A novel anti-proliferative pentapeptide (ILYMP) isolated from *Cyclina sinensis* protein hydrolysate induces apoptosis of DU-145 prostate cancer cells

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Abstract. Prostate cancer is the main causes of cancer associated mortality in men worldwide, cancer patients often suffer serious side effects when treated with chemotherapy or radiotherapy, therefore novel drugs are in high demand to treat prostate cancer. In the present study, a pentapeptide (Ile-Leu-Tyr-Met-Pro; ILYMP) with a molecular weight of 635.71 Da was isolated from the protein hydrolysate of *Cyclina sinensis* via ultrafiltration and chromatographic methods, and subsequently named *Cyclina sinensis* pentapeptide (CSP). The activity of CSP was first investigated in prostate cancer (PCa) DU-145 cells. CSP was demonstrated to significantly inhibit DU-145 cell proliferation at a half-maximal inhibitory concentration of 11.25 mM at a 72 h time interval. In addition, the results of acridine orange/ethidium bromide double staining, scanning electron microscopy and flow cytometry analyses suggested that CSP inhibited DU-145 cell proliferation via the induction of apoptosis. Following treatment with CSP, Bcl-2-associated X (Bax), cleaved caspase-3 and cleaved caspase-9 protein expression levels were enhanced in DU-145 cells; whereas B-cell lymphoma 2 expression was suppressed in DU-145 cells. In conclusion, to the best of the authors' knowledge, this is the first study to investigate the effects of an anti-proliferative peptide derived from *Cyclina sinensis* on DU-145 cells, and the results suggested that CSP may represent a therapeutic nutraceutical agent for the treatment of patients with PCa.

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Introduction

Prostate cancer (PCa) is one of the most prevalent malignancies in men in the USA and worldwide, and is the second leading cause of cancer associated mortality in men (1,2). Each year, more than 913,000 novel cases are diagnosed worldwide, resulting in the mortalities of >261,000 patients (3). In addition, the incidence and mortality rate of PCa in China continues to rapidly increase, particularly in patients suffering from obesity or diabetes (4,5). Androgen deprivation therapy has been previously demonstrated to be effective in ~90% of patients with PCa. However, androgen deprivation therapy may develop into androgen-independent PCa and eventually into lethal, castration-resistant PCa (6). Researchers have developed second generations of hormone therapy, such as abiraterone and enzalutamide for the treatment of PCa (7,8). Despite such therapies improving overall survival, the majority of patients with PCa develop resistance following initial treatment, and so further investigation is required to develop novel therapeutic treatment strategies with a low toxicity.

Biofunctional peptides (2-20 amino acids) are protein fragments that may exhibit numerous physiological effects, such as anticancer (9,10), anti-thrombosis (11), antioxidant (12,13), anti-fatigue (14,15) and antimicrobial (16,17). Recently, numerous antitumor peptides have been isolated from marine derived protein hydrolysates to induce PCa cell apoptosis (18-20). Huang *et al* (18) demonstrated that a tripeptide (Gln-Pro-Lys; QPK) isolated from sepia ink inhibits the proliferation of numerous human PCa lines (DU-145, PC-3 and LNCaP); whereas Song *et al* (19) revealed that a hexapeptide (Tyr-Ala-Leu-Arg-Ala-His; YALRAH), obtained from the heated products of protein hydrolysates isolated from *Setipinna taty*, inhibits PC-3 cell proliferation. Furthermore, Kim *et al* (20) demonstrated that a decapeptide (Ala-Val-Leu-Val-Asp-Lys-Gln-Cys-Pro-Asp; AVLVDKQCPD), isolated from the protein hydrolysates of *Ruditapes philippinarum*, inhibits the proliferation of PCa cells. Notably, numerous biofunctional peptides have been purified from fish sources, whereas only a number of studies have investigated proteins obtained from crustacean and mollusk sources. *Cyclina sinensis*, a bivalve mollusk belonging to the Veneridae family, has been

used in traditional Chinese medicine for the treatment of inflammation, asthma and dental ulcers (21,22). Furthermore, it has been revealed that *Cyclina sinensis* has a high quantity of protein, polysaccharides and lipids, which may attribute to its therapeutic effects, such as anticancer, antioxidant and hepatoprotective activities (21-23).

Jiang *et al* (22,23) reported that the polysaccharide fraction of *Cyclina sinensis* (CSPS) exhibits significant inhibitory effects against human gastric cancer BGC-823 cells *in vivo*. In our previous study (24), the anti-proliferative potential of protein hydrolysates isolated from *Cyclina sinensis* was determined; however, the active component in the protein hydrolysates was not investigated further. Therefore, the present study aimed to isolate the pentapeptide (Ile-Leu-Tyr-Met-Pro; ILYMP) of interest from *Cyclina sinensis* protein hydrolyastes via ultrafiltration as well as chromatographic methods, and was subsequently named CSP. The effect of CSP on the PCa cell line DU-145 was investigated using methylthiazolyldiphenyl-tetrazolium bromide (MTT) assays, acridine orange/ethidium bromide double staining (AO/EB), scanning electron microscopy and flow cytometry. Furthermore, western blotting was performed, and the results demonstrated that the Bcl-2-associated X (Bax), cleaved caspase-3 and cleaved caspase-9 proteins were activated in CSP-treated DU-145 cells; whereas B-cell lymphoma-2 (Bcl-2) was suppressed in CSP-treated DU-145 cells. These results suggest that CSP can inhibit the proliferation of human PCa cells and may represent a therapeutic nutraceutical agent for the treatment and prevention of PCa.

