Original Article

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Isolation and characterization of phosphoglycerate kinase and creatine kinase from bighead carp (*Aristichthys nobilis*): Potential sources for antitumor agents

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SUMMARY: Bighead carp (*Aristichthys nobilis*) has garnered significant attention due to its potential health benefits, yet its bioactive protein components remain largely unexplored. In this study, two proteins S3 and Z1 were isolated from *Aristichthys nobilis* using ammonium sulfate precipitation and serial column chromatography guided by their *in vitro* antitumor activity. Both proteins were found to be homogeneous in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a purity exceeding 95% as confirmed by reverse-phase high performance liquid chromatography (RP-HPLC). Electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis revealed their precise molecular weights to be 44.335 kDa for S3 and 43.028 kDa for Z1. Their amino acid sequences were elucidated through tandem mass spectroscopy and transcriptome unigene analysis, identifying S3 as phosphoglycerate kinase and Z1 as creatine kinase. Furthermore, secondary structure was measured by circular dichroism and three-dimensional structure was predicted by modeling software. The antitumor potential of S3 was evaluated by an *in vitro* assay, yielding an IC_{50} value of 26.3 ± 2.9 μM against the HT-29 cell line. Z1 demonstrated antiproliferative activity *in vitro* with IC_{50} values of 21.8 ± 1.4, 22.3 ± 2.1, and 22.3 ± 2.5 μM against HT-29, HeLa, and HepG2 cell lines, respectively. Notably, Z1 was found to enhance glucose metabolism and significantly increase the production of lactic acid and CO_2 in tumor cells. These findings suggest that bighead carp (*A. nobilis*) could serve as a promising source for both antitumor agents and functional food ingredients.

Keywords: *Aristichthys nobilis* proteins; purification and characterization; antitumor activities

1. Introduction

With the advancement of science and technology, human beings' living standards have been gradually improved, and our life expectancy has been significantly extended. Due to the incomplete understanding of the pathogenesis of cancer as well as limited treatment drugs and means, malignant tumors have become one of the serious threats to human life and health (*1*). There were close to 20 million new cases of cancer in the year 2022 (including nonmelanoma skin cancers [NMSCs]) alongside 9.7 million deaths from cancer (including NMSC) (*2*). Despite the massive efforts of modern pharmacology and the substantial advances made, such as surgical removal and chemotherapy, tumors are still the main cause of mortality around the world (*3*). With demographics-based predictions indicating that the number of new cases of cancer will reach 35 million by 2050, investments in prevention, including the targeting of key risk factors for cancer (including smoking, overweight and obesity, and infection), could avert millions of future cancer diagnoses and save many lives worldwide, bringing huge economic as well as societal dividends to countries over the forthcoming decades (*2*). Hence, searching for antitumor agents with high efficiency and low toxicity is an ongoing challenge (*4*).

Oceans cover 70% of the earth's surface, and are a rich source of bioactive substances as well as potential therapeutic agents (*5*). Because of their competitive, exigent, and aggressive living environment, many marine organisms produce unique and effective molecules, including bioactive proteins and polypeptides,

polyunsaturated fatty acids (PUFAs), vitamins, polysaccharides, enzymes and antioxidants (*6*).

Marine bioactive proteins have attracted increasing attention and research because of their excellent biological activity, especially antitumor activity. Various marine proteins and their derived peptides possess antitumor activities both *in vitro* and *in vivo* (*7-10*), such as geodiamolide H (*11*), dolastatin 10 (*12*), TZT-1027 (*13*), kahalalide F (*14,15*) and *Eucheuma serra* agglutinin (*16*). Most marine antitumor proteins and peptides that exhibit antiproliferative effect may cause tumor apoptosis by binding target proteins, and inducing apoptotic process *via* both intracellular and extracellular pathways (*17,18*).

Fish flesh, rich in proteins and peptides, is one of the main foods for most people around the world. There are approximately 140 million tons of fish harvested per year all over the world (*19*). Several investigations have researched the relationship between fish intake and tumor incidence. Through meta-analysis, Song concluded that fish intake was negatively correlated with the incidence of lung cancer, with a relative risk of 0.79 and a 95% confidence interval of 0.69-0.92 (*20*). Another meta-analysis showed that fish intake reduced 12% incidence of colorectal cancer, and the relative risk was 0.88 (95% CI = 0.80-0.95) (*21*). In addition, previous studies have identified the polypeptides from tuna dark muscle (*8*), and half-fin anchovy (*7*) possessed good antiproliferative effect.

Bighead carp (*Aristichthys nobilis*), belonging to Cyprinidae family, is an important commercial freshwater fish in China and also a source of bioactive substances. The annual worldwide production of bighead carp was over four million tons. Recently, a novel fucose-binding lectin from the gill of bighead carp has been characterized (*22*). Furthermore, a pyrophosphatase responsible for the hydrolysis of pyrophosphate (PPi) in muscle was purified (*23*). Parvalbumin, an allergen protein, was cloned and expressed, and its allergic activity was detected (*24*). Microcystin-detoxifizyme from bighead carp was also cloned and analyzed (*25*). However, few attempts were made on antitumor activity of substances from bighead carp. The only published study reported a lectin from bighead carp exhibiting antitumor and mitogenic activities (*25*). Little concern was concentrated on proteins and their antiproliferative effect from bighead carp.

