Original Article

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Purification and characterization of a novel antioxidant protein from *Arca subcrenata* Lischke

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SUMMARY: A novel protein, G1H2GC2, was isolated from *Arca subcrenata* Lischke using homogenization and ammonium sulfate precipitation, and further purified by several column chromatography techniques including diethylaminoethanol (DEAE) Sepharose Fast Flow anion exchange, gel filtration chromatography (Sephadex G-25), Phenyl Sepharose CL-4B hydrophobic chromatography and reversed-phase high-performance liquid chromatography (RP-HPLC). The purity of G1H2GC2 was over 97% in RP-HPLC, and its high purity was further verified by the appearance of a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein content of G1H2GC2 was found to be over 99% by Bradford assay. The molecular weight was determined as 25.6 kDa by electrospray ionization-mass spectrometry (ESI-MS/MS). The isoelectric point of G1H2GC2 was measured to be 7.71 by isoelectric focusing-polyacrylamide gel electrophoresis (IEF-PAGE). Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS/MS) was employed to detect and identify the protein by mass fingerprinting coupled with fragmentation patterns. No matched protein was found, confirming that G1H2GC2 was a novel protein. An attempt was made to detect the N-terminal amino acid sequence of G1H2GC2 by Edman degradation, but the sequence of G1H2GC2 was found to be blocked. The scavenging percentage of G1H2GC2 at 15 mg/mL against 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) was 52.84%. The median effective concentration (EC_{50}) value of G1H2GC2 against $ABTS^{\bullet+}$ was 17.67 mg/mL. The results showed that G1H2GC2 might be developed as a potential food additive agent.

Keywords: *Arca subcrenata* protein, purification and characterization, antioxidant activity

1. Introduction

Proteins are the molecular carriers of biological functions and occupy a pivotal position within living organisms. Nutritionally, they are sources of essential amino acids, are indispensable for growth and maintenance, and are a source of energy (*1*). Structurally, they are composed of a sequence of amino acids beginning with an N-terminal and ending with a C-terminal amino acid residue (*2*). Isolating proteins from plants and animals, followed by in-depth investigations into their structures and functions, holds significant scientific value for elucidating the essential roles these biomolecules play in human physiological processes. Bioactive proteins hold broad application prospects as drugs, healthcare products, and food ingredients. Natural proteins exhibit diverse biological activities, including antioxidant (*3-5*), antihypertensive (*6-8*), antidiabetes (*9,10*), antiobesity (*11,12*), immunomodulatory (*13,14*), and antitumor (*15,16*). Marine organisms are crucial resources for the discovery and development of marine biological resources. They inhabit extremely harsh, competitive, and aggressive environments, exhibiting significant differences from terrestrial organisms in many aspects. The unique amino acid sequences of their proteins may arise from adaptations to these specialized ecological niches (*17,18*). Recently, increasing investigations have focused on bioactive proteins from marine organisms. Numerous bioactive proteins have been isolated from various marine animals, including *Spongia officinalis*, *Chrysaora quinquecirrha*, *Hippospongia communis*, bryozoans, nudibranchs, and other marine species (*19,20*).

Several methods are used to obtain bioactive proteins and peptides, including isolation, fermentation, enzymatic hydrolysis and a combination of these (*1*). Isolation and purification are the most common techniques (*2*). By exploiting the variability in

molecular weights, charges and affinities of bioactive proteins, a series of advances in methodology and instrumentation has enabled the accurate separation of specific bioactive proteins and peptides (*2*). Among the main techniques used to extract proteins are solvent extraction, which is used to isolate proteins from biological sources, and ultrafiltration and nanofiltration, which separate mixtures of proteins by size (*21,22*). Chromatography has also become an indispensable tool for the purification of proteins. Advances in the understanding of protein structure/function relationships have also driven the generation of more sophisticated chromatographic techniques for protein separation (*23*), including size-exclusion chromatography and reversed-phase high-performance liquid chromatography (RP-HPLC) (*2*). Given the special marine environment, various proteins and peptides with therapeutic application have been generated from marine organisms by the isolation and purification techniques above (*1,24*).

