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# Human Colon and Liver Cancer Cell Proliferation Inhibition by Peptide Hydrolysates Derived from Heat-Stabilized Defatted Rice Bran

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Rice bran, an economical, underutilized coproduct of rough rice milling, was used to produce peptide hydrolysates, which were investigated for anticancer activity. Protein hydrolysates prepared by Alcalase hydrolysis under 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 liver (HepG2) cancer cell lines by Trypan blue dye exclusion assay. GI-resistant <5 and 5−10 kDa sized peptide fractions inhibited growth of Caco-2 cells by 80%, and the <5 kDa fraction inhibited growth of HepG2 cells by ∼50% compared to controls and nonresistant fractions. An MTS cell titer assay confirmed antiproliferative effects of the peptide fractions. The results demonstrated that 5−10 and <5 kDa sized GI-resistant fractions promoted significant ($p < 0.05$) inhibitory activities on both cancer cell lines compared to controls. More investigations are needed to show such value-added effects on the technofunctional and sensorial properties of the food protein and peptide matrices.

**KEYWORDS: Heat-stabilized defatted rice bran; peptide hydrolysate; colon cancer; liver cancer; MTS**

## 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 (*1*). 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 (*2*). 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 there has been no improvement in the mortality rate. Treatment and preventive options for colorectal cancer mainly focus on early detection. There is evidence that dietary components are 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 biofunctional form can serve as bioactive compounds in reducing the incidence of colorectal cancers. Liver cancer is the most common cancer type in developing countries, but is less common (2% of cancer deaths) in the 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 (*3*). Chemotherapy, radiotherapy, and liver transplantation are the treatment options available for treating liver cancer. As alternative treatment, to reduce the risk of developing cancer, the focus is on identifying compounds present in natural foods that could bear anti-liver cancer properties.

Bioactive peptides comprising two to nine amino acids typically possess specific amino acid sequences comprising mainly hydrophobic groups in addition to proline, arginine, or lysine (*4, 5, 1*). Several bioactive peptides (*1, 6*−*11*) have demonstrated antioxidant, antiobesity, antiangiogenic, and antihypertensive activities and hypocholesterolemic and immunomodulatory effects. Peptides and proteins from food sources have aided in cancer prevention and treatment. For example, whey proteins and α-lactalbumin have been shown to inhibit colon cell proliferation (*12*). Cereal grains including soybean, rice, and wheat and their components have been investigated for the presence of bioactive proteins and peptides (*13*). For example, Oryzatensin, an ileum-contracting bioactive peptide obtained from rice albumin, was shown to have an immunostimulatory role (*14*). Similarly, proteolytic hydrolysis of soybean protein using Alcalase and Proteinase S enzymes resulted in peptides that were antihypertensive and antioxidative, respectively (*5*). Rice and its components have also been studied to exert specific antidisease properties such as antioxidative, anticarcinogenic, and antimutagenic (*15*). However, constituents such as proteins and peptides from rice or coproducts of rice milling have been studied to a limited extent and found to confer antidisease characteristics.

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Rice bran is a cheap coproduct of rough rice milling having nutrients including B vitamins, minerals, and fiber (*16*), including oil, which has health benefits (*17*). It is being used as a low-cost animal feed, and the state of Arkansas contributes >50% of the overall rice production in the country (*18*). Defatting the bran and directly hydrolyzing the high-quality protein using endoprotease can sustainably release peptides in a consistent manner. 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 anticancer activities. This approach not only was unique but also can prove to be an economical way of producing anticancer peptides from rice bran on a large scale.

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

## MATERIALS AND METHODS

**Materials.** HDRB was obtained from Riceland Foods (Stuttgart, AR), the Romicon ultrafiltration system from Koch Membrane Systems, USA, and food-grade Alcalase enzyme from a bacterial strain purchased from Novozyme, USA. Human colon (Caco-2) epithelial cancer cell line and liver (HepG-2) epithelial cancer cell line were purchased from ATCC. Dulbecco's modified Eagle's medium, fetal bovine serum, and gentamycin were purchased from Hyclone. The MTS kit was purchased from Promega, Madison, WI. All other chemicals were purchased from Sigma, St. Louis, MO.