In the present study, two proteins, S3 and Z1 were purified from *A. nobilis*, and their physicochemical characterizations were well determined. Otherwise, antitumor activity of two purified proteins was also evaluated using MTT assay, which indicated that *A. nobilis* might be one of the potential sources of antitumor agents and functional foods.

2. Materials and Methods

2.1. Materials

Samples of *A. nobilis* were collected from the Suishi market, college town of Guangzhou, China. Phenyl sepharose CL-4B was purchased from GE Healthcare, Chicago, PO, USA. Tris, sodium dodecyl sulfate (SDS), Coomassie brilliant blue R-250, 3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), cisplatin, penicillin, and streptomycin were obtained from Genview Corporation (Beijing, China). RPMI-1640 and fetal bovine serum (FBS) were obtained from GIBCO Invitrogen Corporation (San Diego, CA, USA). Other commercially available chemicals and reagents were of analytical grade.

2.2. Preparation of crude proteins

The raw material from *A. nobilis* was obtained by removing the skin, bone and tail, and then cut into small pieces. After washing it with tap water and distilled water in turn, phosphate buffer (pH 8.0, 30 mM) was used to extract the total proteins at a ratio of 1:4 (w/v) from the raw material. After homogenizing for 2 min with DS-1 high-speed tissue homogenizer (Jingxin Co., Shanghai, China), SB25-12 ultrasonic cleaner (Ningbo Xingzhi Co., Zhejiang, China) with a straight probe and continuous pulse was used to ultrasonicate the sample for 40 min. Then the sample was centrifuged at 10,000× *g* for 30 min. The supernatant was collected and precipitated with 0–70% and 70–100% saturated ammonium sulfate (*26*). The precipitates were collected by centrifugation (10,000× *g* for 30 min), re-dissolved in phosphate buffer, and dialyzed against distilled water at 4°C for 48 h to remove residual ammonium sulfate (*27*). Finally, two fractions, SNP1 and SNP2, were lyophilized and collected. The ability to inhibit cell growth was determined by the MTT assay. All extraction procedures were carried out at 4°C.

2.3. Isolation of proteins

The lyophilized fraction with the higher anti-proliferative activity as well as higher protein content was further separated on phenyl sepharose CL-4B hydrophobic chromatography column (2.5 × 40 cm), which had previously been equilibrated with the 1.0 M $(NH_4)_2SO_4$ prepared in 30 mM phosphate buffer (pH 8.0). SNP2 was dissolved in the above buffer and loaded onto the column, followed by stepwise elution with $(NH_4)_2SO_4$ (1.0, 0.5 and 0 M) prepared in 30 mM phosphate buffer (pH 8.0) at a flow rate of 0.7 mL/min. Each fraction was eluted and monitored at 280 nm. Finally, the three fractions, CL1, CL2 and CL3, were collected, lyophilized, and their ability to inhibit cell growth was measured by MTT assay.

The active fractions, CL2 and CL3, were further purified using high-performance gel permeation chromatography (HP-GPC) coupled with TSK G2000SWXL column (4.6 × 300 mm, Tosoh Co.,

Yamaguchi, Japan), eluted with 0.5 M sodium sulfate buffer over 30 min at a flow rate of 0.5 mL/min. The chromatographic profile was measured at 280 nm. The column temperature was 25°C. One single peak eluting from CL2 fraction was named as S3, while another peak from CL3 fraction was collected as Z1. Cytotoxicity of the purified peaks was measured by MTT assay after lyophilization.

2.4. Characterization of purified proteins

2.4.1. Identification of proteins by tricine-SDS-PAGE

The obtained proteins were analyzed by SDS-PAGE (*28*) using stacking gels (5% concentration of acrylamide) and running gel (16% concentration of acrylamide) (*29*) to check the purity and determine the molecular weight of the purified proteins. Protein bands were detected by the Coomassie blue staining method (*30*). A middle molecular weight calibration kit (Thermo Scientific, Waltham, MA, USA) was used as the standard markers.

2.4.2. Purity determination of proteins by HPLC

The purity of the purified proteins was measured by RP-HPLC on a Shimadzu series LC-20AB HPLC system (Shimadzu Co., Kyoto, Japan) fitted with a ZORBAX® 300SB-C8 column (4.6 × 250 mm, Agilent Co., Palo Alto, CA, USA). The elution solvent system was composed of water–trifluoroacetic acid (solvent A; 100:0.1, v/v) and acetonitrile–trifluoroacetic acid (solvent B; 100:0.1, v/v) (*27*). The polypeptides were eluted with a linear gradient of acetonitrile (15–55%) over 30 min at a flow rate of 1.0 mL/min. The chromatographic profile was measured at 280 nm. The column temperature was 35°C.

2.4.3. Protein and carbohydrate content assay

The protein content of the polypeptides was measured using Bradford method (*31*) where bovine serum albumin (BSA) was used as the standard protein. The protein content was evaluated by monitoring the absorbance of the samples at 595 nm. The saccharide content of the proteins was measured by the phenol–sulfuric acid method (*32-34*) in which glucose was used as standard. The saccharide content of the detected proteins was evaluated through the absorbance of the samples at 490 nm (*35*).

