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Antioxidant and Antiproliferative Activities of Loach (*Misgurnus anguillicaudatus*) Peptides Prepared by Papain Digestion

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ABSTRACT: Loach protein hydrolysates (LPH) prepared by papain digestion were fractionated into four fractions, LPH-I (MW > 10 kDa), LPH-II (MW = 5−10 kDa), LPH-III (MW = 3−5 kDa), LPH-IV (MW < 3 kDa), and the in vitro antioxidant and antiproliferative (anticancer) activities of all fractions were determined. LPH-IV showed the lowest IC_{50} value (16.9 ± 0.21 mg/mL) for hydroxyl radical scavenging activity and the highest oxygen radical scavenging capacity (ORAC) value (reaching 215 ± 5.9 mM Trolox/100 g loach peptide when the concentration was 60 μg/mL). Compared with other fractions, LPH-IV also exhibited stronger antiproliferative activity for human liver (HepG2), breast (MCF-7), and colon (Caco-2) cancer cell lines in a dose-dependent manner. When the protein concentration was 40 mg/mL, the HepG2 and MCF-7 cell proliferation of LPH-IV reached 7 and 4%, respectively, with no significant difference from those of LPH (8 and 7%, $p > 0.05$), with significantly less growth than those of LPH-I, LPH-II, and LPH-III, respectively ($p < 0.05$). The Caco-2 colon cell proliferation of LPH-IV was 12.8- and 8.7-fold smaller than those of LPH-I and LPH-II, respectively ($p < 0.05$). All of the fractions had a greater ability to inhibit Caco-2 colon cancer cell proliferation than to inhibit HepG2 liver cancer and MCF-7 breast cancer cell proliferation. The ORAC values of most of the fractions correlated ($R^2 > 0.86$, $p < 0.01$) with the antiproliferative activity of the three cancer cell lines, suggesting that higher antioxidant activity leads to better antiproliferative activity. However, further mechanistic and human clinical studies of the anticancer activity of loach protein hydrolysate fractions are needed.

KEYWORDS: loach peptide, antioxidant activity, antiproliferative activity, free radicals

INTRODUCTION

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. The global burden of cancer continues to increase largely because of the aging and growth of the world population along with an increasing adoption of cancer-causing behaviors. On the basis of GLOBOCAN 2008 estimates, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008.[1] Hepatocellular carcinoma is a form of cancer that arises in hepatocytes, the major cell type of the liver. Hepatocellular carcinoma is among the top causes of cancer deaths worldwide and is especially prevalent in parts of Asia and Africa.[2,3] Breast cancer is the most frequently diagnosed cancer in women. Approximately 1 million women are estimated to be newly diagnosed with breast cancer each year worldwide.[4] Breast cancers are extremely difficult to treat due to the several distinct classes of tumors that exhibit different treatment responses.[5,6] Colorectal cancer (colon cancer) is one of the most prevalent cancers in Western countries. Many colorectal cancers are thought to arise from adenomatous polyps in the colon.[7] Although a great deal of work has been done to prevent and treat cancer, the results remain unsatisfactory, and much needs to be done to stop cancer.

The present cancer treatments such as the use of chemotherapeutic agents, surgery, and radiation have not been effective in increasing the currently low survival rate for most forms of cancer.[5] Therefore, new approaches to prevent and treat cancer need to be explored.[4]

A variety of natural compounds have been shown to inhibit cancer cell growth, such as flavonoids, phenolic acids, and carotenoids.[8,9] Moreover, some peptides and proteins from foods have been reported to aid in cancer prevention and treatment. For example, the peptides prepared from the proteolytic hydrolysis of soybean protein using Alcalase and Proteinase S enzymes had antihypertensive and antioxidative activities, respectively.[10] Oryzatensin, an ileum-contracting bioactive peptide obtained from rice albumin, was shown to have an immunostimulatory role.[11] Whey proteins and R-lactalbumin have been shown to inhibit colon cell proliferation.[12,13] However, there have been very few studies that have tested the ability of peptides from fish proteins to suppress human cancer cell proliferation.