Materials and methods

Materials. *Cyclina sinensis* were purchased from a local fish market in Zhoushan, China. Trypsin and neutral protease were purchased from YTHX Biotechnology Co., Ltd. (Beijing, China). MTT and Annexin V-FITC apoptosis detection kits were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The DU-145 PCa cell lines and NIH-3T3 cell lines were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Antibodies against β-actin (cat. no. AA132), Bax (cat. no. AB026), Bcl-2 (cat. no. AB112), caspase-3 (cat. no. AC030) and caspase-9 (cat. no. AC062) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Horseradish peroxidase-conjugated goat-anti-rabbit secondary antibodies (cat. no. A0208) were purchased from Beyotime Institute of Biotechnology. All other reagents used were of analytical grade.

Fractionation of protein hydrolysates by ultrafiltration. *Cyclina sinensis* were hydrolyzed using neutral protease under conditions: 1,200 U/g, solid-liquid ratio 1:4, (pH 7.0) at 45˚C for 6 h. The protein hydrolysates were then fractionated using ultrafiltration (Amicon 8400; EMD Millipore, Billerica, MA, USA) with 3, 5 and 8 kDa molecular weight cut-off membranes at 0.30 MPa, 20˚C. The fractions were then collected as follows: >8, 8-5, 5-3, and <3 kDa. Fractions were then lyophilized at -60˚C to further investigate whether *Cyclina sinensis* exhibits an antitumor effect on DU-145 cells.

Gel filtration chromatography. The elution with the highest antitumor activity following ultrafiltration was dissolved in

0.1 M Tris-HCl (0.05 g/ml, pH 7.0), 500 μl sample was added to a Superose 12 10/300 GL (GE Healthcare, Chicago, IL, USA; 10x300 mm) pre-equilibrated with Tris-HCl (0.1 M, pH 7.0) and then eluted at a flow rate of 1 ml/min using an AKTA purifier 100 (GE Healthcare) at room temperature. Fractions were isolated and detected at 280 nm, and the elution peaks were then isolated and lyophilized at -60˚C to investigate antitumor activity analysis.

High performance liquid chromatography (HPLC). The elution with the highest antitumor activity was further separated using reverse phase (RP)-HPLC (Agilent 1260; Agilent Technologies, Inc., Santa Clara, CA, USA) on a Agilent Zorbax SB-C18 (4.6x250 mm; 5 μm) column with a linear gradient of acetonitrile (0-7%) containing 0.06% trifluoroacetic acid at a flow rate of 1.0 ml/min. The purification was repeated >20 times at the same elution conditions and the final purified peptide (CSP) was collected and subsequently lyophilized at -60˚C to determine its amino acid sequence as well as its antitumor activity against DU-145 cells.

Determination of amino acid sequence and molecular mass of CSP. CSP was dissolved in 15 μl 37% CH_3CN (v/v) solution and applied to TFA-treated glass fiber filters (Shimadzu Corporation, Kyoto, Japan) and then sequenced at the N-terminus using a PPSQ-31A protein sequencer (Shimadzu Corporation). A mass spectrometer (Waters ZQ 2000; Waters GmbH, Eschborn, Germany) combined with an electrospray ionization source was used to determine the molecular weight of the final purified peptide. Ionization was carried out in positive ion mode with a capillary voltage of 3.5 kV, a nebulizer gas (N_2) temperature of 250˚C and flow rate of 1.5 l/min.

Anti-proliferative activity against DU-145 cells. The effects of CSP on cell proliferation were determined using the MTT colorimetric assay according to the protocol detailed by Tang *et al* (25), with a number of modifications. Briefly, DU-145 cells were seeded in a 96-well plate ($1x10^4$ cells/well) and incubated at 37˚C overnight. Cells were then treated with different concentrations of CSP (0, 3.0, 6.0, 12.0, 18.0 and 22.5 mM) for 24, 48 and 72 h time intervals at 37˚C. Following this, PBS (200 μl) with 10% MTT was added to each well at room temperature. The medium was then removed, DMSO (150 μl) was added and the plates were then incubated at 37˚C for a further 4 h. The plates was transferred into a TYZD-I oscillator (Beijing BILON Co., Ltd., Beijing, China) and incubated for 15 min at room temperature. The cell proliferation inhibition rate (%) was calculated using the following equation: Inhibition percentage (%)=[($OD_{control}$-$OD_{treated}$)/($OD_{control}$-OD_{blank})] x100%