2.4.4. Molecular weight determination

The accurate molecular weight of the purified proteins was measured as reference (*27*). S3, and Z1 were dissolved in ultrapure water and loaded into quadrupole linear ion trap (QTRAP) mass spectrometer (Applied Biosystem, Foster City, CA, USA), respectively. The detection was performed in the positive electrospray ionization (ESI + ve) mode. High-purity nitrogen was used for drying (35 psi) and ESI nebulization (45 psi) process.

2.4.5. Mass spectrometry measurement

The purified proteins were dissolved and loaded on 1D SDS-PAGE with a 5-16% polyacrylamide gradient gel. After SDS-PAGE analysis, the target protein spots on the Coomassie Brilliant Blue-stained gels were manually excised, washed, and mixed with 1 M ammonium bicarbonate for maintaining reaction conditions. Then, 50 mM DTT and 100 mM iodoacetamide were added for reduction reaction. The sample was then desalted and further digested with sequencing-grade trypsin (Promega Co.,, Madison, WI, USA) for 20 h at 37°C. The digested samples were finally dried in a vacuum centrifuge, and then dissolved in 40 µL of 1% (v/v) formic acid.

The resulting samples was loaded onto RP-HPLC column for pre-fractionation and analyzed by nanoESI-orbitrap mass spectrometry for identification. A reverse phase PepMap column (ES802 75 µm × 25 cm, Thermo Co., Waltham, MA, USA) coupled with EASY-nLC 1200 instrument (Thermo Co., Waltham, MA, USA) was used for sample pre-fractionation. Mobile phase A consisted of 0.1% (v/v) formic acid in ultrapure water while mobile phase B consisted of 0.1% (v/v) formic acid in 80% acetonitrile were applied at the flow rate of 300 nL/min to separate hydrolyzed peptides. Afterwards, the eluted peptides were directly introduced into a nanoESI Q-Exactive mass spectrometer (Thermo Co., Waltham, MA, USA). The data were acquired and processed using the MASCOT program (*http://www.matrixscience.com*). The transcriptome sequencing of *A. nobilis* has been previously completed (NCBI Short Read Archive accession number: SRX1950353). MS/MS data were examined and matched both in the assembled transcriptome database of *A. nobilis* and National Center for Biotechnology Information (NCBI) basic local alignment search tool (BLAST) database.

2.4.6. Structure elucidation

The secondary structures of the two proteins were analyzed using circular dichroism (CD) spectroscopy based on the reported method (*36*). The purified proteins at the concentration of 0.05 mg/mL in distilled water were filtered through 0.22 µm membrane before CD analysis. Afterwards, the samples were loaded onto Jasco J-810 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan) with the parameters as a scan range of 250–190 nm, scan speed of 50 nm/min, the data interval of 1 nm, the bandwidth of 2 nm. Each spectrum was detected triplicated and recorded as the average of 3 scans. Three-dimensional structures of two purified proteins were elucidated using multiple template

modeling by MODELER 4.0 software and visualized in Pymol 2.7 software.

2.5. *In vitro* cell growth inhibition assay

Three tumor cell lines were used in this study including Hela (cervical carcinoma cell line), HepG2 (human liver carcinoma cell line), and HT-29 (human colon carcinoma cell line). Cell lines were purchased from the Zhongshan School of Medicine, Sun Yat-sen University, and maintained in RPMI 1640 medium supplemented with heat-inactivated 10% FBS, 1.0 mg/mL $NaHCO_3$, 0.2 mg/L L-glutamine, 100 units/mL streptomycin, and 100 units/mL penicillin in a humidified incubator at 37°C and 5% CO_2.

The cytotoxicity of the two purified proteins was measured by MTT assay. The S3 samples were dissolved and diluted to 0.1, 0.4, 1.4, 5.8, and 23.0 μM with RPMI-1640 medium.The Z1 samples were dissolved and diluted to 0.3, 1.3, 5.3, 21.3, and 42.6 μM with RPMI-1640 medium. Cisplatin was used as the positive control. *In vitro* cytotoxic activity was evaluated using MTT assay according to the published procedure (*37*) with some modifications.

Cells in the logarithmic phase were detached with 0.25% trypsin to make a single cell suspension using RPMI-1640 complete culture solution. After counting the cells, they were inoculated into a 96-well culture plate and incubated at 37°C in a humidified incubator with 5% CO_2. After 24 h, the different concentrations of samples were added into the wells and the plate was incubated for a set time. Then, 20 μL of MTT solution was added to each well and the plate was incubated for another 4 h at 37°C. The supernatant was removed and the MTT-formazan crystals were dissolved in 200 μL of DMSO and the absorbance was read at 570 nm. The percentage inhibition of cell growth was calculated according to the following equation:

$$\text{Inhibitory rate\%} = (A_{570,\text{control}} - A_{570,\text{sample}}) \div (A_{570,\text{control}} - A_{570,\text{blank}}) \times 100\% \quad (1)$$