Arca subcrenata Lischke, a bivalve mollusk that lives in the muddy sediment of the shallow coastal waters of the north-western Pacific, belongs to the Arcidae family under phylum Mollusca, class Bivalvia (*25*). A traditional Chinese remedy, wa leng zi (also known as Concha Arcae), is made from *A. subcrenata* (*26*). Furthermore, the body of this species has been used for centuries to treat tumors, anemia, inflammation and dyspepsia in traditional Chinese medicine (*26*). Proteins derived from *A. subcrenata* have been reported to be highly bioactive, with significant pharmacological and medicinal value. Modern research demonstrates that *A. subcrenata* exhibits diverse biological activities, including antitumor, antibacterial, antioxidant, immunomodulatory, and anti-inflammatory properties (*27-33*). A polypeptide fraction from *A. subcrenata*, P2, had antiproliferative capability against seven human tumor cell lines, especially HeLa, which was much more sensitive to P2 than the other tumor cell lines. Furthermore, a purified protein (H3), isolated from P2, exhibited significant antitumor and antioxidant activity (*27*). A polypeptide fraction from *A. subcrenata* (PAS) inhibit HT-29 cells proliferation *via* suppression of insulin-like growth factor 1 receptor (IGF-1R)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling and adenosine triphosphate (ATP) production (*28*). And a novel protein (ASP-3) with unique antitumor activity from *A. subcrenata* Lischke (*29,30*).

Continuing this work, here we report a novel protein, G1H2GC2, isolated from *A. subcrenata* Lischke using a variety of purification techniques. The protein was shown to possess $ABTS^{\bullet+}$ radical-scavenging activity. The complete identification and characterization of G1H2GC2 will help the future investigation of its biological activity.

2. Materials and Methods

2.1. Materials

Samples of *A. subcrenata* Lischke were collected from Huangsha seafood market, Guangzhou, China. All samples were positively identified by Rongmin Yu (Jinan University, Guangzhou, China). The visceral mass was dissected from the samples, weighed and stored at −20 °C until used. Diethyl-aminoethanol (DEAE) Sepharose Fast Flow and Phenyl Sepharose CL-4B were purchased from GE Healthcare (Chicago, PO, USA). Sephadex G-25, Tris, sodium dodecyl sulfate (SDS), and Coomassie Brilliant Blue R-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Coomassie Brilliant Blue G-250 and bovine serum albumin were obtained from Sino-American Biotechnology Co., USA. Molecular weight markers were obtained from Shanghai Puyi Biotechnology Co., Ltd., China. Other commercially available chemicals and reagents were of analytical grade.

2.2. Extraction of crude protein

The crude protein of *A. subcrenata* Lischke was extracted by the ammonium sulfate precipitation-centrifugation method of Chen *et al.* (*27*). Briefly, the visceral mass of *A. subcrenata* Lischke was washed with 4°C tap water three times, followed by 4°C distilled water three times. It was then homogenized with triple volume of phosphate-buffered saline (PBS) (0.03 M, pH 8.0). After extraction with ultrasound at 4°C for 40 min, the homogenate was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was precipitated with 70%-100% saturation of solid ammonium sulfate. The precipitate was obtained by centrifugation (10,000 × g for 30 min), then re-dissolved in 30 mM Tris-HCl buffer (pH 8.0) and dialyzed against distilled water for 48 h to remove ammonium sulfate. The dialyzed solution was then lyophilized for further use to obtain crude protein JNY-1.

2.3. Purification of protein

2.3.1. DEAE Sepharose Fast Flow chromatography

The crude protein (JNY-1) was dissolved in 0.03 M Tris-HCl buffer (pH 8.0) at a proportion of 1:10 (w/v), and loaded onto a DEAE Sepharose Fast Flow anion exchange column (2.5 cm × 40 cm), which was pre-equilibrated with the aforementioned Tris-HCl buffer. The column was stepwise eluted with 0, 0.1, 0.3 and 2 M NaCl prepared in the same buffer at a flow rate of 7 mL/min to obtain the fraction P2.

2.3.2. Sephadex G-25 exclusion chromatography

The fraction P2 was collected, dialyzed, freeze-dried and loaded onto a Sephadex G-25 column (1 cm × 80 cm)

that was pre-equilibrated with distilled water. A stepwise elution was carried out with distilled water at a flow rate of 1 mL/min to obtain the fraction G1.