**Methods.** *Preparation of Protein Hydrolysates from Heat-Stabilized Defatted Rice Bran.* Heat-stabilized defatted rice bran was ground and passed through a 60 mesh sieve. It was stored at 4 °C until further use. For extraction of protein hydrolysates, the sample was allowed to attain room temperature overnight. Approximately half a kilogram of the ground and sieved HDRB was dissolved and homogenized with 0.6 L of deionized water and stirred for 30 min at room temperature. Samples from the mixture were used for enzyme treatments as outlined in the following section.

*Response Surface Optimization.* A response surface model 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.

To arrest proteolytic digestion the enzyme was inactivated by incubating the suspension at 85 °C for 10 min (*19*). The suspension was then centrifuged at 3000*g* for 15 min to obtain soluble hydrolysates in the supernatant.

*Degree of Hydrolysis.* The degree of hydrolysis was determined according to the OPA method (*20*) using serine as standard. The sample solution was prepared by dissolving 0.1−1.0 g of freeze-dried hydrolysate in 100 mL of deionized water. Serine standard/sample solution (400 $\mu$L) was added to the test tube (time 0) containing 3 mL of OPA reagent, mixed for 5 s, and allowed to stand for exactly 2 min, and then the absorbance was read at 340 nm in the spectrophotometer.

Degree of hydrolysis was calculated as follows: DH = $h/h_{tot} \times 100\%$, where $h$ is the number of cleaved peptide bonds and $h_{tot}$ is the total number of peptide bonds per protein calculated as $h_{tot} =$ (serine-$NH_2$ − 0.4), where serine-$NH_2$ is the meqv of serine $NH_2$ per gram of protein.

$$\text{serine-NH}_2 = (\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}) \times 0.9516 \text{ meqv/L} \times \frac{(\text{sample vol in L}) \times 100/(\text{sample wt in g} \times \text{protein \%})}{(\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}})} \quad (1)$$

*Protein Content.* Protein contents were determined according to the Kjeldahl method. A Kjeldahl 2006 Digester (Foss Tecator, Hoganas, Sweden) was used to digest samples.

*Treatment with Simulated Gastric Juice.* Simulated gastric juice was prepared as follows: To deionized water (90 mL) in a 100 mL volumetric flask were added sodium chloride (0.2 g) and concentrated hydrochloric acid (0.7 mL) 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 was dissolved in the simulated gastric juice and allowed to incubate at 37 °C with constant shaking. After 120 min, the pH was adjusted to 7.2 to inactivate the enzyme (*21*). The reaction mixture was centrifuged at 3000*g* for 20 min to obtain soluble peptide hydrolysates in the supernatant. In the in vitro digestibility studies, the samples are typically examined up to 120 min (*22*). 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 were added potassium phosphate monobasic (0.68 g) and sodium hydroxide 0.2 N (7.7 mL) 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 was maintained at 37 °C. Pancreatin (Sigma-Aldrich Corp.) at a final concentration of 0.1% (*23*) 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 (*22*), enzyme was inactivated by heating at 85 °C for 10 min (*23*). The reaction mixture was centrifuged at 3000*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 in. diameter hollow-fiber polysulfone membrane cartridges. The filtered soluble GI-resistant peptide hydrolysate was run through sequential ultrafiltration columns with membrane cartridges having nominal molecular weight cutoffs (MWCO) of 50000, 10000, and 5000 Da. In each MWCO cartridge, the peptide hydrolysate was ultrafiltered at a dilution factor of 5. Immediately after the first ultrafiltration, the retentate was diafiltered twice with 2 volumes of deionized water. The permeates of the first (50000 Da) ultrafiltration (UF) and the second diafiltration step (DF) were pooled and subjected to the second run through the 10000 Da and then the 5000 Da MWCOs, respectively. The resulting retentates from each of the MWCO were freeze-dried and stored at 4 °C until used for anticancer bioactivity. Only GI-resistant peptide hydrolysates were subjected to fractionation.