2.6. Effect of purified proteins on the metabolic rate of tumor cells

Before adding MTT, samples of the cell supernatant in each well at the end of the cell proliferation inhibition experiment were taken, and the detection was carried out according to the operating instructions of the GLU kit (Nanjing Jiancheng Bioengineering Institute, China). The 3 μL of sample and calibrator was mixed with 300 μL of the reaction solution R respectively. Distilled water was used as the blank control. The reaction was carried out at 37°C for 10 min and the absorbance was read at 500 nm. The initial glucose concentration in the culture medium, which is 16.65 mmol/L. The glucose consumption was calculated according to the following equation:

$$\text{GLU (mmol/L)} = A_{500,\text{sample}}/A_{500,\text{calibrator}} \times \text{Calibrator (mmol/L)}$$

$$\Delta\text{GLU (mmol/L)} = \text{GLU (mmol/L, initial)} - \text{GLU (mmol/L, end)}$$

Similarly, samples of the cell supernatant in each well at the end of the cell proliferation inhibition experiment were taken, and the detection was carried out according to the operating instructions of the Lactic Acid LD kit (Guangzhou Kofa Biotechnology Corporation, China). A total of 20 μL of the standard (3 mmol/L), 20 μL of the sample, 1,000 μL of the enzyme reaction solution, and 200 μL of the chromogenic solution were added successively and mixed. Distilled water was used as the blank control. The reaction was carried out at 37°C for 10 min and 2,000 μL of the stop solution was added. The absorbance was read at 530 nm and the content of lactic acid was calculated according to the following equation:

$$\text{Lactic acid (mmol/L)} = A_{530,\text{sample}}/A_{530,\text{standard}} \times \text{Standard (mmol/L)}$$

2.7. Statistical analysis

All of the assays were conducted in triplicate and the experimental data were expressed as the mean ± standard deviation. GraphPad Prism 5.0 was used for statistical analysis. Two-sample comparisons were performed using Student's *t*-test; $P < 0.05$ was considered statistically significant.

3. Results

3.1. Purification of proteins

The total proteins were firstly released from the flesh of *A. nobilis*, and afterwards fractionated by salting-out at increasing levels of saturation of ammonium sulfate at 4°C. Two fractions, named as SPN1 and SPN2, were obtained at an ammonium sulfate saturation of 0–70% and 70–100%, respectively. Since it possessed higher protein concentration and better antiproliferative activity, SPN2 was loaded onto a phenyl sepharose CL-4B hydrophobic chromatography column and eluted with decreasing concentrations of $(NH_4)_2SO_4$ for further purification. Three resulting peaks, namely as CL1, CL2 and CL3, were obtained (Figure 1A). Due to the protein and saccharide content determination, CL1 contained the highest protein concentration (Table S1, *https://www.ddtjournal.com/action/getSupplementalData.php?ID=243*), then followed with CL3. CL2 contained only 31.6% protein content and 8.3% saccharide content. With the guidance of bioassay, CL2 and CL3 were

further purified using high-performance gel permeation chromatography (HP-GPC). Finally, a protein, designated as S3, was purified from CL2. And another protein Z1 was obtained from CL3 (Figures 1B and 1C).

3.2. Characterization of purified proteins

3.2.1. Purity determination

SDS-PAGE is a pervasive method to characterize the purity of proteins according to the protein molecular weights (*36,37*). The electrophoresis technique also provides a foundation for further evaluation of proteins. In the present investigation, the two purified proteins were determined by SDS-PAGE (Figures 2A and 2B). SDS-PAGE analysis revealed that S3 and Z1 were composed of a diffuse single band respectively, which indicated both of them are monomeric, homogeneous proteins after purification. In addition, S3 and Z1 similarly shared an approximate molecular weight about 43 kDa.

RP-HPLC was another common technique to determine the purity of the purified proteins. As shown in Figures 2C and 2D, the RP-HPLC elution profiles

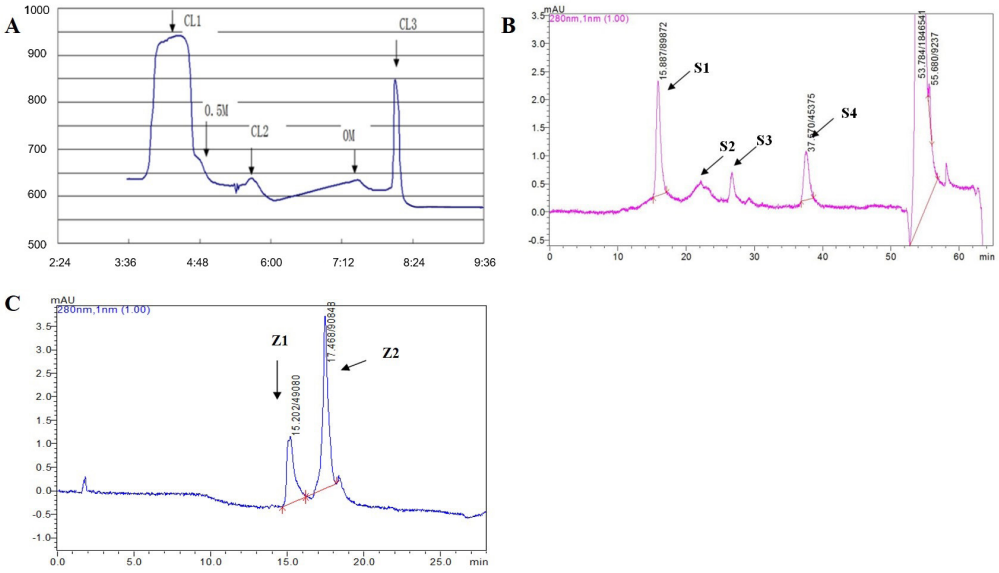

Figure 1. Purification of proteins from *A. nobilis*. (A) Elution profile of SPN2 by phenyl sepharose CL-4B hydrophobic chromatography; **(B)** Elution profile of CL2 by HP-GPC; **(C)** Elution profile of CL3 by HP-GPC.

