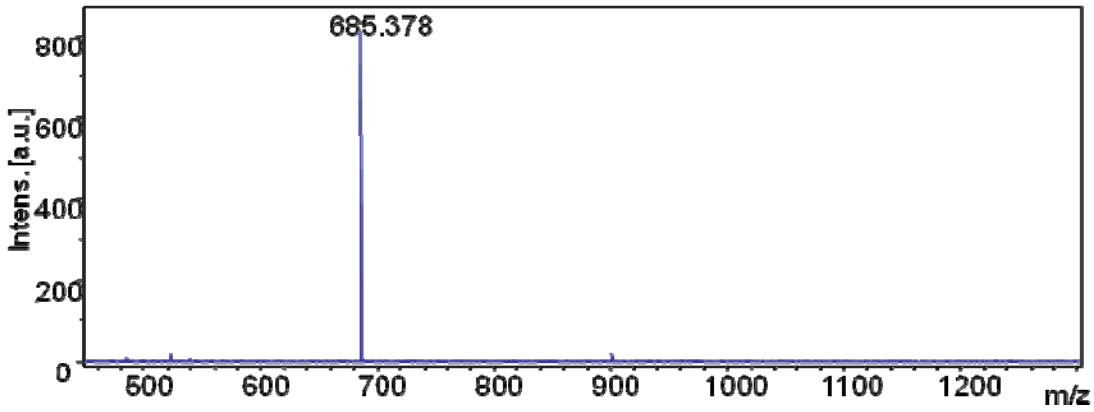

Figure 4.6 MALDI profile of the pure peptide obtained after HPLC purification.

x-axis: mass to charge ratio (m/z); y-axis: intensity
Molecular mass of the peptide identified to be 685.378 daltons.
Inset: Purified peak at 62min elution from HPLC.

Amino acids	Peptide **nmol/ml**
Glutamic Acid	**1.50**
Proline	**0.99**
Arginine	**2.65**

Table 4.1. Amino acid analysis of the pure peptide obtained after HPLC purification

Sequence of the peptide: Glu-Gln-Arg-Pro-Arg (EQRPR)

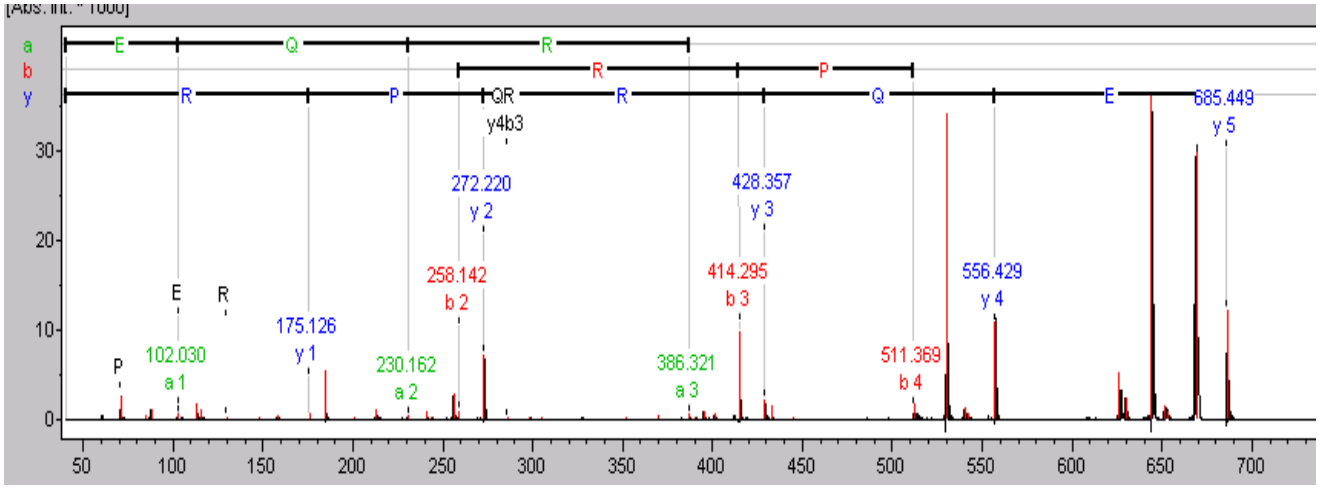

Figure 4.7: Tandem MS-MS PSD fragmentation of peptide 685.37 Da

	E	Q	R	P	R	Glu	Gln	Arg	Pro	Arg
Ion	1	2	3	4	5	1	2	3	4	5
a	E	Q	R	P	R	102.055	230.114	386.215	483.267	639.369
b	E	Q	R	P	R	130.050	258.108	414.210	511.262	667.363
y	E	Q	R	P	R	175.119	272.172	428.273	556.331	685.374
i	E	Q	R	P	R	102.055	101.070	129.113	70.065	129.113
	5	4	3	2	1	Arg	Pro	Arg	Gln	Glu

Table 4.2. *de novo* sequencing of peptide matched for probable sequence based on exact mass of 685.378 Da

Conclusion

This study has provided the basis for developing novel gastrointestinal environment resistant bioactive peptides active against different cancer cell types from heat-stabilized de-fatted rice bran (HDRB)., an underutilized and inexpensive co-product of rough rice processing.

HDRB can be a good starting material for creating new bioactive peptides that can be active against several chronic disease conditions. Owing to difficulty in protein extraction from bran, use of food-grade enzymes is preferred to directly hydrolyze bran protein to obtain peptides. Evaluating peptides' resistances to gastrointestinal juices, fractionating and testing for anti-cancer activities on multiple-cancer sites formed a novel aspect of this research. Although lower molecular weight fractions and a five-amino acid peptide exhibited significant anti-cancer properties, it would be interesting to evaluate the possibility of peptides from higher molecular weight fractions that show substantial activity.

A pure pentapeptide was isolated and fully characterized with the help of proteomic tools. Information obtained from characterizing structural components of the pentapeptide can be used to chemically synthesize the anticancer peptide for food ingredient or pharmaceutical use.