PEPTIDES DERIVED FROM RICE BRAN PROTECT CELLS FROM OBESITY AND ALZHEIMER'S DISEASE

A. Kannan[1], **N.S. Hettiarachchy*[1]** and M. Mahedevan[2]
[1]Department of Food Science, University of Arkansas, Fayetteville, Arkansas 72704
[2]Department of Obstetrics and Gynecology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

***Corresponding Author:** nhettiar@uark.edu

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ABSTRACT
Bioactive food-derived peptides possess the ability to promote wellness through health benefits by reducing the risk of chronic complications including obesity or age-related diseases such as Alzheimer's disease. Their large demand in the global food market has promoted intense research on bioactive peptides able to prevent disease propagation. Our research involved in isolating and characterizing fractions of peptides from rice bran for possible inhibitory effects against Alzheimer's and obesity. Minimal hydrolysis and gastrointestinal juices treatment followed by fractionation resulted in <5 and 5-10kDa fractions. With <5 and 5-10kDa fractions, preadipocytes showed differentiation and proliferation (60%) significantly compared to undifferentiated cells (controls) (25%). Approximately 35% reduction in cytotoxicity of amyloid-induced neuroblastoma cells was observed with peptide fractions, <5 and 5-10kDa compared to the cytotoxicity observed with amyloid-induced cells (control) that were not treated with peptides. A pentapeptide, Glu-Gln-Arg-Pro-Arg (EQRPR) was characterized from the <5kDa fraction showing bioactive effects. It showed nearly 70% adipocyte viability more than control possibly signifying insulin-like differentiation to confer protective role against obesity. It also showed nearly 45% reduction in cell cytotoxicity on amyloid-induced neuronal cells. An efficient and reproducible biocatalytic technology to utilize underutilized co-product such as rice bran to produce anti-Alzheimer and anti-obese value-added bioactive peptide has been established. This should promote further research as applicable to food industry as a natural nutraceutical ingredient.

Keywords: Rice bran; Peptides; anti-obesity; anti-Alzheimer's

1. INTRODUCTION
The growing epidemics of type 2 diabetes and cardiovascular disease is linked to obesity. In nearly 90% of diabetics, obesity can be attributed as a cause. Consequently obesity has triggered impaired glucose tolerance in nearly 197 million people worldwide[1]. Estimated cases of diabetes in the United States currently stand at 19.2 million comprising 30-40% of World's type 2 diabetics[2]. Roughly 63% of Americans have been identified to be obese with a basal metabolic index (BMI) of at least 25. Particularly childhood obesity in the United States has tripled since the last two decades[3]. Following the ranks of cardiovascular disease and its complications in causing death, is a neurological disorder, Alzheimer's disease (AD).

It has been thought that blood vessel damage in the brain, most likely to occur in patients with diabetes and high cholesterol, can lead to symptoms of Alzheimer's disease[4] and, preventing these states can reduce the risk of developing AD[5]. One in eight persons aged 65-85 and nearly half of persons over age 85 have Alzheimer's disease. In 2011, baby boomers (those born between 1946 and 1964) will begin turning 65, reaching the age that stratifies greatest risk for Alzheimer's disease[6]. The treatments of these age related chronic diseases are life-long and can have side effects. Americans are supplementing traditional health care by turning to nutraceuticals and functional foods. Nutraceuticals are considered safe, effective, and ingestible for a lifetime without toxicity, reversibly inhibiting type and, also amenable to clinical trials. These, when proven effective can possibly function as low cost prescription alternatives[7, 8].

Enzymatic hydrolysis has been one of the main approaches to produce bioactive peptides from soybean, wheat, corn, rice, barley, buckwheat, and sunflower protein[9]. However, the potential bioactive nature of components of rice is not well documented including the bran portion, which is nutritionally beneficial but used as a low cost animal feed. With studies suggesting whole bran extracts to confer bioactivity on cancer cells and mutagenic models, we found it necessary to test rice bran peptides for reducing the risk of obesity and Alzheimer's disease on cell culture models. For complete value it is important that the peptides be resistant to gastrointestinal environment when ingested for being metabolically bioactive. Rice bran is a cheap co-product of rough rice milling having nutrients including B vitamins, minerals and fiber[10]. From an agricultural stand point rice bran production could be boosted particularly in the State of Arkansas (ranked number one in U.S rice production) if peptides derived from defatted rice bran (currently used as animal feed) can prove effective against complex disease manifestations. Rice bran has approximately 20% protein and could be a source of bioactive peptides possibly due to the presence of unique amino acid types and sequence. The presence of unique sequences could also render bioactive nature that can enhance glucose uptake into cells, or act to sequester bad cholesterol and fatty acids accumulating in excessive BMI states, or even mitigate molecular events taking place in neuronal cells during the onset of Alzheimer's disease. However studies on specific components like peptides within rice bran that could possibly contribute to such anti-disease characteristics are lacking.

Therefore, the identification and isolation of bioactive peptides from rice bran that confer bioactive effects on obesity and Alzheimer's disease had been the focus of this research hoping to advance the use of this rice component that has low value.