*Human Colon (Caco-2) and Liver (HepG2) Cancer Cell Culture and Anticancer Activity Testing.* Human colon epithelial cancer cell line Caco-2 and liver epithelial cancer cell line HepG2 were cultured separately at 37 °C in DMEM in the presence of 10% fetal bovine serum, supplemented with 1 mM L-glutamine, sodium pyruvate, 1 mM sodium bicarbonate, and 50 $\mu$g/mL gentamycin. After cell growth reached 70%, cell viability was monitored by 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. One hundred microliters of rice bran peptide fractions at 1 mg/mL protein content was added to the cultures. For positive control genistein at 200 and 400 $\mu$M in saline and for negative control saline alone was used. After 24−48 h of treatments, the medium was removed, and the cells were briefly dissociated with 0.1% trypsin−0.53 mM ethylenediaminetetraacetic acid (EDTA) solution. Following this, 0.5% Trypan blue dye mixed in growth medium was added to each well.

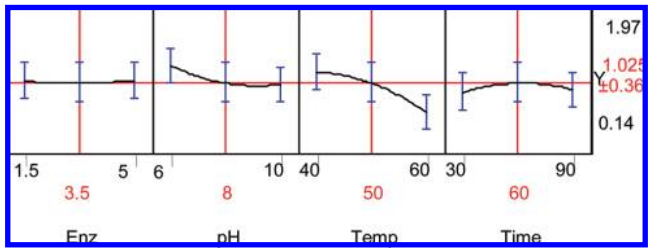


**Figure 1.** Box−Behnken RSM 3 points prediction profiler.

Samples were then aspirated from each well and loaded onto chambers in a hemocytometer 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 determined 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 Caco-2 and HepG2 cell growth, respectively, cells were trypsinized, loaded onto a hemocytometer, and counted. Approximately 1000 cells per well was used for growth onto 96-well flat-bottom plates. The cells were allowed to attach and grow for 36 h. After 36 h, old medium was replaced with fresh medium, and samples of bran peptide fractions were treated with the cells. After 2−4 h of exposure of the rice bran peptides to the monolayer, the MTS mix was added at a final volume of 20 $\mu$L/100 $\mu$L of 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 was measured at 490 nm (*24*). Positive and negative controls were used similarly as used in the Trypan blue dye exclusion assay. All assays were performed in duplicates, and the results are 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 $P < 0.05$. 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.** A four-factorial response surface design optimization with optimum degree of hydrolysis as well as digested protein contents as response values was determined. The four parameters, enzyme concentration [1.5, 3.5, and 5 Alcalase units (AU)], pH (6, 8, and 10), temperature (40, 50, and 60 °C), and incubation time for digestion (30, 60, and 90 min), were fitted to generate optimum concentrations of enzyme, pH, time, and temperature for achieving optimum degree of hydrolysis. Box−Behnken surface response using the JMP 7.0 statistical software was used to evaluate the interactions between parameters to generate optimum values for enzymatic hydrolysis. A degree of hydrolysis at 23.4% was considered to be optimum, with an E/S ratio of 0.01.

Digested protein contents (in mg/mL) were obtained for each factorial combination. For example, at 1.5 AU enzyme concentration, pH 8, and 40 °C 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** enabled selection of the optimum conditions needed for proteolytic digestion. The prediction profile designated 3.5 AU enzyme concentration at pH 8, 50 °C, and 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 toward optimizing the extraction of proteins from rice and rice bran using enzymatic and chemical approaches. Tang et al. (*25*) 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 (*26*). 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 to be 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 sites for action, was chosen. In this particular study an endoprotease was used instead of chemical treatments to attain specificity and also to maintain the protein functionality at its optimal level.