Loach (*Misgurnus anguillicaudatus*) is a common freshwater fish in East Asia. It has been used in China since ancient times and is enjoyed as a food for its desirable taste and flavor. It has also traditionally been used as a folk remedy to treat many diseases. Therefore, the objectives for this study are (1) to determine the in vitro antioxidant activity of loach peptide hydrolysates of different molecular weights, (2) to evaluate the antiproliferative activity of loach peptides on the growth of HepG2 human liver cancer cells, MCF-7 breast cancer cells, and Caco-2 colon cancer cells, and (3) to examine the correlations between antioxidant and antiproliferative activities.

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MATERIALS AND METHODS

Loach Protein Hydrolysates (LPH) Preparation. Live loaches (*M. anguilliacaudatus*, 8.4 ± 1.1 g body weight and 9.8 ± 3 cm body length) were purchased from a local market in Guangzhou, China, and transported to the laboratory within 10 min. After killing, all of the removable muscle tissue of the body was collected and ground twice through a meat grinder with a plate with 4 mm holes (MM12, Shaoguan Food Machine Co., Shaoguan, China). The ground meat was stored in a polyethylene bag at −18 °C until use, a maximum of 4 months. Fifty grams of loach meat was mixed with 100 mL of distilled water and homogenized at a speed of 10000 rpm for 1 min using a homogenizer (T25, Ika, Staufen, Germany). The homogenate was hydrolyzed with papain (7×10^5 U/g) (Baiao Biochemistry Co., Jiangmen, China) at 55 °C for 4 h in a water bath shaker (New Brunswick Scientifics C24, Jintan, China). The enzyme to substrate ratio was 3:1000 (w/w). Hydrolysis was conducted at pH 7.0 (SL1-PHS-3B pH-meter, Wuhan Midwest Instrument Co. Ltd., Wuhan, China). After hydrolysis, the enzyme was inactivated by placing the samples in boiling water for 15 min. The hydrolysates were centrifuged in a GL-21 M refrigerated centrifuge (Xiangyi Instrument Co. Ltd., Changsha, China) at 5000*g* for 20 min at 4 °C, and the supernatants were lyophilized (R2L-100KPS, Kyowa Vacuum Engineering, Tokyo, Japan) and stored in a desiccator for further use. The LPH powder was 85% protein as determined using the Kjeldahl method[14] with a conversion factor of 6.25.

Ultrafiltration. The lyophilized LPH was dissolved in distilled water (30 mg/mL) and fractionated through ultrafiltration membranes using a bioreactor system (Vivaflow 200, Vivascience, Sartorius, Goettingen, Germany). The loach peptide solution was pumped through a range of nominal molecular weight cutoff (MWCO) membranes of 10, 5, and 3 kDa, respectively, in the order of decreasing pore size. The >10 kDa fraction (LPH-1, retentate from 10 kDa membrane), 5−10 kDa fraction (LPH-II, retentate from 5 kDa membrane), 3−5 kDa fraction (LPH-III, retentate from 3 kDa membrane), and <3 kDa hydrolysate (LPH-IV, permeate from 3 kDa membrane) were collected and lyophilized (R2L-100KPS, Kyowa Vacuum Engineering) and then stored in a desiccator and used within 90 days. LPH-I, LPH-II, LPH-III, and LPH-IV represent the fractions with molecular weight (MW) distributions of >10, 5−10, 3−5, and <3 kDa, respectively.

Hydroxyl Radical Scavenging Activity Assay. The hydroxyl radical scavenging activity was assayed using the method of Li et al.[15] with some modifications. A mix of 600 μL of 1,10-phenanthroline (5.0 mM), 600 μL of $FeSO_4$ (5.0 mM), and 600 μL of ethylenediaminetetraacetic acid (EDTA) (15 mM) was mixed with 400 μL of sodium phosphate buffer (0.2 M, pH 7.4). Then 600 μL of samples (0−25 mg/mL) and 800 μL of H_2O_2 (0.01%) were added. The mixture was incubated at 37 °C for 60 min, and the absorbance was measured at 536 nm (UV754, Xianjian Scientific Instrument Co., Shanghai, China). The following equation was used:

$$\text{hydroxyl radical scavenging activity } (\%) = (A_S - A_0) \times 100/(A_c - A_0)$$

A_S is the absorbance of the sample, A_0 is the absorbance of the blank solution using distilled water instead of the sample, and A_c is the absorbance of a control solution in the absence of H_2O_2. The plot of scavenging activity versus concentration of hydrolysate was prepared, and the IC_{50} (concentration of samples to decrease the scavenging activity by 50%) was obtained.