Thus, the objectives involved were preparation of protein/peptide hydrolysates from rice bran, treating the hydrolysates with simulated gastrointestinal environments (GIE) to obtain GIE resistant peptides fractionate the resistant peptides to ultrafiltration to generate peptide fractions based on molecular size, evaluate the roles of resistant peptide fractions for bioactivities against obesity and Alzheimer's disease using cell culture models, and finally characterize peptide(s) using chromatography and mass spectrometry.

2. MATERIALS AND METHODS

2.1 Materials: HDRB (Heat stabilized De-fatted Rice Bran) (Riceland foods (Stuttgart, AR)), Human pre-adipocytes and adipocyte basal growth medium and differentiation medium (Lonza, USA), Neuroblastoma cell line (IMR-32) and growth medium (ATCC, Manassas, USA), and media supplements including fetal bovine serum, gentamycin, were purchased from Hyclone, USA. MTS kit was purchased from Promega USA. Preparative liquid chromatography system LC-8A was purchased from Shimadzu, USA, Sephadex G-75 resin from Pharmacia biotech AB, Uppsala Sweden, biopore C-18 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.

2.2 Methods

2.2.1 Preparation of rice bran peptide fractions: Heat stabilized de-fatted rice bran (Riceland, Arkansas) was hydrolyzed with food grade Alcalase under optimum degree of hydrolysis following a response surface design. The hydrolysates were treated with simulated gastrointestinal resistant solutions to generate GIE resistant peptides, which were then fractionated into <5, 5-10, 10-50, and >50 kDa fractions as previously done[11].

2.2.2 Anti-obesity effect: Evaluation of degree of differentiation of adipocytes for accumulation of lipids: Undifferentiated human preadipocytes (Lonza, USA) were allowed to differentiate upon treatment with a known differentiating agent [0.25µmol/L DEX (IS-IBMX-DEX) mixture] (Sigma USA). To determine the role of peptide fractions on adipocyte differentiation, peptide fractions were added to substitute for insulin or supplemented to DEX mixture. Microscopic observation of accumulation of lipids using a phase contrast microscope (Tissue culture facility, University of Arkansas) as well as degree of differentiation and hence, anti-obese property, if any on the peptide fractions were observed. A trypan blue dye exclusion assay was conducted to determine the adipocyte cell viabilities before and after differentiation upon treatments with the peptides.

2.2.3 Evaluation of anti-Alzheimer's disease activity: Human neuroblastoma cells (IMR-32 - ATCC Number CCL-127) was used as model system to evaluate the protective role of rice bran peptide fractions against amyloid beta (1–42)-(Cat.No.PP69, EMD Chemicals, Inc.,San Diego,CA) dependent toxicity. Human neuroblastoma cells were grown in the presence of beta amyloid peptide with and without bran peptide fractions at different levels. Cell survival to the amyloid-induced neuroblastoma cells upon bran peptide treatments was examined by the MTS [(3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium,inner salt] assay, and compared to the cytotoxicity observed with amyloid-induced neuroblastoma cells.

2.2.4 Purification and characterization of a pentapeptide isolated from the <5kDa fraction: Ion-exchange chromatography and reverse phase HPLC were employed to purify a pentapeptide fully characterized using mass spectrometry and amino acid sequencing by previously published methodologies[12].

3. RESULTS

3.1 Anti-obesity effect: Obesity results in lipid accumulation and differentiation in adipocytes. Observation of lipid accumulation and rate of differentiation of adipocytes[13] upon treatment with peptide fractions can determine anti-obese property (non or reduced differentiation of adipocytes) of peptide fractions if any. Visceral preadipocytes were allowed to grow in the presence of peptide fractions without the differentiating factor. The degree of differentiation into adipocytes was observed visually, and cell counts taken by trypan blue dye exclusion assay. With <5 and 5-10kDa fractions the preadipocytes showed differentiation and proliferation significantly more compared to undifferentiated cells (controls). Figure 1 shows that nearly 60% cells proliferated better than control (undifferentiated cells (~25% proliferation)), possibly signifying differentiation-inducing characteristics of the HDRB peptide fractions. Moreover, in our study a pentapeptide was isolated from the <5kDa fraction showed nearly 70% adipocyte viability more than control possibly signifying insulin-like differentiation to confer obesity protective role.

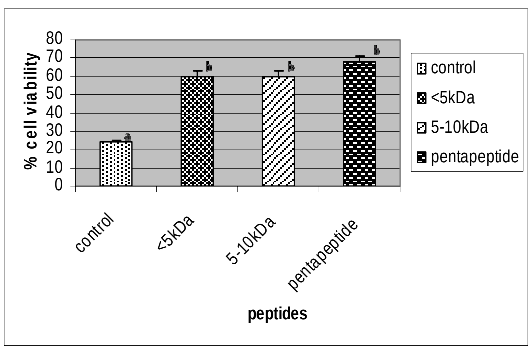

Fig 1. Cell viability of adipocytes upon treatment with peptide fractions. Values are means of three replications with standard deviations. Cell viability was measured using the trypan blue dye exclusion assay.