Acridine orange/ethidium bromide (AO/EB) staining. Cell morphology was investigated using AO/EB double staining as described by Tang *et al* (25). To accurately distinguish cells in different stages of apoptosis, DU-145 cells in exponential phase were digested using 0.25% trypsin, suspended at a final concentration of $1x10^5$ cells/well in a 6-well flat-bottomed plate and then cultured at 37˚C in a 5% CO_2 incubator. Cells were then treated with 0, 3.0, 12.0 and 18.0 mM CSP for a further 24 h. Finally, cells were stained using the AO/EB dye mixture (100 μg/ml) and the morphology of apoptotic cells was then
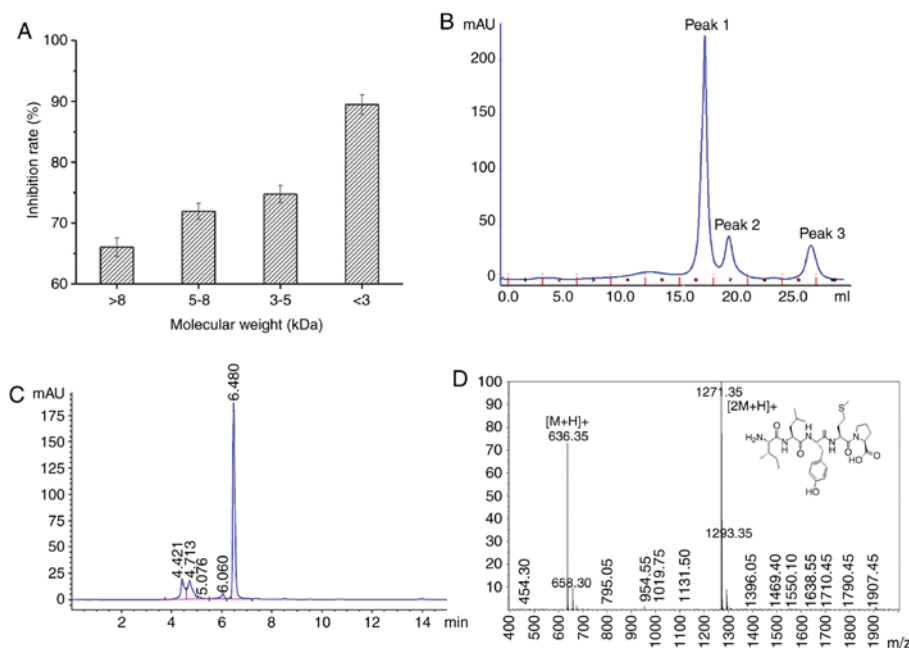
Figure 1. Purification and identification of activity peptide from protein hydrolysates isolated from *Cyclina sinensis*. (A) *Cyclina sinensis* were hydrolyzed using neutral protease, and protein hydrolysates (>8, 8-5, 5-3 and <3 kDa) were fractionated via ultrafiltration. Following this, protein hydrolysates were lyophilized to determine the anti-proliferative ability of CSP towards DU-145 cells. (B) The elution (peak 1) exhibiting the greatest inhibitory effect following ultrafiltration was then isolated via gel filtration chromatography. (C) The elution with highest antitumor activity (peak 1) was further separated using reverse phase high performance liquid chromatography on a Zorbax SB-C18 column with a linear gradient of acetonitrile (0-7%) containing 0.06% trifluoroacetic acid at a flow rate of 1.0 ml/min. (D) A mass spectrometer combined with an electrospray ionization source was used to determine the molecular weight of the CSP (635.71 Da).

immediately investigated using a fluorescent microscope (Leica DM 3000; Leica Microsystems GmbH, Wetzlar, Germany).

Scanning electron microscope. DU-145 cells were seeded in a 25 ml culture vessel and treated with 0, 3.0 and 12.0 mM CSP at 37°C. When cells reached a final concentration of $1x10^5$ cells/ml, they were subsequently cultured at 37°C in a 5% CO_2 incubator for 24 h. The harvested cells were fixed with 2.5% glutaraldehyde for 24 h and 1.5% osmium acid for 2 h at room temperature, dried and coated with gold by an ion sputtering coating machine. Finally, the cells were observed using a scanning electron microscope (Hitachi H-7650; Hitachi, Ltd., Tokyo, Japan).

Cell apoptosis analysis. Cell apoptosis rates were determined using an Annexin V fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining assay and flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, DU-145 cells were cultured in 6-well flat-bottomed plates at 37°C for 24 h and then treated with 0, 3.0, 12.0 and 18.0 mM CSP for a further 24 h. Harvested cells were then digested with 0.25% trypsin, resuspended in phosphate buffer and then collected by centrifugation (1,000 x g, 4°C for 5 min). Finally, the cells were incubated with 5 μl of Annexin V-FITC and 10 μl of PI for 15 min at room temperature in the dark and then immediately analyzed by flow cytometry using a flow cytometer.