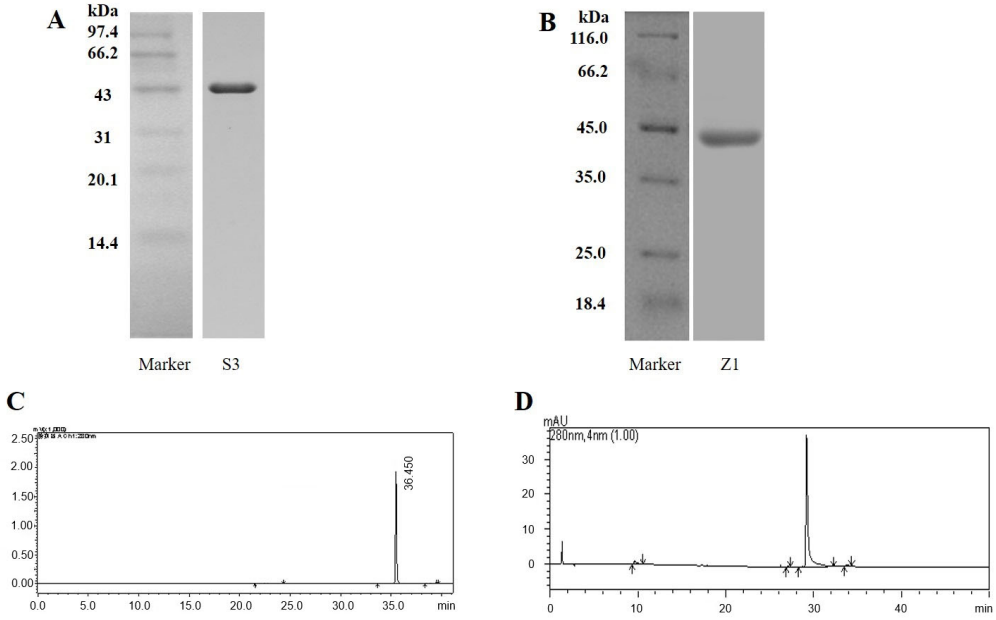

Figure 2. The purity determination of proteins from *A. nobilis*. (A) SDS-PAGE of S3 from CL2 fraction. **(B)** Z1 from CL3 fraction. **(C)** RP-HPLC profile of S3; **(D)** RP-HPLC profile of Z1.

of S3 and Z1 with elution time at 35.450 and 29.190 min showed a symmetric single peak with the purity of 97.6% and 95.6%, respectively (Table S2, *https://www.ddtjournal.com/action/getSupplementalData.php?ID=243*).

3.2.2. Molecular weight

A number of researches have shown that marine antitumor proteins are macromolecular proteins (*38-40*). For example, two proteins with 27.1 kDa and 20.5 kDa purified from *Arca inflata* showed high anti-proliferation effect against human tumor cells *in vitro (41,42)*. At the present investigation, the accurate molecular weights of S3 and Z1 were determined by ESI-MS/MS as 44.335 and 43.028 kDa, respectively.

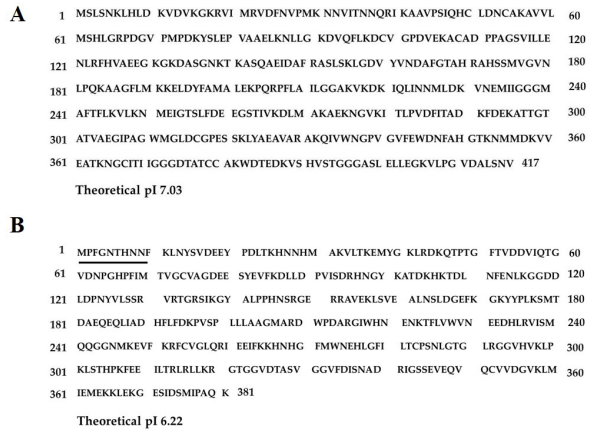

Figure 3. Amino acid sequences of S3 (A) and Z1 (B). N-terminal sequence of Z1 determined by Edman degradation was underlined, nevertheless, the N-terminal sequence of S3 was blocked.