2.3.3. Phenyl Sepharose CL-4B hydrophobic chromatography

G1 was freeze-dried, dissolved in 1.0 M $(NH_4)_2SO_4$ prepared with 30 mM phosphate buffer (pH 8.0) and loaded onto a Phenyl Sepharose CL-4B hydrophobic chromatography column (2.5 cm × 40 cm), which had previously been equilibrated with the same buffer. Then the column was stepwise eluted with decreasing concentrations of 1.0, 0.5 and 0 M $(NH_4)_2SO_4$ mixed into the above buffer and finally eluted with distilled water at a flow rate of 1 mL/min to obtain the fraction G1H2.

2.3.4. Sephadex G-25 exclusion chromatography and HPLC

After being dialyzed and freeze-dried, G1H2 was purified again on a Sephadex G-25 column (1 cm × 80 cm) to obtain the fraction G1H2G, and G1H2G was separated twice on a Shimadzu series LC-20AB HPLC system (Shimadzu Co., Kyoto, Japan) fitted with a ZORBAX® 300SB-C8 column (Agilent Co., Palo Alto, CA, USA; 4.6 mm × 250 mm). The elution solvent was composed of water-trifluoroacetic acid (TFA) (solvent A; 100:0.1, v/v) and acetonitrile-TFA (solvent B; 100:0.1, v/v) (*34*). Firstly, gradient elution was performed from 10% to 16% of solvent B at 0-7.5 min, from 16% to 37% (7.5-10 min), from 37% to 50% (10-50 min), from 50% to 100% (50-55 min), and then held at 100% of solvent B (55-63 min). Secondly, the protein obtained from RP-HPLC was further separated by gradient elution from 10% to 10% at 0-10 min, from 10% to 35% (10-15 min), from 35% to 55% (15-40 min), from 55% to 100% (40-55 min), and then held at 100% of solvent B (55-60 min). Gradient elution was performed with a flow rate of 1 mL/min and column temperature of 25°C. The detection wavelength was set at 280 nm in all experiments.

2.4. Characterization of protein

2.4.1. Determination of protein and saccharide content

The protein content was estimated using the Bradford method. Bovine serum albumin (BSA) was used as a standard protein. The absorbance at 595 nm was used to determine the amount of protein in each sample. The saccharide content was measured by the phenol-sulfuric acid method with a 100 μg/mL glucose solution as standard. The absorbance at 490 nm was used to determine the amount of carbohydrate in each sample.

2.4.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The purity and molecular weight of the protein were determined by SDS-PAGE according to the method described by Laemmli, using an acrylamide concentration of 5% for the stacking gel and 16% for the separating gel. The separation was first manipulated with a voltage of 60 V for 0.5 h, and then 80 V for approximately 2.5 h. The gel was stained with Coomassie Brilliant Blue R-250 for 1 h and then destained using 40% methanol and 10% acetic acid for 2 h. The molecular weight of the purified protein was determined by comparison of its electrophoretic mobility with that of the molecular weight marker proteins, using a middle-molecular-weight calibration kit (Thermo Scientific, Waltham, MA, USA).

2.4.3. Isoelectric focusing-polyacrylamide gel electrophoresis (IEF-PAGE)

The isoelectric point of the protein sample was determined by immobilized pH gradient (IPG) IEF-PAGE. Ampholyte (40%, pH 3.5-10.0) was used to prepare isoelectric focusing gel with an acrylamide concentration of 5%. The experiment was carried out at 150 V for 0.5 h, then at 200 V for 2.5 h in an electrophoresis apparatus (Protean II, BioRAD, Hercules, CA, USA). The IEF-PAGE gel-unloaded samples were washed with double-distilled water, and sliced into pieces 0.5 cm in length from acidic terminus to basic terminus, then separately dipped into glass tubes containing 2.0 mL of 10 mM KCl for 30 min. The pH value of the liquid around each slice was measured. The gel-loaded sample was fixed with 10% trichloroacetic acid for 30 min, stained with Coomassie Brilliant Blue R-250 overnight, and then destained until faded from the background. Data were derived from the calibration curve of the isoelectric points with the length of gel as abscissa and pH value as ordinate (*33*).