Western blot analysis. Total protein lysates from the different cell groups were extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China), quantified by using BCA protein assay kit and equal amounts of protein (50 μg) were loaded per well on a 10% SDS-PAGE gel and then separated. Following SDS-PAGE, proteins were

transferred to a polyvinylidene difluoride membrane and the membrane was then blocked using 10% non-fat milk for 1.5 h at room temperature. The membrane was incubated with specific primary antibodies (β-actin, Bax, Bcl-2, caspase-3 and caspase-9; 1:1,000) at 4°C overnight and then washed three times using Tris-HCl with 0.05% Tween-20. Following this, the membrane was incubated with horseradish peroxidase-conjugated goat-anti-rabbit secondary antibodies (1:3,000) at room temperature for 2 h. The intensity of specific bands was visualized using enhanced chemiluminescence (FluorChem FC3, Protein Simple, Inc., San Jose, CA, USA) and then quantified using Quantity One software (version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data are presented as the mean ± standard deviation of at least four independent experiments. Data were analyzed by one-way analysis of variance with Turkey's test using the SPSS 19.0 software (IBM Corps., Armonk, NY, USA). $P<0.05$ was considered to indicate a significant difference.

Results and Discussion

Purification of activity peptide and peptide identification. The target peptide was purified from *Cyclina sinensis* protein hydrolysates via ultrafiltration, gel filtration chromatography and RP-HPLC. The anti-proliferative activity of the peptide against DU-145 cells was used to monitor the purification process. The <3 kDa peptide fraction was revealed to demonstrate the greatest anti-proliferative activity towards DU-145 cells (89.46±4.47%; Fig. 1A). Chi *et al* (9) reported that <1 kDa peptide fractions isolated from blood clam protein hydrolysates exhibit the greatest anti-proliferative activity against
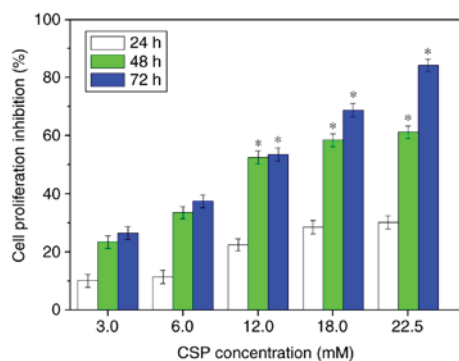
Figure 2. Inhibition of DU-145 cell proliferation treated with numerous concentrations of CSP. DU-145 cells were treated with numerous concentrations of CSP (0, 3.0, 6.0, 12.0, 18.0 and 22.5 mM) for 24, 48 and 72 h time intervals at 37˚C, and the inhibition of cell proliferation was investigated using a MTT assay. The half-maximal inhibitory concentration of CSP was ~11.25 mM at 72 h. The results were normalized to the 0 mM CSP group. P<0.05 vs. 24 h.
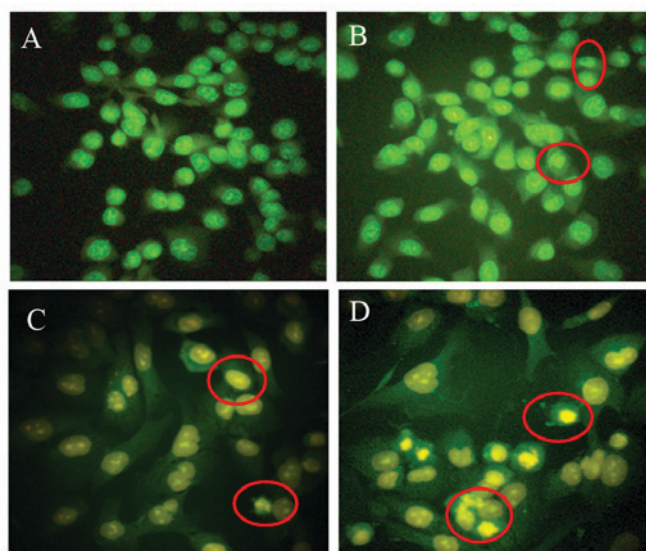

Figure 3. Morphological observation via acridine orange/ethidium bromid-estaining (magnification, x400). (A) Cells were treated with (A) 0, (B) 3.0, (C) 12.0 and (D) 18.0 mM CSP. Red circles represent (B) viable cells, (C) early apoptotic cells (D) and late apoptotic cells. Each experiment was performed in triplicate (n=3) and generated similar morphologic features.

PC-3, DU-145 and H1299 cells. Similar results were observed in protein hydrolysates obtained from *Setipinna taty* and *Ruditapes philippinarum* (26,27). The results of the present study were consistent with previous findings suggesting that short peptides exhibited greater anticancerous activities, and thus <3 kDa peptide fractions were subsequently collected and lyophilized for further investigation.

Following this, fractions <3 kDa were separated into three sub-fractions (peak 1, peak 2 and peak 3). The results of the MTT assay revealed that peak 3 exhibited the greatest anti-proliferative activity (12 mM; 59.46±3.67% CSP treatment for 24 h) towards DU-145 cells compared with peak 1 and peak 2 (Fig. 1B). Subsequently, peak 3 was collected, lyophilized and further purified via preparative RP-HPLC. The peak with a retention time of 6.480 min demonstrated the greatest inhibitory effect on DU-145 cell proliferation (Fig. 1C). Following this, this peak was further purified ~20 times via preparative RP-HPLC and subsequently subjected to MS and amino acid sequence analysis. Following analysis using a Shimadzu PPSQ-31A protein sequencer and electrospray ionization-mass spectrometry, the amino acid sequence of CSP was determined to be ILYMP (Fig. 1C) with a molecular weight of 635.35 Da [(M+H)+; Fig 1D], which was consistent with a theoretical mass of CSP (635.71 Da).