**Colon and Liver Anticancer Activity Evaluation of GI-Resistant and Nonresistant Rice Bran Peptide Hydrolysate Fractions.** The 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 to Caco-2 as well as HepG2 cells. Both GI-resistant peptides and non-GI-resistant peptides were tested on colon and liver cancer cell lines.

**Figure 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 and 5−10 kDa were found to significantly inhibit the proliferation of viable cells compared to higher molecular weight fractions (>10 and >50 kDa), non-GI-resistant fractions, and also negative control. There is an approximately 3-fold reduction in viable cells between GI-resistant and non-GI-resistant fractions that are below 10000 Da. Similar patterns of inhibition between fractions that are above 10000 Da were not observed. The positive control used was genistein at concentrations of 200 and 400 $\mu$M. It is an isoflavone that is a known anticancer agent (*27*). At 200 $\mu$M concentration there were 20000 viable cells/mL on the Caco-2 cell line, which was significantly less than the negative control (lacking genistein) that resulted in over 100000 viable cells/mL. Proteins and peptides have been shown to possess antiproliferative effects (1) 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 antiproliferative/anticancer effects on colon cancer cell lines. A study reported inhibitory effects of a casein-derived peptide, $\beta$-casomorphin-7, on human colonic lymphocytes (*28*), and in general casein-derived peptides could have the ability to arrest colonic epithelial cell proliferation and also induce apoptosis (*29*).

**Figure 3** 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. From experimental data it was found that the resistant fractions were more bioactive than the nonresistant 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, causing them to expose more side chains, but also may imply their suitability in the digestive tract, rendering absorptive and hence consumable properties. In the human body peptides are usually generated when proteins pass through the

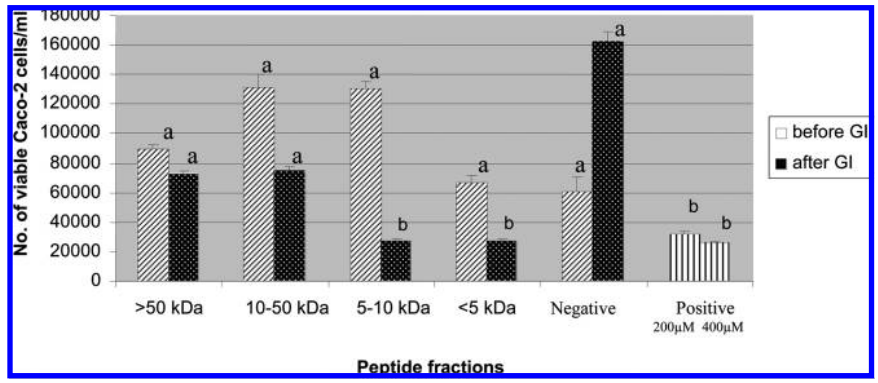


**Figure 2.** Viability of Caco-2 cells after exposure to rice bran peptide fractions measured by Trypan blue dye exclusion assay.