2.4.4. Reversed-phase high-performance liquid chromatography (RP-HPLC)

The sample was prepared for RP-HPLC by dissolving the protein in distilled water, which was then filtered and loaded into a Shimadzu series LC-20AB HPLC system fitted with a ZORBAX® 300SB-C8 column (Agilent Co., Palo Alto, CA, USA; 4.6 mm × 250 mm). The mobile phase was composed of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The column was eluted with a gradient of 10% to 100% solvent B for 30 min with a flow rate of 1 mL/min and a detection wavelength of 280 nm.

2.4.5. UV-wavelength scanning by photodiode array (PDA) detector

The protein was dissolved in distilled water to a concentration of 1.0 mg/mL and scanned by a PDA detector (Shimadzu Co., Kyoto, Japan). The scan range

was 190-700 nm, and distilled water was used as the blank control.

2.4.6. Molecular weight determination

To determine the precise molecular weight of the purified protein, the protein was dissolved in water of HPLC grade and loaded into an API type 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA). The sample was passed at a flow rate of 20 μL/min *via* the electrospray interface, which was operated in the positive electrospray ionization (ESI +ve) mode. The gas used for drying (35 psi) and ESI nebulization (45 psi) was high-purity nitrogen.

2.4.7. Identification of protein by MALDI-TOF-MS

The Coomassie Blue-stained protein spots were cut out from the gels and sent to GuangZhou FitGene Biological Technology Co. Ltd, Guangzhou, China, for trypsin in-gel digestion and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Thermo Co., Waltham, MA, USA) analysis. Peptide mass fingerprints coupled with peptide fragmentation patterns were used to identify the protein in the non-redundant sequence database UniProt (Homo sapiens–animal database: Mascot Search Results: Protein View and Homo sapiens-Arcidae database: Mascot Search Results: Protein View) using the MASCOT search engine (*http://www.matrixscience.com*).

2.5. Assay of $ABTS^{\bullet+}$ radical-scavenging activity

$ABTS^{\bullet+}$ was generated by mixing an ABTS stock solution (7 mM) with potassium persulfate (2.45 mM). The mixture was left in the dark at room temperature for 12-16 h. The $ABTS^{\bullet+}$ radical cation solution was diluted in 5 mM PBS at pH 7.4 until an absorbance of 0.7 (± 0.02) at 734 nm was reached. 190 μL $ABTS^{\bullet+}$ solution was mixed with 10 μL samples of the protein at different concentrations (0.01, 2.50, 5.00, 10.00, 15.00, 20.00 and 25.00 mg/mL). Ascorbic acid (AA) was used as the positive control. Ten min later, the absorbances were measured at 734 nm against the corresponding blanks. The $ABTS^{\bullet+}$-scavenging activities of the samples were calculated using the following equation:

$$ABTS^{\bullet+}\text{-scavenging activity (\%)} = [(A0 - A1)/A0] \times 100$$

where A0 is the absorbance (without sample) and A1 is the absorbance in the presence of the sample.

2.6. Statistical analysis

The experimental data are expressed as the mean ± standard deviation obtained from triplicate experiments in all figures. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Extraction and purification of protein from *A. subcrenata* Lischke

The crude proteins were released from the muscle of *A. subcrenata* Lischke and fractionated by salting-out at increasing saturation levels of ammonium sulfate. The crude protein extracted at 70%-100% saturation of ammonium sulfate was named JNY-1.

JNY-1 was separated into four fractions (P1-P4) on the DEAE Sepharose Fast Flow column. P1-P4 were eluted with increasing concentrations of NaCl in 0.03 M Tris-HCl buffer (pH 8.0), *i.e.*, 0, 0.1, 0.3, and 2 M NaCl (Figure 1A). Then, as depicted in Figure 1B, P2 was separated into three subfractions (G1, G2, and G3) by size exclusion chromatography with Sephadex G-25. After the SDS-PAGE analysis and determination of the protein content of G1-G3 (Table 1), G1, which had the highest protein content, was further purified with a Phenyl Sepharose CL-4B hydrophobic chromatography column. Through stepwise elution with decreasing concentrations of 1.0, 0.5 and 0 M $(NH_4)_2SO_4$ and distilled water, as shown in Figure 1C, four proteins with different hydrophobicity were isolated and named consecutively G1H1-G1H4. G1H2 was purified again on a Sephadex G-25 column to obtain the fraction G1H2G (Figure 1D), which contained two proteins, G1H2-1 and G1H2-2, that had identical electrophoretograms in SDS-PAGE (Figure 2E). The protein content of G1H2 was only 14.03%, while after purification on the Sephadex G-25 column, the protein content of G1H2G (combining G1H2-1 and G1H2-2) increased to 87.12% (Table 1). Then, G1H2G was separated twice by RP-HPLC to obtain the single protein G1H2GC2 (Figures 1E and 1F).