3.2.3 Amino acid sequence analysis

High resolution tandem mass spectrometry, state of the art technique for the characterization and sequencing of proteins, was used in this study. Trypsin hydrolysis was applied to produce amounts of peptides at lysine and arginine cleavage sites. Those peptides were pre-separated by EASY-nLC 1200 and further determined by nano ESI-MS/MS instrument. The tested peptide fragments were identified and aligned with transcriptome of *A. nobilis* (Tables S3 and S4, *https://www.ddtjournal.com/action/getSupplementalData.php?ID=243*). According to the tandem mass spectrometry, the complete amino acid sequences of the two purified proteins were obtained (Figure 3). Aligning with NCBI BLAST database, S3 and Z1 were identified as phosphoglycerate kinase and creatine kinase, respectively (Figure 4).

3.2.4. Structure elucidation

CD spectroscopy provides an exquisitely sensitive and rapid method for the analysis of protein secondary structures. It is widely used in protein folding and protein conformation studies. In the circular dichroic ultraviolet wavelength zone (190-240 nm), the main chromophore is a peptide chain, and the CD spectrum of this wavelength range contains information on the conformation of the main chain of a protein. The CD spectrum of the α-helical conformation has a negative peak at 222 nm and 208 nm and a positive peak at around 190 nm. The CD spectrum

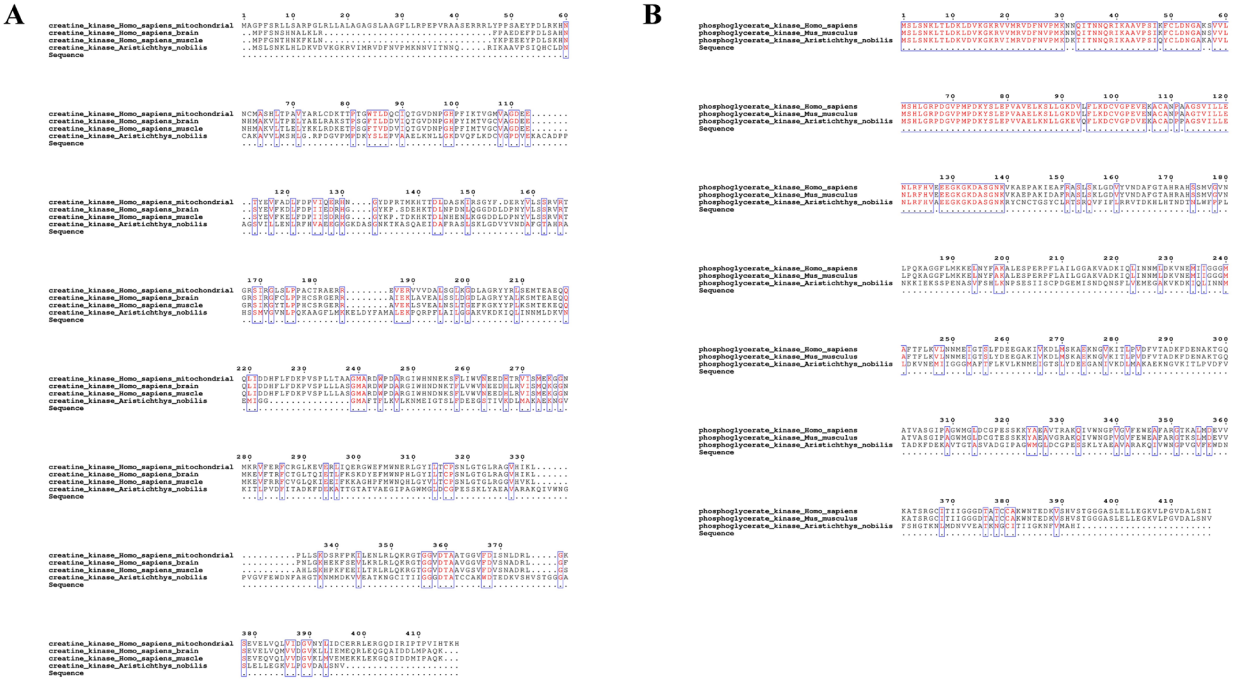

Figure 4. Sequence alignment among Z1 (A) and S3 (B), respectively.

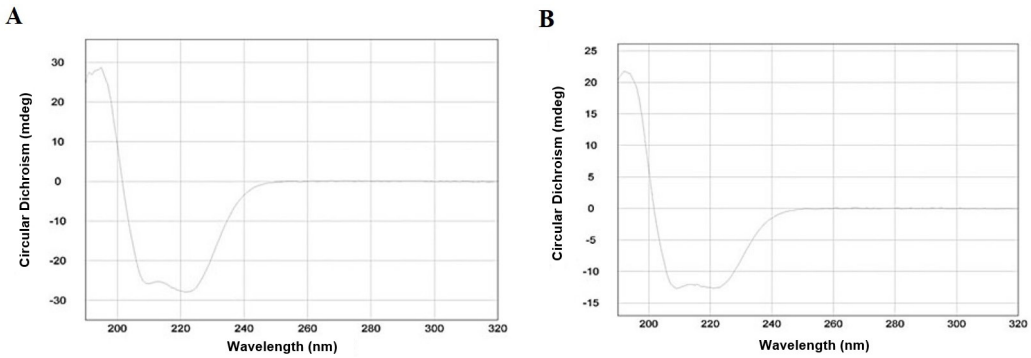

Figure 5. Secondary structure determination of S3 (A) and Z1 (B) by circular dichroism spectroscopy.