CSP exhibits an anti-proliferative effect against DU-145 cells. Cell proliferation occurs in almost all tissues and regulates cell proliferation and programmed cell death in order to ensure tissue and organ integrity. However, uncontrolled cell division may result in tissue hyperplasia as well as the development of diseases, such as cancer (10,28). Thus, inhibition of cell proliferation is considered to represent an effective therapeutic strategy for the treatment of cancer. In the present study, MTT assays were used to determine the inhibitory effect of CSP on DU-145 cell proliferation. The results revealed that CSP exhibited a marked increase in cytotoxicity against DU-145 cells in a dose-dependent manner, with an inhibition rate of ~84.17% at 22.5 mM at the 72 h time interval (Fig. 2). The half-maximal inhibitory concentration (IC_{50}) of CSP was ~11.25 mM at the 72 h time interval. Furthermore, CSP did not exhibit any toxicity

towards the normal NIH-3T3 cells (data not shown), which are frequently used as a healthy control cell line (8). Therefore, the results suggested that CSP exhibited selective toxicity towards cancer cells compared with normal cells.

Morphological observations. AO/EB staining has been previously used to investigate apoptosis in cancer cells (25,29). The present study aimed to determine whether CSP-induced inhibition of cell proliferation occurs via apoptosis by treating DU-145 cells with 3.0, 12.0 and 18.0 mM CSP and observing changes in cell morphology using AO/EB staining (Fig. 3). Early-stage apoptotic cells, marked by crescent-shaped or granular yellow green AO nuclear staining, were observed following treatment with 3.0 and 12.0 mM CSP for 24 h, which suggested that DU-145 cells were undergoing early stage apoptosis (Fig. 3A, B and C). Late-stage apoptotic cells, with concentrated and asymmetrically localized nuclear AO/EB staining, were observed following treatment with 18.0 mM CSP for 24 h (Fig. 3D). The morphological characteristics of apoptotic DU-145 cells in the present study were similar to the results of AO/EB staining in previous studies using DU-145, PC-3 and LNCaP cells that were treated with sepia ink oligopeptide QPK (18), HeLa cells treated with a hexapeptide (Phe-Ile-Met-Gly-Pro-Tyr; FIMGPY) isolated from *Raja porosa* cartilage protein hydrolysates (10) and PC-3 cells treated with a peptide (Arg-Asp-Gly-Asp-Ser-Cys-Arg-Gly-Gly-Gly-Pro-Val; RDGDSCRGGGPV) isolated from *Bullacta exarata* (30).

Scanning electron microscopy was used to further investigate the effects of CSP on DU-145 cells. Cells in the control group did not exhibit any typical morphological changes (microvilli reduction or disappearance, chromatin condensation or margination) (Fig. 4A and B). However, typical morphological changes were observed when cells were treated with 3.0 (Fig. 4C and D) and 12.0 mM CSP for
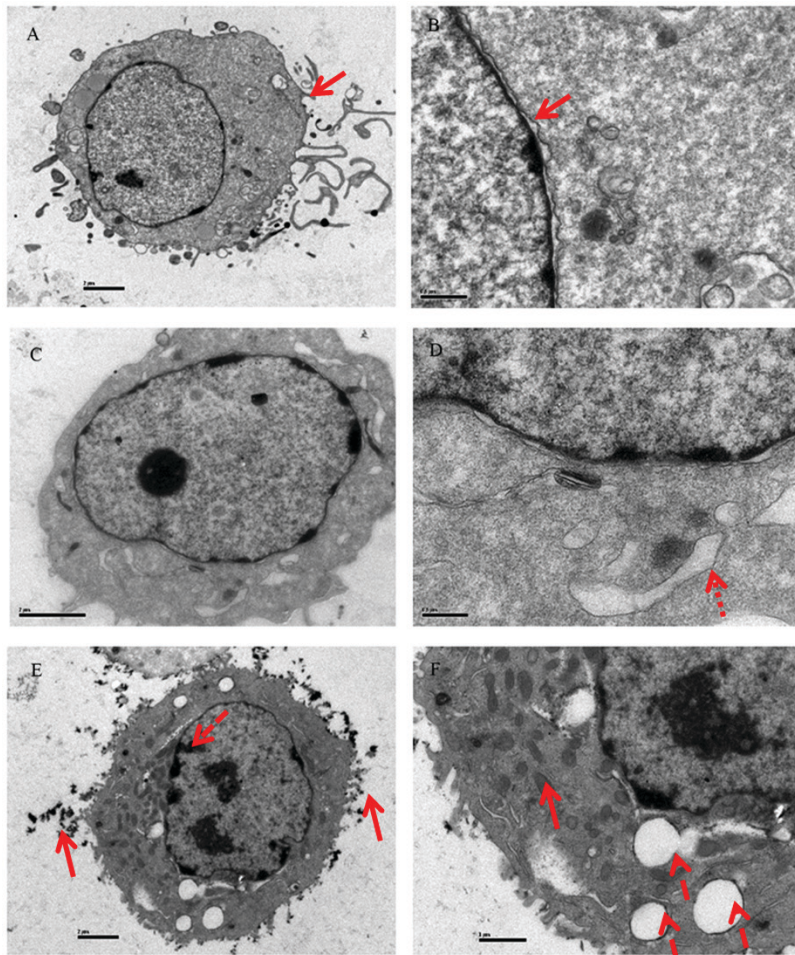
Figure 4. Morphological observation of DU-145 cells revealed by scanning electron microscopy. Untreated cells did not exhibit any typical morphological changes at (A) magnification, x3,700, the red arrow with the solid line in (A) indicates abundant microvilli are observed on the surface and (B) magnification, x17,500, the red arrow with the solid line in (B) indicates nuclear membrane was intact. Cells treated with 3.0 mM CSP at (C) magnification, x6,200 and (D) magnification, x17,500, the red arrow with the small dots in (D) indicates a smooth endoplasmic reticulum extension. Cells treated with 12.0 mM CSP at (E) magnification, x3,700 and (F) magnification, x8,900. The red arrow with the solid line in (E) indicates heterochromatin aggregation and the red arrow with long dashes indicates microvilli loss. The red arrow with the long dash in (F) indicates vacuoles appearing in the cytoplasm and the red arrow with the solid line indicates mitochondrial ridges disappearing.

