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Purification of a Novel Peptide Derived from a Shellfish, *Crassostrea gigas*, and Evaluation of Its Anticancer Property

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ABSTRACT: A novel anticancer peptide was purified from *Crassostrea gigas* and investigated for its cytotoxic activity. To prepare the peptide, eight proteases were employed for enzymatic hydrolysis. Flavourzyme hydrolysate, which showed clearly superior cytotoxic activity on prostate cancer cells, was further purified using a membrane system and consecutive chromatographic methods. Finally, a novel anticancer peptide was obtained, and the sequence was partially purified as His-Phe-Asn-Ile-Gly-Asn-Arg-Cys-Leu-Cys at the N-terminus. The peptide purified from *C. gigas* effectively induced the death of prostate, breast, and lung cancer cells but not normal liver cells. This is the first report of an anticancer peptide derived from the enzymatic hydrolysates of *C. gigas*.

KEYWORDS: *Crassostrea gigas, anticancer peptide, cancer cells, enzymatic hydrolysis, flavourzyme*

INTRODUCTION

Crassostrea gigas, known as the Pacific oyster, is a widely cultivated aquatic species with an annual world production of 4.6 million metric tons in 2006.[1] There are more than 100 species of oysters worldwide. Oysters are a nutrient-rich food source that includes minerals such as calcium, phosphorus, and iron, various vitamins and amino acids, ands proteins, lipids, and glycogen. Taurine and glycogen from oysters affect many chronic diseases, hepatitis, and eye strength recovery. Further, the oyster is rich in selenium, which supports a number of cellular functions, including heavy metal detoxification.[2]

C. gigas has been thoroughly researched in areas related to the food industry, and much is known about their culture conditions, cDNA cloning or gene expression during development, and environment.[2−4] Recently, it has been reported that oyster extracts have several bioactive peptides, including angiotensin-converting enzyme (ACE) inhibitory peptides and antifungal peptides.[5,6] However, the anticancer effects of *C. gigas* have not been evaluated.

In recent years, peptides generated by the digestion of various proteins, including animal and plant sources, have been found to possess biofunctional activity.[7] These peptides are inactive within the sequences of their parent proteins and are released during gastrointestinal digestion or food processing.[8] Once such bioactive peptides are liberated, depending on their structural, compositional, and sequential properties, they may exhibit various biofunctional activities. The functional properties of a protein can be improved by enzymatic hydrolysis under controlled conditions. Indeed, hydrolytic processes have been developed to convert underutilized materials into marketable and acceptable forms, which can then be widely used in food rather than as animal feed or fertilizer. Hydrolysis potentially influences the molecular size, hydrophobicity, and polar groups of hydrolysates.[9] Further, the characteristics of a hydrolysate directly affect its functional properties and potential as a food ingredient.

Despite the intensive efforts and substantial advances that have occurred by focusing on improving treatments, cancer is still a leading cause of death worldwide. Chemotherapy is one of the major approaches for treating cancer by delivering a cytotoxic agent to the cancer cells. The main problem with chemotherapy is the inability to deliver the correct amount of drug directly to cancer cells without affecting normal cells.[10] Apoptosis as a form of programmed cell death is one of the major mechanisms of cell death in response to cancer therapies.[11] A growing body of evidence shows that most marine anticancer peptides that exhibit cytotoxicity may trigger apoptosis by targeting many cellular proteins, and the induced apoptotic process involves both intracellular and extracellular pathways.[12,13] The objective of this work was, therefore, to identify and investigate the effect of the cancer toxicity components hydrolysated from *C. gigas*.

MATERIALS AND METHODS

Materials. *C. gigas* was obtained from a local market (Jeonnam, Korea). Dulbecco's modified Eagle's medium (DMEM), RPMI medium 1640, fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Invitrogen Corp. (Carlsbad, CA). RNase A and Tween 20 were purchased from Novagen (Darmstadt, Germany) and USB (Cleveland, OH), respectively. Four enzymes (papain, pepsin, α-chymotrypsin, and trypsin) were obtained from Sigma Chemical Co. (St. Louis, MO), and four other enzymes (flavourzyme, neutrase,

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Table 1. Apoptosis Rates of Various Enzymatic Hydrolysates from *C. gigas* on PC-3 Prostate Cancer Cells

	flavourzyme	neutrase	protamex	alcalase	papain	pepsin	α-chymotrypsin	trypsin
apoptosis (LC_{50}, mg/mL)	2.41 ± 0.43[a]	4.19 ± 0.21	3.34 ± 0.34	6.89 ± 0.37	5.20 ± 0.54	4.08 ± 0.98	5.33 ± 0.93	4.38 ± 0.22

[a]Mean ± SD of determinations made in triplicate experiments. Significantly different at the $p < 0.05$ level, as analyzed by Bonferroni's multiple-comparison tests.

protamex, and alcalase) were donated by Novozyme Co. (Bagsvaerd, Denmark). All other reagents were of the highest grade commercially available.