Cu Ion Chelating Activity. The ability of loach peptide to chelate pro-oxidative Cu^{2+} was investigated according to the method of Zhu et al.[16] In the chelation test, 1 mL of 2 mM $CuSO_4$ was mixed with 1 mL of pyridine (pH 7.0) and 20 μL of 0.1% pyrocatechol violet. After the addition of 1 mL of sample (0−5 mg/mL), the disappearance of the blue color, due to dissociation of Cu^{2+}, was recorded by measuring the absorbance at 632 nm after 5 min of reaction. An equivalent volume of distilled water instead of the sample was used for the blank. The Cu^{2+} chelating activity of the loach peptide were calculated as

$$\text{Cu chelating activity} = [(A_0 - A_s)/A_0] \times 100\%$$

where A_S is the absorbance of the sample and A_0 is the absorbance of the blank solution using distilled water instead of sample. The plot of scavenging activity against concentration of sample was prepared and the IC_{50} was obtained.

Measurement of the Lipid Peroxidation Inhibition Activity in a Linoleic Acid Emulsion System. The lipid peroxidation inhibition activity of loach peptide was measured in a linoleic acid emulsion system according to the method of Qian et al.[17] Briefly, a 2.0 mL sample (0−20 mg/mL) was mixed with 2 mL of 2.5% linoleic acid dissolved in 95% ethanol. Then 4 mL of 50 mM sodium phosphate buffer (pH 7.0) and 2 mL of distilled water were added. The mixture was incubated in a 50 mL conical flask with a screw cap at 40 ± 1 °C in a dark room, and the degree of oxidation was evaluated by measuring the FeSCN value described below. The reaction solution (100 μL) incubated in the linoleic acid model system was mixed at different time intervals during the incubation period with 9.7 mL of 75% ethanol, 0.1 mL of 30% NH_4SCN, and 0.1 mL of 20 mM $FeCl_2$ solution in 3.5% HCl. After 3 min, the SCN value was measured by reading the absorbance at 500 nm. An equivalent volume of distilled water instead of the sample was used for the blank.

$$\text{lipid peroxidation inhibition activity } (\%) = [1 - (A_{S,t=144h} - A_{S,t=0h})] \times 100/(A_{0,t=144h} - A_{0,t=0h})$$

$A_{S,t=144h}$ and $A_{S,t=0h}$ are the absorbances for the sample at 144 and 0 h, respectively; $A_{0,t=144h}$ and $A_{0,t=0h}$ are the absorbances for the blank at 144 and 0 h, respectively. The plot of scavenging activity against concentration of sample was prepared, and the IC_{50} was obtained.

Oxygen Radical Scavenging Capacity (ORAC) Assay. The total antioxidant activity of LPH and its fractions was measured using the ORAC assay according to the method of Song et al.[18] Briefly, 20 μL of blank, Trolox standard (Sigma-Aldrich Co., St. Louis, MO), or samples in 75 mM potassium phosphate buffer (pH 7.4, working buffer) was added to triplicate wells in a black, clear-bottom, 96-well microplate. A volume of 200 μL of 0.96 μM fluorescein (in the working buffer) was added to each well and incubated at 37 °C for 20 min, with intermittent shaking, before the addition of 20 μL of freshly prepared 119 mM 2,2′-diazobis(2-aminodinopropane) dihydrochloride (ABAP) (Sigma-Aldrich Co.) in the working buffer using a 12-channel pipet. The microplate was immediately inserted into a Fluoroskan Ascent FL plate reader (Thermo Lab Systems, Franklin, MA) at 37 °C. The decay of fluorescence at 538 nm was measured with excitation at 485 nm every 4.5 min for 2.5 h. The areas under the fluorescence versus the time curve for the samples minus the area under the curve for the blank were calculated and compared to a standard curve of the areas under the curve for 6.25, 12.5, 25, and 50 μM Trolox standards minus the area under the curve for the blank. ORAC values were expressed as mean micromoles of Trolox equivalents (TE) per 100 g of loach peptide.