24 h (Fig. 4E and F), such as a loss of microvilli structures on the surface of the cell membrane, chromatin condensation and the presence of apoptosis bodies. Furthermore, an expansion of the smooth endoplasmic reticulum, loss of mitochondrial cristae and an appearance of numerous cytoplasmic vacuoles were observed in CSP-treated DU-145 cells. In conclusion, these results suggested that apoptosis is enhanced in cells following treatment with CSP, and the morphological features observed were similar to those observed in DU-145 cells following treatment with an oligopeptide (Asp-Trp-Pro, DWP) isolated from *Ruditapes philippinarum* (31).

Cell apoptotic rate is enhanced in DU-145 cells following treatment with CSP. Cancer is a disease state characterized by disordered cell proliferation and inhibition of apoptosis. Previous therapeutic strategies for the treatment of tumors have focused on the inhibition of cell proliferation and the induction of apoptosis. In order to further investigate the induction of apoptosis by CSP, flow cytometric analysis was performed and the rate of cellular apoptosis was determined using Annexin V-FITC/PI double staining. The results revealed that

the percentage of Annexin V-FITC stained DU-145 cells in the control group was $6.10\pm1.1\%$ (Fig. 5A). Following 24 h exposure to 3.0, 12.0, and 18.0 mM CSP, the percentage of apoptotic cells increased to $9.01\pm1.3\%$ (Fig. 5B), $12.36\pm1.8\%$ (Fig. 5C) and $20.18\pm1.9\%$ (Fig. 5D), respectively; thus demonstrating a marked increase in apoptosis rates following treatment with CSP in a dose-dependent manner. Therefore, the results suggested that CSP induced apoptosis in DU-145 cells.