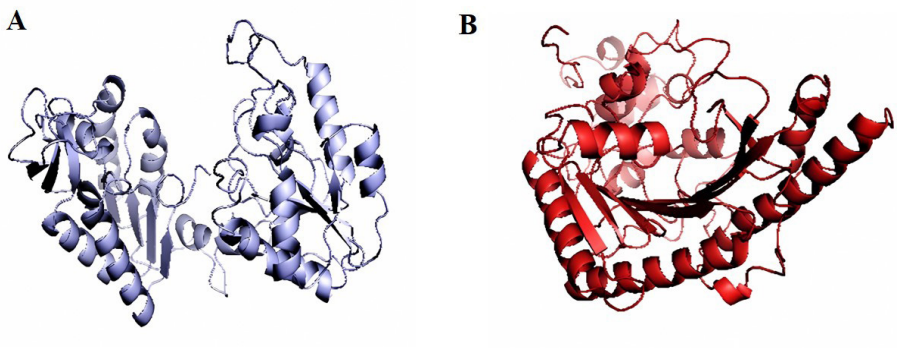

Figure 6. Three-dimensional predicted structures of S3 (A) and Z1 (B) using multiple templates modeling. Three templates (2xe6, 2y3i, and 5m1r) were used for modeling S3 structure and three other templates (1i0e, 1vrp, and 2crk) were used for modeling Z1 structure.

of the β-sheet conformation has a negative peak at 217-218 nm and a strong positive peak at 195-198 nm. The CD spectrum of the random coil conformation has a negative peak around 198 nm and a small and broad positive peak around 220 nm (*43,44*). As shown in Figure 5, the CD spectrum of two proteins exhibited one strong positive peak at 195 nm, as well as two negative ellipticity signals at 210 and 222 nm, respectively, which were typical features of α-helical and β-sheet conformations of folded proteins. Proposed secondary structure conformations were acquired using the tested CD data and Jasco secondary structure estimation program software. S3 displayed 46.0% α-helix, 34.4% β-sheet, and 19.6% random coil of secondary structure. In addition, Z1 showed 56.2% α-helix, 23.6% β-sheet, and 20.2% random coil of secondary structure. Tertiary structures predicted by homology modeling of S3 and Z1 were shown in Figure 6.

3.3. Cytotoxicity of purified proteins

The cytotoxicity of two purified proteins against three human tumor cell lines including HepG2, HeLa and HT-29 were examined using MTT assay (Table 1). Cisplatin was used as a positive control and performed anti-proliferative activity against HepG2, HeLa and HT-29 cells with IC_{50} values of 4.3, 5.0 and 4.7 μM, respectively. Z1 possessed the highest cytotoxicity and displayed anti-proliferative effect against HepG2, HeLa and HT-29 cell lines with IC_{50} values of 22.3, 22.3 and 21.8 μM, respectively.

Table 1. Anti-proliferative activities of two purified proteins against three tumor cell lines

Polypeptides	IC_{50} (μM) (mean ± SD, n = 3)		
	HeLa	HepG2	HT-29
Z1	22.3 ± 2.1	22.3 ± 2.5	21.8 ± 1.4
S3	> 30	> 30	26.3 ± 2.9
Cisplatin	5.0 ± 1.0	4.3 ± 1.0	4.7 ± 0.3

In addition, the effect of Z1 on the metabolic rate of tumor cells was determined. When Z1 was applied to HepG2, HeLa and HT-29 tumor cells at 22 μM for 72 h, the cell growth was about half of the blank group, but the actual consumption of glucose of Z1 group was about the same as the blank group. As shown in Figure 7, the glucose consumption of those tumor cells was 1.49, 2.21 and 2.68 mmol/L, respectively. Combining the lower growth of Z1 group, the rates of glucose consumption of Z1 group were 1.48, 2.43 and 2.36 times that of the non-administered group in HepG2, HeLa and HT-29 cells. Similarly, the amounts of lactic acid were 0.62, 0.74, and 0.86 mmol/L, which was 2.21, 2.33, and 2.48 times of the control group, respectively. The production of CO_2 was 4.32, 6.25 and 8.53 mmol/L, which was 2.18, 2.87 and 2.91 times compared with that of the non-administered group respectively based

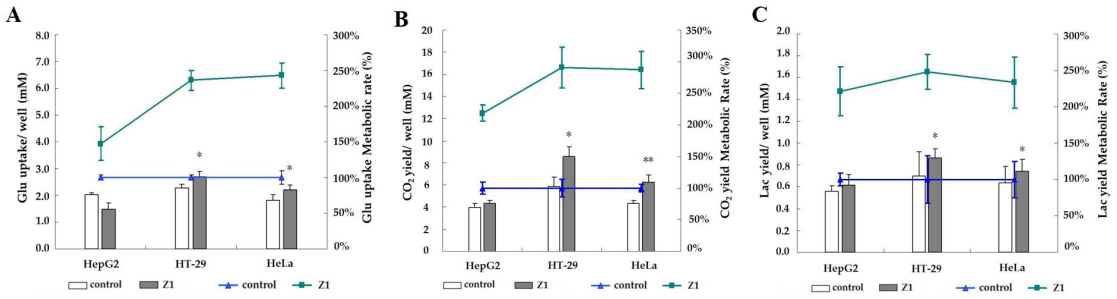

Figure 7. Effect of Z1 on glucose (A), lactic acid (B) and CO_2 (C) consumption of three tumor cells (HepG2, HeLa and HT-29) at IC_{50} concentration (n = 3, *$P < 0.05$, **$P < 0.01$)

on the growth rate.