Preparation of Enzymatic Hydrolysates from *C. gigas*. Prior to enzymatic hydrolysis, *C. gigas* was pulverized into a powder using a grinder, and the enzymatic hydrolysates were obtained following the method described by Park et al.[14] Briefly, 100 mL of buffer solution was added to 2 g of powder sample, and then 40 μL (or 40 mg) of each enzyme was added after a 30 min preincubation. The enzymatic hydrolysis reactions were conducted for 8 h to achieve an optimal hydrolytic level and the mixtures immediately heated to 100 °C for 10 min to inactivate the enzyme. The mixture was then rapidly cooled to 20−25 °C in an ice bath. The resulting solution was filtered with Whatman filter paper No. 41 (GE Healthcare, Piscataway, NJ).

Purification of an Anticancer Peptide from *C. gigas*. *Fractions from a TFF System.* The enzymatic hydrolysates from *C. gigas* were fractionated through ultrafiltration (UF) membranes with a range molecular weight cutoffs (MWCOs) of 30, 10, and 5 kDa, using a TFF system. The fractions were designated as follows. CGEH (*C. gigas* enzymatic hydrolysates) I refers to the filtrates that were not passed through a 30 kDa MWCO membrane. CGEH II refers to the filtrates that were passed through a 30 kDa MWCO membrane but not through a 10 kDa MWCO membrane. CGEH III refers to the filtrates that were passed through a 10 kDa MWCO membrane but not through a 5 kDa MWCO membrane. CGEH IV refers to filtrates that were passed through a 5 kDa MWCO membrane. All fractions recovered were lyophilized in a freeze drier for 3 days.

Ion-Exchange Chromatography Using a Diethylaminoethyl (DEAE)-Sephacel. Among the eight enzymatic hydrolysates, CGEH IV filtrates separated from flavourzyme hydrolysates, showing toxicity activity in PC-3 cells, were selected for further purification. Four milliliters of CGEH IV filtrates (250 mg/mL) was loaded onto a DEAE-Sephacel ion-exchange column (74 mm × 280 mm), equilibrated with 1.0 M Tris-HCl buffer (pH 8.0) and eluted with a linear gradient of NaCl (0 to 1.0 M) in the same buffer at a flow rate of 80 mL/h by open column chromatography. Each fraction was monitored at 280 nm, collected with a volume of 3.0 mL, and pooled as three fractions. The pooled solution was desalted overnight using a dialysis membrane and then lyophilized, and the cytotoxic activity of each fraction was investigated. The chromatography was conducted as the next step with the strongest anticancer fraction.

High-Performance Liquid Chromatography (HPLC). The fraction exhibiting the highest cytotoxic activity was further purified by HPLC on a GPC-SB802.5 column (4 mm × 300 mm) with distilled water at a flow rate of 2.0 mL/min. The elution peaks were detected at 215 nm, pooled and concentrated using a rotary evaporator, and lyophilized for 3 days. The fraction exhibiting the highest cytotoxic activity was further purified using reversed-phase HPLC (RP-HPLC) on a C_{18} column (20 mm × 250 mm) with a linear gradient of acetonitrile (0 to 80%) at a flow rate of 2.0 mL/min. The elution peaks were detected at 215 nm, pooled, concentrated using a rotary evaporator, and lyophilized for 3 days. For further purification, the fraction with the highest toxicity from the first RP-HPLC run was loaded onto a C_{18} column (4.0 mm × 250 mm) with a linear gradient of acetonitrile (0 to 80%) at a flow rate of 0.5 mL/min. The fraction with the highest toxicity activity from the second RP-HPLC run was further purified by being loaded onto a C_{18} column (4.0 mm × 250 mm) with a linear gradient of acetonitrile (0 to 40%) at a flow rate of 0.2 mL/min. Potent peaks were collected, evaluated for cytotoxic activity, and then lyophilized. The final purified peptide was analyzed for amino acid sequence.