All the elution profiles were shown in Figure 1. Throughout the process of purification, every fraction obtained from every purification step was analyzed by SDS-PAGE to determine its molecular weight and purity, and the electrophoretograms from SDS-PAGE were shown in Figure 2. The protein and saccharide contents of the fractions were shown in Table 1.

3.2. Characterization of purified protein from *A. subcrenata* Lischke

As shown in Figure 2G, G1H2GC2 gave a single band in SDS-PAGE, indicating that it was electrophoretically homogeneous. Furthermore, the RP-HPLC elution profile (Figure 3A) showed a single sharp peak, from which the purity of G1H2GC2 was calculated to be over 97.2% by the normalized peak area method. Thus, both techniques demonstrated that G1H2GC2 had been purified to homogeneity. Furthermore, in IEF-PAGE, G1H2GC2 again showed a single band, and its isoelectric point was

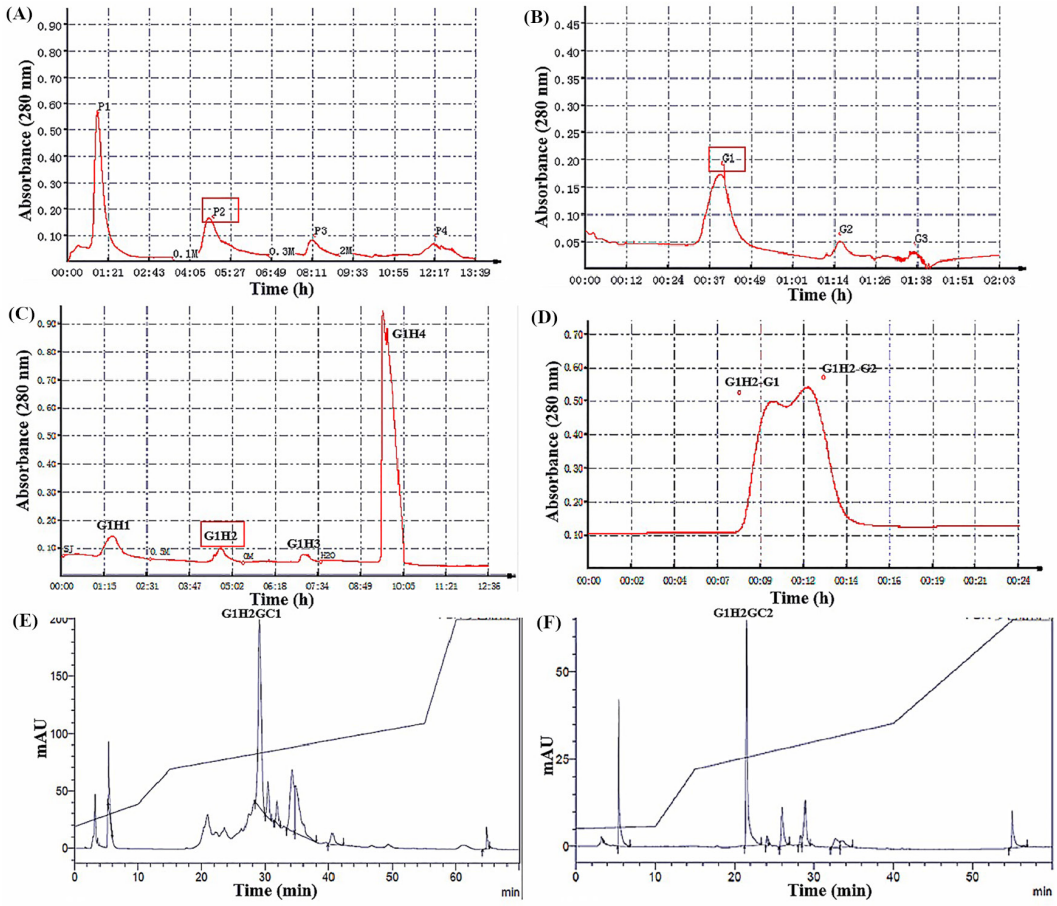

Figure 1. Purification of the proteins from *A. subcrenata* Lischke. (A) P2: separated from JNY-1 by anion exchange chromatography on a DEAE-Sepharose Fast Flow column. **(B)** G1: separated from P2 by size exclusion chromatography on Sephadex G-25. **(C)** G1H1-G1H4: separated from G1 on a Phenyl Sepharose CL-4B hydrophobic chromatography column. **(D)** G1H2G: (combining G1H2-1 and G1H2-2) enriched by Sephadex G-25. **(E)** G1H2GC1: separated from G1H2G by RP-HPLC. **(F)** G1H2GC2: separated from G1H2GC1 by RP-HPLC.

Table 1. Protein and saccharide content of different fractions

Sample	JNY-1	P2	G1	G2	G3	G1H2	G1H2G	G1H2GC2
Protein (%)	47.06	77.14	89.76	3.81	4.21	14.03	87.12	99.8
Saccharide (%)	49.88	21.06	9.81	4.96	3.57	3.84	4.06	n.d.

n.d. not detected.