Measurement of Inhibition of HepG2, MCF-7, and Caco-2 Cell Proliferation. The antiproliferative activities of LPH and its fractions were assessed by the measurement of the inhibition of HepG2, MCF-7, and Caco-2 (American Type Culture Collection, ATCC, Rockville, MD) human cancer cell proliferation. Antiproliferative activity was determined by the MTS colorimetric assay (MTS-based cell titer 96 nonradioactivity cell proliferation assay; Promega, Madison, WI) using the method of Yang et al.[19] HepG2 human liver cancer cells were cultured in Williams medium E (WME), containing 10 mM Hepes, 5 mg/mL insulin, 0.05 mg/mL hydrocortisone, 2 mg/mL glucagon, 5% fetal bovine serum (FBS) (Gibco, Life Technologies, Grand Island, NY), 50 units/mL penicillin, 50 mg/mL streptomycin, and 100 mg/mL

Table 1. Ultrafiltration Separation of Loach Protein Hydrolysates Prepared by Papain Digestion and Their Antioxidant Activities

			antioxidant activity (IC_{50}, mg/mL)		
sample	molecular weight (kDa)	recovery (%)	hydroxyl[a]	Cu ion[b]	lipid peroxidation inhibition[c]
LPH-I	>10	1.04 ± 0.21	18.2 ± 0.68	11.5 ± 0.58	15.4 ± 0.65
LPH-II	5−10	2.91 ± 0.54	17.9 ± 0.24	5.43 ± 0.64	16.8 ± 0.57
LPH-III	3−5	8.06 ± 1.12	17.1 ± 0.11	1.98 ± 0.69	17.8 ± 0.82
LPH-IV	<3	88.0 ± 2.43	16.9 ± 0.21	2.17 ± 0.53	13.4 ± 0.79
LPH		100	17.0 ± 0.54	2.89 ± 0.33	12.3 ± 0.98
GSH			4.7 ± 0.63	1.03 ± 0.21	10.1 ± 1.47

[a] The scavenging activity for the hydroxyl radical. [b] The chelating activity of Cu ion. [c] The lipid peroxidation inhibition activity in a linoleic acid emulsion system.

gentamicin. MCF-7 cells were maintained in Alpha minimum essential medium (MEM-R), containing 10 mM Hepes, 0.01 mg/mL insulin, 50 units/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL gentamicin, and 10% FBS. Caco-2 human colon cancer cells were maintained in Dulbecco's modified Eagle's medium (DMEM), containing 10 mM Hepes, 5% FBS, 50 units/mL penicillin, 50 mg/mL streptomycin, and 100 mg/mL gentamicin. All of the above reagents, except FBS, were purchased from Sigma-Aldrich Co. HepG2, MCF-7, and Caco-2 cells were maintained in a 5% CO_2, 37 °C incubator. A total of 2.5×10^4 cancer cells in growth media were placed in each well of a 96-well flat-bottom plate. Cell proliferation was measured by the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfenyl)-2*H*-tetrazolium (MTS) to formazan. After 4 h of incubation, the growth medium was removed by using a 12-channel pipet and media containing various concentrations of loach peptides were added to the cells. Cell proliferation (percent) was determined at 96 h from the MTS absorbance (490 nm) reading for each concentration compared to the control.

Statistical Analysis. All of the tests were conducted in triplicate. The experimental data were expressed as the mean ± standard deviation. The results were subjected to one-way analysis of variance (ANOVA). Least significant differences (LSD) and Dunnett's T3 tests were performed to determine the significant differences between samples within the 95% confidence interval using SPSS 13.0 software (SPSS Inc., Chicago, IL). The linear correlations between various parameters were also investigated using the SPSS 13.0 software.

RESULTS AND DISCUSSION

Ultrafiltration. Ultrafiltration membranes were used to separate the LPH into four fractions, LPH-I (MW > 10 kDa), LPH-II (MW = 5−10 kDa), LPH-III (MW = 3−5 kDa), and LPH-IV (MW < 3 kDa), which represented 1.04, 2.91, 8.06, and 88.0% of the loach peptide, respectively. As shown in Table 1, the fraction with MW < 3 kDa accounted for about 88% of the protein, indicating that the LPH papain hydrolysates were mainly low molecular weight peptides.