Identification of the Amino Acid Sequence of the Purified Peptides by Edman Degradation. Amino acid sequences of the purified peptide were partially sequenced at the N-terminus using automated Edman degradation using a Milligen 6600 protein sequencer (Milligen, Watford, U.K.) by the Korea Basic Science Institute. The purified peptide was searched against the NCBI nonredundant peptide database (http://www.ncbi.nlm.nih.gov/blast).

Cell Culture. Prostate cancer cells were cultured and maintained in RPMI medium 1640, and normal liver cells and breast, lung, and liver cancer cells were cultured and maintained in DMEM supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% FBS and maintained at 37 °C under a humidified atmosphere with 5% CO_2. All the treatments were performed at 30% confluence.

Apoptosis Analysis. For apoptosis analysis, the harvested cells were fixed with ethanol (containing 0.5% Tween 20) for 24 h, incubated with 50 μg/mL propidium iodide (PI) and 1 μg/mL RNase A at 37 °C for 30 min, and analyzed by flow cytometry, using a FACScan instrument (BD, Franklin Lakes, NJ). The cells that belong to the sub-G1 population were considered apoptotic cells, and the percentage of each phase of the cell cycle was determined. The apoptosis rate was expressed as LC_{50} (milligrams per milliliter), the concentration of the anticancer peptide that results in the death of 50% of cancer cells.

Statistical Analysis. Statistical analysis of data was conducted with GraphPad Prism (GraphPad Software, Inc., San Diego, CA). Multigroup data were analyzed using a one-way analysis of variance followed by Bonferroni's multiple-comparison tests. All results are expressed as means ± the standard deviation (SD) of comparative fold differences. Data are representative of three independent experiments.

RESULTS AND DISCUSSION

Preparation of *C. gigas* Protein Hydrolysates and Selection Based on Their Anticancer Properties. Prostate cancer is one of the most common malignant tumors; surgical removal and chemotherapy have been the mainstays of prostate cancer treatment. For the treatment of cancer, limited numbers of effective anticancer drugs are currently in use, even though they have some side effects such as nausea, vomiting, diarrhea, skin rashes, and headache. There is a need for new, side-effect safe, cheap, and effective anticancer drugs to combat this dreaded disease.[15] Natural products continue to be a major source of pharmaceuticals and for the discovery of new molecular structures. It has been reported that some compounds derived from marine organisms have antioxidant properties and anticancer activities, but they are largely unexplored.[16] In this study, we investigated the apoptosis

Table 2. Apoptosis Rates of Four Fractions Separated from Flavourzyme Hydrolysates of *C. gigas* with Various Molecular Weights

MWCO[a] (Da)	LC_{50} (mg/mL)	recovery rate (%)
CGEH[b] I (≥30000)	5.48 ± 0.84[c]	38.6
CGEH II (10000−30000)	4.25 ± 0.58	5.4
CGEH III (5000−10000)	6.57 ± 0.97	11.9
CGEH IV (<5000)	2.06 ± 0.54[d]	33.1

[a]Molecular weight cutoff. [b]*C. gigas* enzymatic hydrolysates. [c]Mean ± SD of determinations made in triplicate experiments. [d]Significantly different at the $p < 0.05$ level, as analyzed by Bonferroni's multiple-comparison tests.