Expression levels of apoptosis associated proteins in DU-145 cells are increased following treatment with CSP. The results of the flow cytometry analysis suggested that apoptosis was increased in DU-145 cells following treatment with CSP in a dose-dependent manner. In order to investigate the underlying mechanisms, expression levels of anti- and pro-apoptosis associated proteins were determined in CSP-treated DU-145 cells via western blotting. The Bcl-2 protein family plays an important role in the regulation of apoptosis, and includes pro-apoptotic proteins (Bax, Bcl-2 associated agonist of cell death and Bcl-X) as well as anti-apoptotic proteins (Bcl-2, Bcl-extra large and Bcl-2-like protein) (32). Thus, the ratio
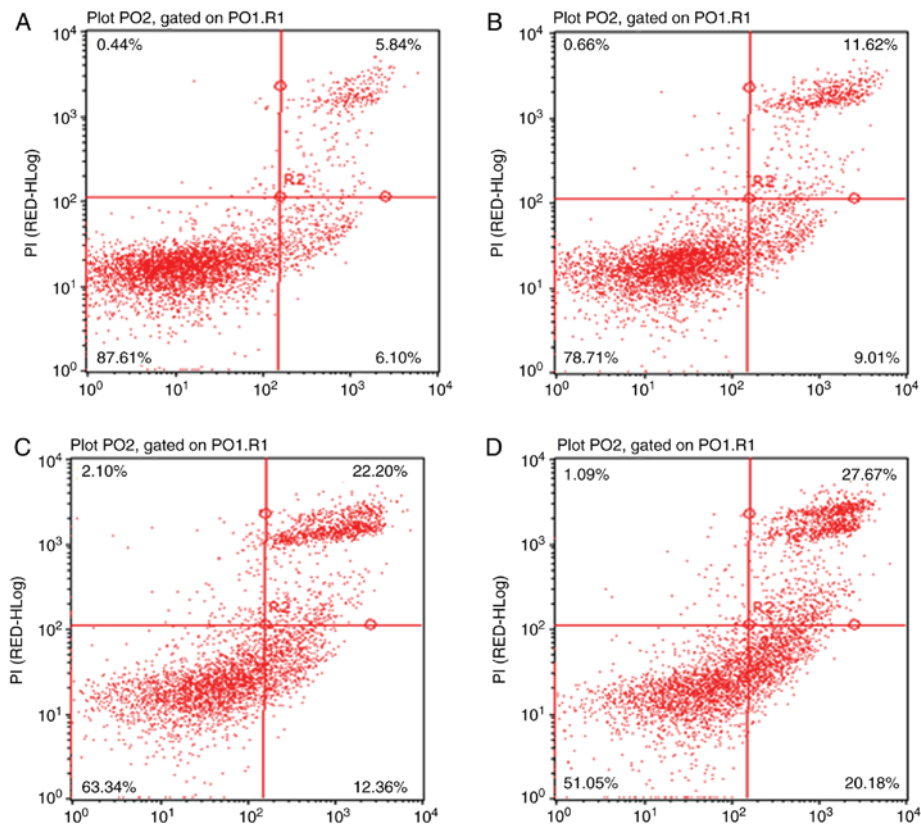
Figure 5. Flow cytometry analysis of DU-145 cells following Annexin V-FITC and PI double staining. The percentage of early apoptotic cells was (A) 6.10% in untreated cells, (B) 9.01% in 3.0 mM CSP-treated cells, (C) 12.36% in 12.0 mM CSP-treated cells and (D) 20.18% in 18.0 mM CSP-treated cells. Quadrants: Lower left, live cells; upper left, necrotic cells; lower right, early apoptotic cells; upper right, late apoptotic cells. Each experiment was performed in triplicate (n=3) and generated similar morphologic features. PI, propidium iodide.
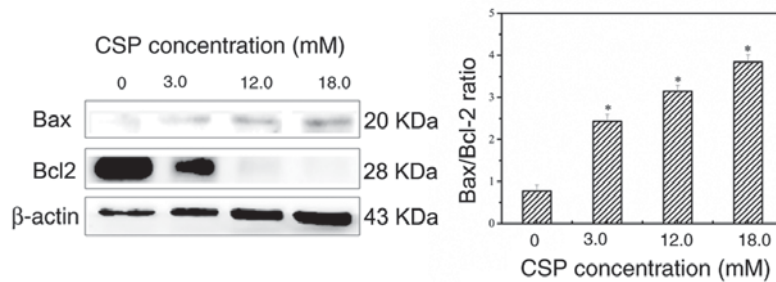

Figure 6. Expression of Bax and Bcl-2 apoptosis-associated proteins in DU-145 cells treated with CSP for 24 h. DU-145 cells were treated with different concentrations of CSP (0.0, 3.0, 12.0 and 18.0 mM) for 24 h at 37˚C and the protein expression levels of Bax and Bcl-2 were determined via western blotting. Bcl-2 expression was significantly decreased and the expression of Bax was significantly increased following treatment with CSP in a dose-dependent manner. CSP, *Cyclina sinensis*; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X. P<0.05 vs. 0 mM CSP.

between Bax and Bcl-2 protein expression is frequently used as an apoptotic index (10,18,25). The western blotting results demonstrated that Bcl-2 expression was significantly decreased, whereas the expression of Bax was significantly increased following treatment with CSP in a dose-dependent manner, thus resulting in a dose-dependent increase in the Bax/Bcl-2 ratio in CSP-treated DU-145 cells (Fig. 6). These results therefore suggested that treatment with CSP significantly enhanced apoptosis via upregulation of the Bax/Bcl-2 ratio in DU-145 cells.