As one of reactive oxygen species generated in the human body, hydroxyl radicals can react easily with biomolecules such as amino acids, proteins, and DNA. This can lead to physiological disorders.[20,21] As shown in Table 1, LPH-IV (MW < 3 kDa) showed the lowest IC_{50} value (16.9 ± 0.21 mg/mL), which indicated the highest hydroxyl radical scavenging activity. The IC_{50} value of LPH for the chelation activity of Cu ion was 2.89 ± 0.33 mg/mL, with no significant difference between fractions LPH-III and LPH-IV ($p > 0.05$), but these were 3- and 0.9-fold lower than those of the fractions LPH-I and LPH-II. Lipid peroxidation is thought to proceed via a radical-mediated abstraction of hydrogen atoms from methylene carbons in polyunsaturated fatty acids.[22] The lipid peroxidation inhibitory activity of LPH with the linoleic acid system gave an IC_{50} value of 12.3 ± 0.98 mg/mL, which was not statistically different from that of the LPH-IV fraction ($p > 0.05$), but much lower than those of the LPH-I, LPH-II, and LPH-III fractions. On the basis of our previous results,[21] the average molecular weight of LPH was 4 times more than that of GSH (glutathione, data not shown), so LPH had stronger antioxidant activities than GSH at the same molar concentration.

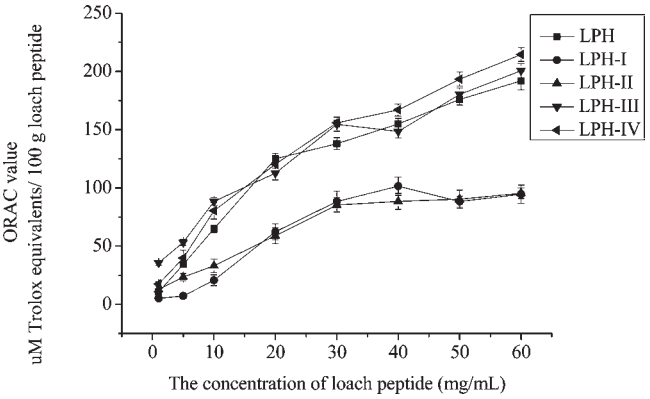

Figure 1. ORAC values of loach peptides (0−60 mg/mL). The error bars represent one standard deviation.

The ORAC values of all fractions increased with increasing concentrations. LPH-IV also showed the highest ORAC value (Figure 1). When the concentration of LPH-IV was 60 mg/mL, the ORAC value reached 215 ± 5.9 μM Trolox/100 g loach peptide, higher than that of control (LPH) (193 ± 4.8 μM Trolox/100 g loach peptide) and the other fractions. The results are consistent with those of Ren et al.,[23] who also found that the peptides with MW < 3 kDa had higher antioxidant activity than the other fractions.

Accumulating evidence suggests that active oxygen and free radicals attack key biological molecules, leading to many degenerative disease conditions.[24,25] Many studies have demonstrated low antioxidant activity in tumor cells of injured organs (e.g., pulmonary, hepatic, pancreatic, and gastric cancer cells).[8,26] In previous work, the authors found that loach peptide could enhance the endogenous cellular defense system (including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) enzymes to eliminate reactive oxygen species when mice had high levels of active oxygen and free

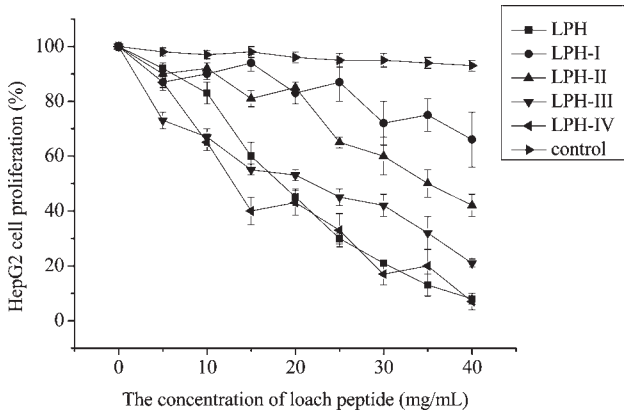

Figure 2. Percent inhibition of HepG2 cell proliferation by loach peptides (0−40 mg/mL). The error bars represent one standard deviation.