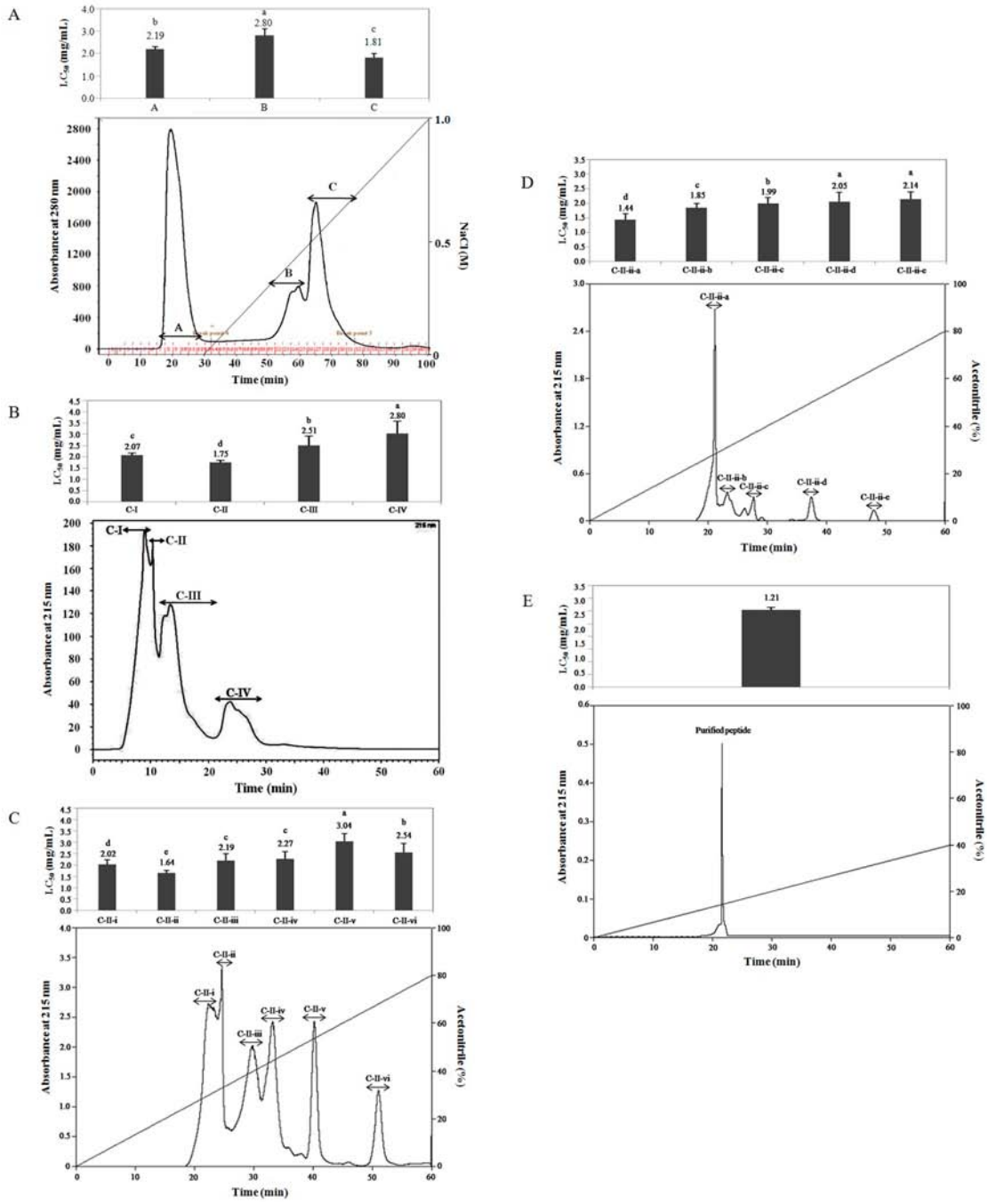

Figure 1. Purification of an anticancer peptide from the flavourzyme hydrolysate of *C. gigas*. (A) Ion-exchange chromatogram of the CGEH IV fraction separated from the flavourzyme hydrolysate by DEAE-Sephacel anion-exchange chromatography. The elution was performed at a flow rate of 80 mL/h with a linear NaCl gradient (0 to 1.0 M) in 1.0 M Tris-HCl buffer (pH 8.0). (B) High-performance liquid chromatography (HPLC) pattern on a GPC-SB802.5 column (4 mm × 300 mm) of active fraction C and toxicity activities in cancer cells (top panel) of the fractions. HPLC was conducted with the same buffer at a flow rate of 2.0 mL/min using an UV detector at 215 nm. (C) First reversed-phase HPLC (RP-HPLC) pattern on a C_{18} column (20 mm × 250 mm) of active fraction C-II and toxicity activities in cancer cells (top panel) of the fractions. HPLC was conducted with a linear CH_3CN gradient (0 to 80%) at a flow rate of 2.0 mL/min using an UV detector at 215 nm. (D) Second RP-HPLC pattern from a C_{18} column (4.0 mm × 250 mm) of active fraction C-II-ii and toxicity activities in cancer cells (top panel) of the fractions. HPLC was conducted with a linear CH_3CN gradient (0 to 80%) at a flow rate of 0.5 mL/min using an UV detector at 215 nm. (E) Third RR-HPLC pattern from a C_{18} column (4.0 mm × 250 mm) of active fraction C-II-ii-a and toxicity activities in cancer cells (top panel) of the fractions. The fourth HPLC was conducted with a linear CH_3CN gradient (0 to 40%) at a flow rate of 0.2 mL/min using an UV detector at 215 nm. Values with different letters (a−e) are significantly different at the $p < 0.05$ level, as analyzed by Bonferroni's multiple-comparison tests.