3.2 Anti-alzheimer's effect: Neurotoxic beta amyloid peptide (1-42) is considered responsible for the formation of senile plaques that accumulate in the brains of patients with Alzheimer's disease[17]. Neuroblastoma cells were treated with amyloid peptide followed by treatment with rice bran peptide fractions. Cytotoxicity to the amyloid-induced neuroblastoma cells was examined by the MTS assay. Figure 2 shows the extent of cytotoxicity observed with amyloid-induced cells with and without peptide treatments.

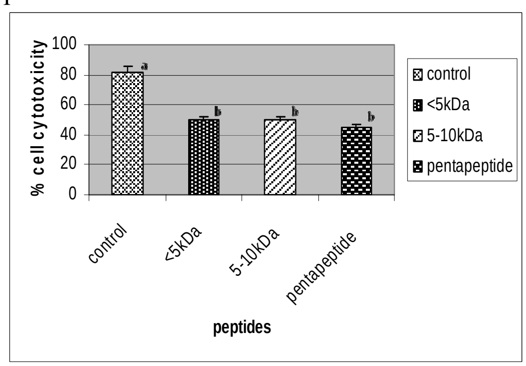

Fig 2. Cell viability of amyloid peptide-induced neuronal cells upon treatment with peptide fractions. Values are means of three replications with standard deviations. Cell viability was measured using the MTS assay. Control represents amyloid-induced neuronal cells without peptide treatment.

We observed nearly 35% reduction in cytotoxicity of amyloid-induced neuroblastoma cells that were treated with peptide fractions <5 and 5-10kDa compared to the cytotoxicity observed with amyloid-induced cells (control) that were not treated with peptides. Studies have suggested presence of certain proteins common to diabetes (possibly its complications) and Alzheimer's conditions possibly influencing insulin resistance and lipid metabolism[18]. It is hence possible that the fractions contain peptide sequences able to reduce amyloid-induced cytotoxicity to neuroblastoma cells. In our study the bran fractions were able to confer protective role against Alzheimer's pathology, and further a pentapeptide isolated from rice bran showed nearly 45% reduction in cell cytotoxicity on amyloid-induced neuronal cells better than the control and the <5kDa fraction.

3.3 Characterization of a pentapeptide from the <5kDa fraction: The <5kDa fraction was characterized to obtain a pure peptide. 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[12]. Peptide was *de novo* sequenced by using the MS spectra monoisotopic mass of the amino acids. 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. De novo sequencing revealed the amino acid sequence as Glu-Gln-Arg-Pro-Arg (EQRPR).

4. DISCUSSION

Very few similar studies have attempted to characterize compounds from cereal grains including rice bran for anti-obesity effect, including a study that has evaluated Coumaric acid derived from stabilized rice bran to inhibit adipogenesis having implications for *in vivo* anti-obesity effects[14]. Choi et al (2006)[15] has enumerated phenolic acids including rutin to bear adipogenesis inhibitory effects via a hepatic PPARγ mechanism. Apart from these studies, literature lacks information about use of proteins and protein components in mitigating formation or accumulation of fat or serving as inhibitory agents against possible obesity. Our study shows that rice bran peptides could be useful as natural alternatives to aid in the management of chronic disease states like obesity. Most of the obesity regulating peptides (YY) are gastrointestinal based, where they act like gut hormones (leptin) in conferring protective roles mainly by improving digestion and nutrient absorption[16]. The significance of testing peptide fractions through simulated gastrointestinal tract in our study, adds importance thus enabling the peptides to resist gastrointestinal degradation, an important aspect of all gut-regulating peptides.

A few studies have evaluated compounds from natural food sources conferring protective effects against Alzheimer's. Curcumin and wine phenolics are known to cause anti-amyloidogenic effecs[19]. There have been several associations between complications favoring or occurring in diabetes including obesity and other chronic illnesses including Alzheimer's. Particularly, blood vessel damage in the brain, most likely to occur in patients with diabetes and high cholesterol, can lead to symptoms of Alzheimer's disease[4] and, preventing these states can reduce the risk of developing AD[5]. In this context we examined the bran peptide fractions for a potential neuroprotective effect. Moreover, the purified pentapeptide showed enhanced anti-Alzheimer's activity. The significance of the sequence of the pentapeptide in conferring protective role depends on what pathological event in amyloidogenesis it influences. Most of the compounds suggested to cause anti-amyloidogenesis exert functions corresponding to interplay of a multitude of proteins and other compounds interfering with associated pathologies, and hence it is difficult to determine an exact protective role. Nevertheless such peptides derived from natural sources able to reduce cytotoxicity induced by amyloid peptide, can promote alternative medicine via nutraceutical interventions.

Very few studies have examined naturally occurring proteins and peptides for such effects. Findings from our research will especially support production of potential nutraceutical co-products like rice bran in large scale and impact both agricultural and pharmaceutical industries. Our research is the first to determine bioactive properties of peptide fractions derived from rice bran against obesity, Alzheimer's and possibly diabetes. These peptides could be useful as natural alternatives (nutraceuticals) to aid in the management of such chronic disease states.

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