4. Discussion

There are a number of fish species identified with potential pharmaceutical values. Bioactive proteins and their derivatives from muscles of various fish species have shown diverse biological functions including antihypertensive, antioxidant, anti-inflammatory, anticoagulant, and antibacterial activities. And they may be a potential material for biomedical and food industries. The proteins derived from sardine and hair tail meat exhibited inhibitory activities against angiotensin I-converting enzyme (ACE) (*45*). Furthermore, another ACE inhibitory protein was derived from bigeye tuna dark muscle (*46*). Hence, fish muscles are valuable natural resources and show the potential use in nutraceutical and pharmaceutical industries. It is necessary to investigate the bighead carp flesh and reveal the hidden functional components.

S3 exhibited 73% homology with predicted phosphoglycerate kinase 1 derived from sheepshead minnow (*Cyprinodon variegatus*) (Figure 4B). This demonstrated that S3 was a newly discovered phosphoglycerate kinase 1 from bighead carp (*A. nobilis*), which had not previously been extensively characterized.

In addition, Z1 showed 100% homology with muscle-type creatine kinase. Z1 from *A. nobilis* displayed high homology with *Cyprinus carpio* and *Carassius auratus*, which indicated the highly similar genetic background among these three species (Figure 4A). The N-terminal amino acid sequences of Z1 determined by protein sequencer (Figure 3B) were well matched with the amino acids detected by tandem mass spectrometry, which implied the accuracy of the results produced by tandem mass spectrometry. Nevertheless, the N-terminal amino acid sequence of S3 was blocked and failed to detect, which indicated that S3 might have posttranslational modification at N-terminal sites.

Diverse bio-active peptides attributes to highly ordered and stable conformations, such as possessing high ratio of β-sheet and α-helix conformation in secondary structures. S3 and Z1 both exhibited highly ratio of α-helix and β-sheet conformation and performed tightly folded conformation. This result is similar to the antitumor protein α-sarcin produced by the mold *Aspergillus giganteus*, which had highly α-helix and β-sheet content and ordered a tightly conformation (*47*). Three-dimensional structures of two proteins were simulated by multiple template modeling (Figure 6). The simulation results possessed highly ordered structures, which were well matched with CD spectrum of two proteins.

Z1 was identified as muscle-type creatine kinase from bighead carp. Creatine kinase is a key enzyme in the process of cellular energy metabolism, and exists in humans in the form of four isoenzymes, CK-BB (brain type), CK-MB (hybrid type), CK-MM (muscle type) and CK-Mt (mitochondrial type). Creatine kinase and creatine kinase isoenzymes are widely used in the diagnosis of clinical myocardial infarction, muscle atrophy and other diseases (*48*). CK-Mt is located in the intracellular mitochondrial membrane and mitochondrial outer membrane space, which promotes ATP production and catalyzes the reversible transfer of phosphate bond between creatine and ATP. CK-Mt plays a very important role in the stability of intracellular ATP pool and the regulation of the energy metabolism (*49*). However, an increasing number of studies have shown that the high expression of CK-Mt levels may be closely related to the strong energy demand of tumor growth. According to the recent investigations, high expression of CK-Mt was involved in cancer energy metabolism and facilitated cancer cell division and inhibited apoptosis. Our experiments show that CK-MM can multiply the metabolism of glucose, and the production of lactic acid and CO_2 also increases exponentially in tumor cells (Figure 7).

Z1, muscle-type creatine kinase from *A. nobilis*, exhibited anti-proliferative activity against multiple tumor cell lines. As illustrated in Figure 4A, Z1 showed high homology with human muscle-type (CK-BB) and brain-type creatine kinase (CK-MM) but low homology with mitochondrial-type creatine kinase (CK-Mt), which indicated that Z1 might exhibit anti-proliferation effect through competitive binding the target of human CK-Mt. Moreover, compared with cytotoxicity against other

tumor cells, S3 showed better anti-proliferation effect on colon tumor cell line HT-29 with an IC_{50} value of 26.3 μM (Table 1), which showed better activity than cecropin B peptide from silkworm pupae (*50*).

5. Conclusion

In summary, two proteins, S3 and Z1 were purified from bighead carp (*A. nobilis*). S3 and Z1 were identified as phosphoglycerate kinase and creatine kinase, respectively. Physicochemical properties of two purified proteins were further determined. Moreover, Z1 and S3 showed anti-proliferative activity against HT-29, HeLa and HepG2 cell lines. The structural and functional characterization of proteins S3 and Z1 is poised to facilitate advancements in therapeutic and nutritional applications. This investigation has significantly broadened the knowledge base regarding the proteome of the bighead carp, thereby necessitating further in-depth studies aimed at the exploration and development of biomacromolecules within this species. Concurrently, the elucidation of the antitumor mechanisms of Z1 and S3 is currently under rigorous investigation.

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