rates of the critical cancer cells. As shown in Table 1, the flavourzyme hydrolysates from *C. gigas* showed the highest cytotoxic activity, resulting in apoptosis rates in PC-3 cells with LC_{50} values of 2.41 mg/mL in a dose-dependent manner.

Purification of an Anticancer Peptide from *C. gigas*. To purify the anticancer peptide, the flavourzyme hydrolysate with the highest cytotoxic activity from *C. gigas* was filtered via various MWCO membranes, consecutively. As shown in Table

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Table 3. Apoptosis Rates of the Anticancer Peptide Purified from *C. gigas* in Various Cancer Cells

	PC-3 prostate	A549 lung	MDA-MB-231 breast
apoptosis (LC_{50}, mg/mL)	1.21 ± 0.18	1.87 ± 0.62	2.07 ± 0.27

Table 4. Cytotoxic Activities and Purification Folds in the Stages of Purification from *C. gigas* Protein Hydrolysates

fraction	apoptosis (LC_{50}, mg/mL)	recovery rate (%)
flavourzyme	2.41 ± 0.43	88.98
MWCO[a] of <5000	2.06 ± 0.54	33.11
DEAE Sephacel	1.81 ± 0.74	10.83
GPC (4 mm × 300 mm)	1.75 ± 0.92	1.94
ODS C_{18} (20 mm × 250 mm)	1.64 ± 0.33	0.38
ODS C_{18} (4 mm × 250 mm)	1.44 ± 0.75	0.16
ODS C_{18} (4 mm × 250 mm)	1.21 ± 0.18	0.13

[a]Molecular weight cutoff.

2, the recovery rates of CGEH I, CGEH II, CGEH III, and CGEH IV were 38.6, 5.4, 11.9, and 33.1%, respectively. The 50% lethal concentrations of CGEH I, CGEH II, CGEH III, and CGEH IV were 5.48, 4.25, 6.57, and 2.06, respectively (Table 2). A previous study has reported that short peptides exert greater anticancer potential than other bioactive properties,[17] and our results agree with the earlier reports.

The CGEH IV fraction exhibited the highest toxicity activity and was selected for further study. CGEH IV was dissolved in deionized water and loaded onto a DEAE-Sephacel anion-exchange column with a linear gradient of NaCl (0 to 1.0 M). The elution peaks were monitored at 280 nm, and each 3.0 mL fraction was collected and fractionated into three subfractions (Figure 1A). Each fraction was pooled, lyophilized, and measured for cytotoxic activity as the apoptosis rate. As a result, fraction C potentially induced toxicity in cancer cells, and the LC_{50} value was 1.81 mg/mL (Figure 1A). Therefore, fraction C was selected for the next step. Lyophilized active fraction C was further separated by HPLC on a GPC-SB802.5 column (4 mm × 300 mm) with distilled water, and the fraction was divided into four subfractions (C-I, C-II, C-III, and C-IV) (Figure 1B). Fraction C-II exhibited stronger cytotoxic activity than the other fractions, with a LC_{50} value of 1.75 mg/mL. Lyophilized active fraction C-II was further separated by RP-HPLC on a C_{18} column (20 mm × 250 mm) using a linear gradient of acetonitrile (0 to 80%), and the fraction was divided into six subfractions from C-II-i to C-II-vi (Figure 1C). Among them, fraction C-II-ii exhibited the strongest cytotoxic activity, with an LC_{50} value of 1.64 mg/mL. Fraction C-II-ii was further subjected to a second separation by RP-HPLC on a C_{18} column (4.0 mm × 250 mm) using a linear gradient of acetonitrile (0 to 80%) and fractionated into five subfractions (Figure 1D). The fractions were pooled and lyophilized. Fraction C-II-ii-a, of the five fractions pooled, exhibited the strongest toxicity activity, with an LC_{50} value of 1.44 mg/mL. Finally, we obtained a purified anticancer peptide (Figure 1E). The amino acid sequences were measured, and the partial sequence of the purified peptide was His-Phe-Asn-Ile-Gly-Asn-Arg-Cys-Leu-Cys at the N-terminus. In addition, the toxicity activity of the peptide was investigated, and the LC_{50} values were 1.21, 1.87, and 2.07 mg/mL for PC-3, A549, and MDA-MB-231 cells, respectively (Table 3). As summarized in Table 4, the purification of the anticancer peptide was accomplished in seven steps: separation into fractions using a membrane system and chromatography techniques, followed by ion exchange on Sephacel and HPLC. The activity of the final fractions increased approximately 2-fold compared to that of the crude hydrolysates. These activities are stronger than those of other enzymatic hydrolysates.[18]