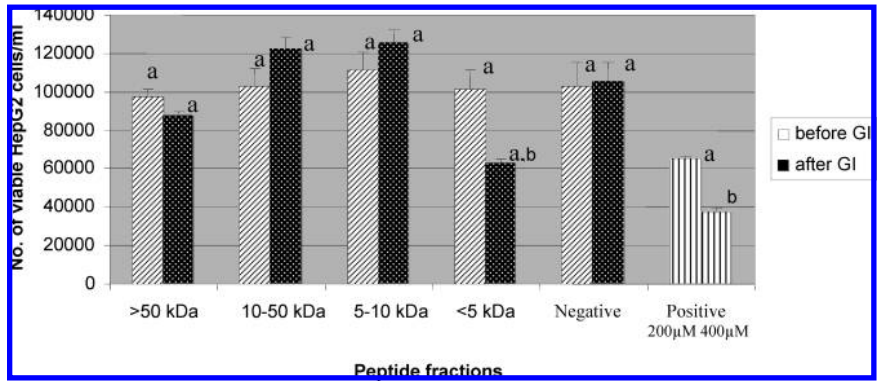


**Figure 3.** Viability of HepG2 cells exposed to rice bran peptide fractions measured by Trypan blue dye exclusion assay. Values are means of two trials ± SE. Values not designated by the same letters are significantly different ($P < 0.05$). Positive control, saline with 0.1% methanol; negative control, genistein (200, 400 $\mu$M) in saline with 0.1% methanol.

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 (*29*). Thus, we support our experimental observations that gastrointestinal resistant peptide fractions (<5 and 5−10 kDa) tend to open up their chains, imparting more bioactivity by effectively inhibiting proliferation of both colon and liver cancer cells more than the nonresistant fractions. Moreover, only soluble peptides were generated in a process of eliminating organic or other constituents of rice bran that could possibly interfere with bioactivity. We believe that although rice bran has organic bioactive components, soluble peptides derived from enzymatic hydrolysis of protein hydrolysates can aid in the inhibitory action of human anticolon and liver cancer cell proliferation and can have true biological activity in terms of bioavailability and delivery.

To confirm the peptide bioactivity experimental findings, a more specific assay, the MTS-based titer assay, was conducted. **Figure 4** depicts MTS-based cell titer assay results for confirming inhibitory actions of peptide fractions on colon 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 >80% cytotoxicity to HepG2 cells with the <5 kDa fraction and nearly 70% cytotoxicity to Caco-2 cells. The 5−10 kDa fraction caused 60% cytotoxicity to HepG2 cells and 50% cytotoxicity to Caco-2 cells. The results of this test confirm that resistant peptide fractions <5 and 5−10 kDa inhibit the growth of colon and liver cancer cells more effectively than the nonresistant fractions. For fractions that were >10 kDa pronounced bioactivity was

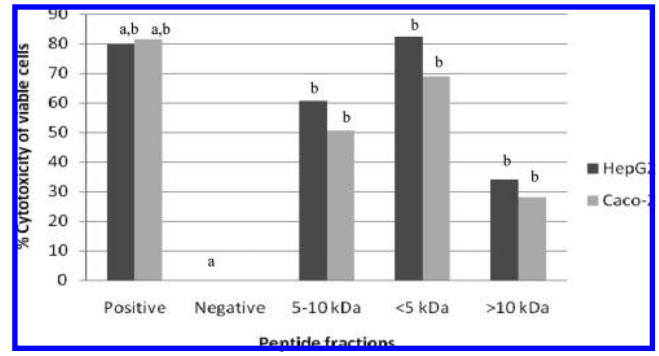


**Figure 4.** MTS confirmatory assay for anticancer activities of rice bran peptide fractions (GI-resistant fractions) on Caco-2 and HepG2 cells. Values are means of two trials ± SE. Values not designated by the same letters are significantly different ($P < 0.05$). MTS, (3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium. Negative, saline; positive, genistein (200 $\mu$M).

not observed, probably because they are longer in length and hence may have needed more time for proteolytic exposure to unfold. Typically, the biopeptides studied so far have had very short sizes, comprising only two to nine amino acids. Hence, it is not surprising to find less or no activity with peptides longer than 10 kDa. Because 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.

Using a presumptive in vitro model such as 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 have potential as human antitumor 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 <5000 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 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 bodies to release bioactive peptides. These could be our next generation of natural antidisease 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 United States (*30*). PeptoPro, muscle cells repairing peptide, lactium, antistress bioactive peptide, and C12, a blood pressure lowering peptide from casein, are available in the U.S. market. As a closing remark, so far peptides from plant proteins have not been commercialized in the United States (*26*), and it would bear significance if peptides produced from rice bran can be commercialized to be incorporated into foods to fight diseases such as cancer.

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