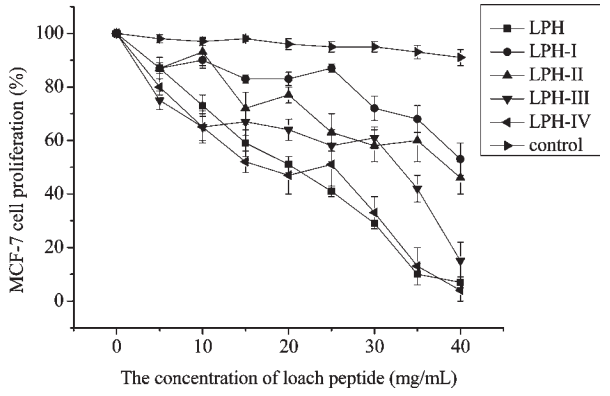

Figure 3. Percent inhibition of MCF-7 cell proliferation by loach peptides (0−40 mg/mL). The error bars represent one standard deviation.

radicals due to fatigue.[21] Therefore, loach peptide can protect cells against reactive oxygen species by scavenging free radicals and, thus, it can be hypothesized that it may serve as a potential source of anticancer agents.

Inhibition of HepG2 Cell Proliferation. The human HepG2 hepatoma cell line is widely used for biochemical and nutritional studies as a cell culture model of human hepatocytes because these cells retain their morphology and most of their function in culture.[27−29] The antiproliferative activity of LPH and its fractions on the growth of HepG2 human hepatoma cells in vitro are presented in Figure 2. All fractions inhibited HepG2 cell proliferation at doses of 5−40 mg/mL ($p < 0.05$), and the inhibition was dose-dependent. The LPH-IV fraction showed significant antiproliferative activity against HepG2 cells at doses of 5−15 mg/mL ($p < 0.05$) in a dose-dependent manner. When the concentration was 40 mg/mL, the HepG2 cell proliferation of LPH-IV was 7% of the control, with no significant difference from that of LPH (8%, $p > 0.05$), but 8.4-, 5-, and 2-fold lower than those of LPH-I, LPH-II, and LPH-III, respectively ($p < 0.05$). Therefore, the LPH and LPH-IV fractions with a strong inhibitory activity of HepG2 cancer cell growth might be studied further to determine if this activity is retained in vivo. The results were similar to those of Kannan,[13] who found that the <5 kDa fraction of gastrointestinal-resistant peptide hydrolysate

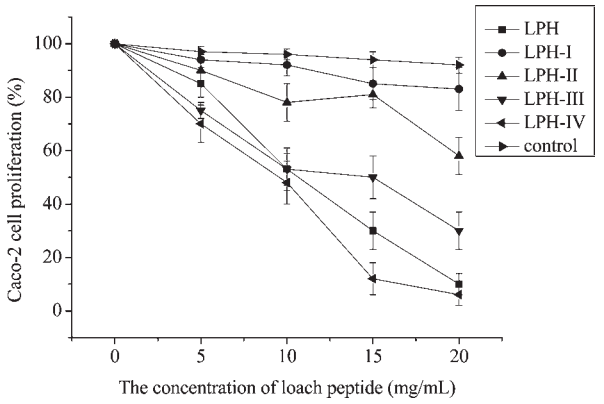

Figure 4. Percent inhibition of Caco-2 cell proliferation by loach peptides (0−20 mg/mL). The error bars represent one standard deviation.

Table 2. Correlation Analysis of the Total Antioxidant Activity and Antiproliferative Activity

	antiproliferative activity[a]		
sample	HepG2 cells	MCF-7 cells	Caco-2 cells
LPH	0.986**	0.978**	0.985**
LPH-I	0.665	0.714*	0.937*
LPH-II	0.868**	0.926**	0.923**
LPH-III	0.945**	0.871**	0.960**
LPH-IV	0.977**	0.964**	0.977**

[a] Correlation coefficient R^2. **, indicates significantly different $p < 0.01$; * indicates significantly different $p < 0.05$.

prepared from heat-stabilized defatted rice bran could inhibit the growth of HepG2 cells by 80%, stronger than that of other fractions with higher molecular weights.

Inhibition of MCF-7 Cell Proliferation. Figure 3 shows the effect of LPH and its fractions on MCF-7 breast cancer cell growth. The LPH and LPH-IV fractions were found to significantly inhibit the proliferation of viable cells compared to the higher molecular weight fractions. When the concentration is 40 mg/mL, the MCF-7 cell proliferation of LPH-IV is 4% of the control, with no significant difference from that of LPH (7%, $p > 0.05$), but 12- and 10-fold smaller than those of LPH-I and LPH-II, respectively ($p < 0.05$).