Many bioactive peptides have been extracted by enzymatic hydrolysis using various enzymes. One of the approaches for the effective release of bioactive peptides from protein sources is enzymatic hydrolysis, which is widely applied to improve and upgrade the functional and nutritional properties of protein.[19] Many of these properties are attributed to physiologically active peptides in protein molecules.[20] Peptides derived from a food source may have diverse biological activities of interest for human health such as antihypertensive, antithrombotic, opioid, immunomodulatory, antimicrobial, and anticancer activities.[20,21] Dietary consumption for anticancers appears to provide further benefits to the endogenous anticancer defense in the fight against oxidative stress.[22] Recent studies have shown that peptides with anticancer properties can be released from several food sources such as soy protein[17] and milk casein.[23] These peptides either are present in the raw material or are generated during food processing and protein hydrolysis by digestive enzymes. In this study, therefore, we utilized eight proteases to extract anticancer peptides from *C. gigas* and evaluated their anticancer activity as determined by their toxicity capacity in cancer cells. Among the eight proteases, flavourzyme hydrolysates showed stronger toxicity in cancer cells than other hydrolysates, which suggests its potential in tumor therapy. Recently, it has been proposed that the potential of food to provide biologically active peptides[21] should also be considered. It has been reported that the growth of transplantable sarcoma-S180 was markedly inhibited in a dose-dependent manner in mice given the oyster hydrolysates.[24]

Dead-end filtration results in a buildup of product on the membrane surface that may damage product, lower recovery, and foul the membrane. Fouling impedes the filtration rate until it eventually stops.[25] On the other hand, TFF involves the recirculation of the retentive across the surface of the membrane. This gentle cross-flow feed acts to minimize membrane fouling, maintains a high filtration rate, and provides higher product recovery because the sample remains safely in solution.[26,27] Accordingly, in this study, we chose a TFF system to separate flavourzyme hydrolysates of different molecular weights. Many researchers have reported that short peptides have more potent bioactivity,[17] and we were able to verify that observation. In particular, Wu et al.[28] reported that the N-terminal sequence of the peptide was more important than the C-terminal sequence from a functional point of view. Anticancer activity has been also attributed to certain amino acid sequences,[23] as well as large amounts of hydrophobic amino acids such as Leu, Met, and Ile.[29] Davalos et al.[30] reported that a peptide containing Met, Trp, and Tyr exhibited the highest anticancer activity. The addition of a Leu or Pro residue to the N-terminus of a His-His dipeptide enhanced anticancer activity and facilitated further synergy with non-peptide anticancers such as BHT. Therefore, we speculated that the toxicity activity of the peptide obtained from the *C. gigas* hydrolysate could be attributed to the presence of His, Ile, and Leu. These results suggest that *C. gigas* hydrolysates have

significant health-promoting effects with excellent cytotoxic activity.

In conclusion, the search for beneficial agents led to a novel anticancer peptide from *C. gigas*. The toxicity ability of enzymatic hydrolysates from *C. gigas* was examined by flow cytometry, and the resulting anticancer peptides were purified using consecutive chromatographic methods. Finally, we obtained an anticancer peptide, and the partial sequence of the purified peptide was found to be His-Phe-Asn-Ile-Gly-Asn-Arg-Cys-Leu-Cys at the N-terminus. The results showed that the novel anticancer peptide efficiently induced toxicity in cancer cells but not in normal liver cells, and this is the first report of an anticancer peptide derived from the enzymatic hydrolysates of *C. gigas*.

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Notes

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