In addition, caspase proteins are important for the maintenance of cellular homeostasis via the regulation of cell death and inflammation, and may be classified as initiator caspases (caspase-8/9) or executioner caspases (caspase-3/6/7) (33). Caspase-9 protein is an important component of the mitochondrial death pathway and caspase-3 is the predominant downstream effector caspase responsible for cleaving the majority of the cellular substrates in apoptotic cells (34). Western blotting analysis revealed that the protein expression of cleavedcaspase-3 and cleavedcaspase-9 were significantly increased following treatment with CSP in a dose-dependent manner (Fig. 7). Therefore, the results suggest that CSP-induced apoptosis is associated with the mitochondrial-mediated death pathway in DU-145 cells.
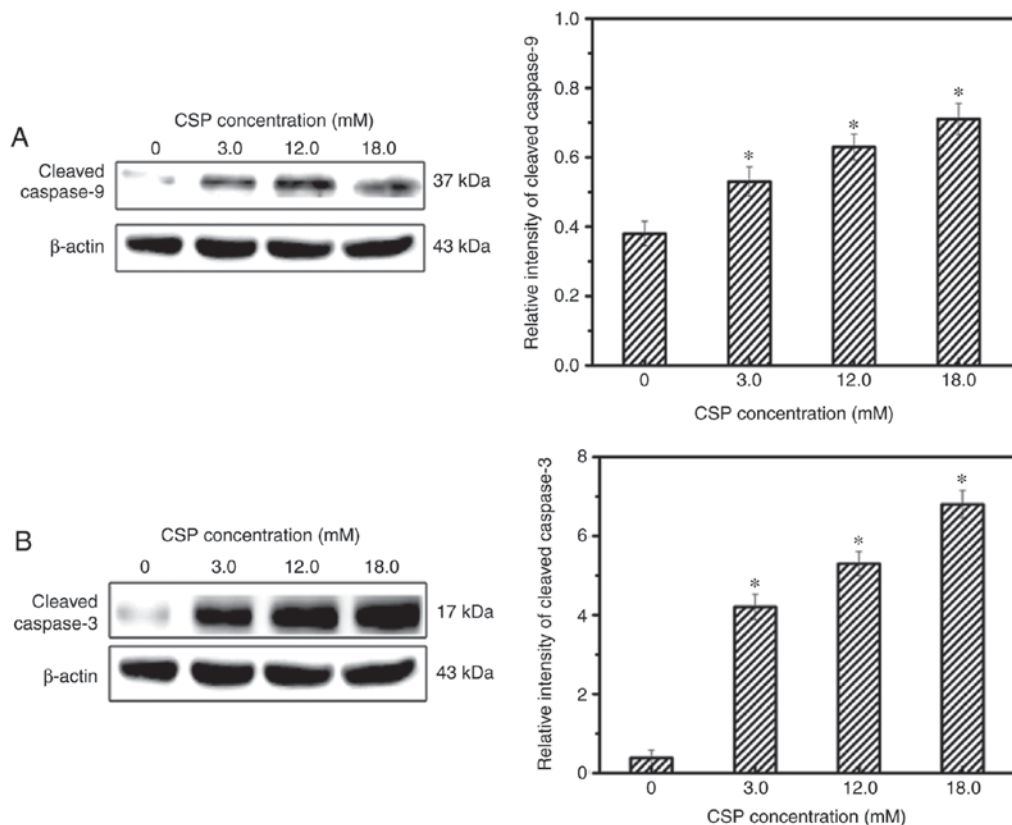
Figure 7. Expression of cleaved caspase-9 and cleaved caspase-3 apoptosis-associated proteins in DU-145 cells treated with CSP for 24 h. DU-145 cells were treated with numerous concentrations of CSP (0, 3.0, 12.0 and 18.0 mM) for 24 h at 37˚C, and the protein expression levels of (A) cleaved caspase-9 and (B) cleaved caspase-3 were determined via western blotting analyses. The protein expression levels of cleaved caspase-3 and cleaved caspase-9 were revealed to be significantly enhanced following treatment with CSP in a dose-dependent manner. The expression of all groups was normalized to β-actin. $P<0.05$ vs. 0 mM CSP.

CSP exhibits anticancer activity. It has been previously established that the hydrophobic properties of amino acids affect their anticancer activities. For example, the A and L residues in the YALPAH peptide isolated from *Setipinna taty* peptide hydrolysates were revealed to be associated with its anti-proliferative activities against PC-3 cells (26). F, I, M, P and Y residues in the sequence of the FIMGPY hexapeptide isolated from *Raja porosa* cartilage were also demonstrated to be associated with its anticancer activities against HeLa cells (10). Therefore, it can be suggested that the amino acid residues in the CSP sequence (ILYMP) may contribute to its anticancer activities. The CSP isolated from *Cyclina sinensis* exhibited anticancer activity to DU-145 cells, with lower toxicity to normal cells. The properties of this pentapeptide make it a promising therapeutic agent for PCa prevention or treatment. Further investigation in future studies is required to fully characterize CSP activity both *in vitro* and *in vivo*.

In conclusion, an anti-proliferation pentapeptide (ILYMP) named CSP was obtained from protein hydrolysates isolated from *Cyclina sinensis* via ultrafiltration and chromatographic methods. The results revealed that CSP inhibited the proliferation of DU-145 cells with an IC_{50} of 11.25 mM at 72 h. Furthermore, AO/EB staining, scanning electron microscopy and flow cytometry analyses demonstrated that CSP suppresses DU-145 cell proliferation via the induction of apoptosis. Enhanced expression of Bax and cleaved caspase-3/9 as well as suppression of Bcl-2 expression was observed in CSP-treated DU-145 cells. To the best of the authors' knowledge, this is the first study to investigate the effects of an antiproliferative peptide on DU-145 cells derived from *Cyclina sinensis*, and the results revealed that the *Cyclina sinensis* extract may have a therapeutic effect on PCa. However, the underlying mechanism of CSP-induced apoptosis was not fully determined, and our future studies will focus on revealing the molecular and proteomic mechanisms associated with this effect, using *in vitro* and *in vivo* studies.

Availability of data and materials

The data used in the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZY and FY conceived and designed the experiments. YZ, LY and YT performed the experiments. GD and XZ conducted data analysis. All authors read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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