Inhibition of Caco-2 Cell Proliferation. Derived from a human colon carcinoma, Caco-2 cells were used as a model cell culture system in this study. As shown in Figure 4, the inhibition of cell proliferation of Caco-2 colon cells occurred in a dose-dependent manner. The LPH, LPH-III, and LPH-IV fractions demonstrated greater antiproliferative activity than the other fractions in a dose-dependent manner (0−20 mg/mL). The Caco-2 colon cell proliferation of LPH-IV is about 6% of the control, 12.8- and 8.7-fold lower than those of fractions LPH-I and LPH-II, respectively ($p < 0.05$). Kannan et al.[13] also found that gastrointestinal-resistant peptide fractions prepared from heat-stabilized defatted rice bran at <5 and 5−10 kDa had 70% and 50 inhibition activities on the growth of Caco-2 cells, respectively, stronger than those of the higher molecular weight fractions (>10 and >50 kDa). All of the fractions had a greater

ability to inhibit Caco-2 colon cancer cell proliferation than they had with HepG2 liver cancer and MCF-7 breast cancer cells.

Correlations. The correlations between total antioxidant activity (ORAC value) and antiproliferative activity toward HepG2, MCF-7, and Caco-2 cancer cells are summarized in Table 2. The ORAC values of all the fractions, except the LPH-I fraction, showed a very high correlation ($R^2 > 0.86$, $p < 0.01$) with their antiproliferative activity with the three cancer cell lines. For the LPH fraction, the correlation coefficient between the ORAC value and its antiproliferative activities toward HepG2, MCF-7, and Caco-2 cells were 0.986, 0.978, and 0.985, respectively ($p < 0.01$). For the LPH-IV fraction, the correlation coefficients between the ORAC value and its antiproliferative activities toward the three cancer cell types were a little lower than those of LPH, but still indicated a highly significant correlation. The positive correlation indicates that the higher ORAC values resulted in a higher inhibition of cancer cell proliferation. There was a weak correlation between the ORAC value of the LPH-I fraction and its inhibition of MCF-7 cell proliferation ($R^2 = 0.714$, $p < 0.05$). However, there was no significant linear relationship between the ORAC value and the antiproliferative activity of HepG2 cells in the LPH-I fraction tested ($R^2 = 0.665$, $p > 0.05$).

The results show that different molecular mass fractions of loach protein hydrolysates have different antioxidant activities in vitro and different abilities to inhibit the proliferation of human HepG2 hepatoma cells, MCF-7 breast cancer cells, and Caco-2 colon cancer cells. Therefore, loach protein hydrolysates could be a potential source of human antitumor bioactive agents if the results can be repeated in vivo. The fraction that had the highest antioxidant capacity in vitro, LPH-IV, also showed the best antiproliferative activity. These results are similar to those of Kannan et al.,[13] who found that the 5−10 and <5 kDa gastrointestinal-resistant fractions prepared from rice bran protein hydrolysates had significant inhibitory activity with both HepG2 and Caco-2 cancer cell lines compared to controls and other fractions. The current conclusion that higher antioxidant activity leads to better antiproliferative activity is also consistent with some previous reports that proposed that antioxidants have the potential to prevent and treat diseases associated with active oxygen species, especially some forms of cancer.[8,30,31] Chinery et al.[32] reported that antioxidants were better than chemotherapy agents such as 5-fluorouracil in causing complete remission of colorectal cancer.[8] Jang et al.[33] synthesized the peptide GFHI and found that it resulted in a slight decrease of MCF-7 cell viability in a dose-dependent manner. When 400 μg/mL of peptide GFHI was applied to the AGS stomach cancer cell system, viability was decreased by 75%. Although the dose of LPH and its fractions applied to the MCF-7 cells seemed to be higher than the GFHI, we have since purified and identified a unique peptide Pro-Ser-Tyr-Val from LPH with a yield of 0.64% of LPH.[34] The calculations then suggest that the concentration of this unique loach peptide was about 250 μg/mL, which is lower than that of GFHI (400 μg/mL) in the same test. The mechanisms that suppress tumor genesis often involve inhibition of tumor cell mediated protease activity,[30] attenuation of tumor angiogenesis,[35] promotion of cell cycle arrest,[36] induction of apoptosis,[37] or immunostimulation.[8,38] Results from this study suggest the necessity of performing clinical studies on the <3 kDa peptide fraction. However, identification and characterization of the specific anticancer peptides prior to the clinical studies might be a valuable intermediate step.

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