found to be 7.71 (Figure 3B). The purified G1H2GC2 had an approximate molecular weight of 28 kDa as determined by comparison of the single band on the SDS-PAGE with the molecular weight markers (Figure 2G). According to a more precise analysis by ESI-MS/MS, the molecular mass of the protein was 25.6 kDa (Figure 3C). Additionally, in UV-wavelength scanning, the spectrum of G1H2GC2 contained an absorption peak at 278 nm (Figure 3D), which is a characteristic absorption of proteins.

3.3. Homology identification of protein from *A. subcrenata* Lischke

The protein G1H2GC2 with a molecular weight of 25.6 kDa was excised from the Coomassie-stained preparative gel for mass spectrometric analysis by MALDI-TOF MS to obtain its peptide mass fingerprints. The resulting primary and secondary mass spectrograms of G1H2GC2 were shown in Figure 4. They were used for protein identification by matching them in the non-redundant sequence database UniProt (Homo sapiens-animal) using the MASCOT search engine, which combines *de novo* peptide sequencing with database identifications. G1H2GC2 was found to be a previously unknown protein: the closest existing match had a match score of only 17 and a sequence coverage of 5%, as shown in Figure 5. Amino acid sequence alignment showed that G1H2GC2 does not share the same amino acid sequence as the proteins ASP-3 and H3 derived from *A. subcrenata* Lischke (*27,29*). Therefore, G1H2GC2 is a novel protein from *A. subcrenata* Lischke.

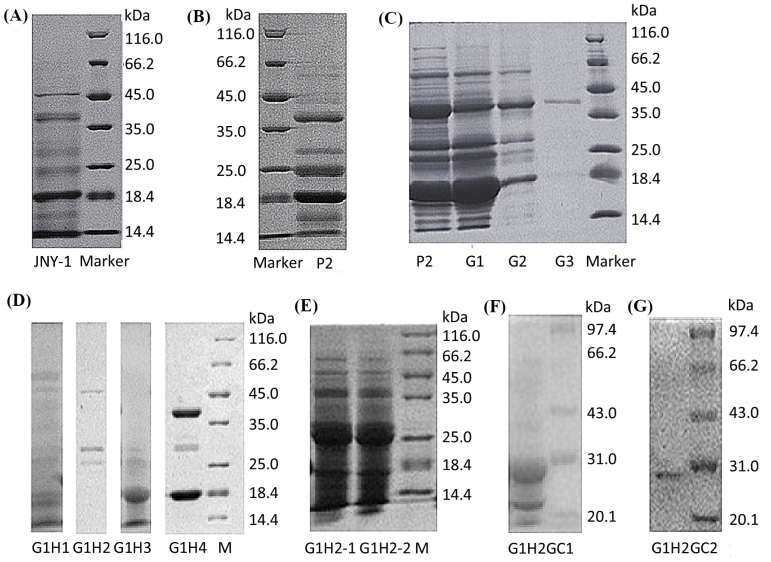

Figure 2. SDS-PAGE electrophoretic analysis of proteins obtained at each purification step. (A) JNY-1: the crude protein extracted from *A. subcrenata* Lischke. **(B)** P2: isolated from JNY-1 on a DEAE Sepharose Fast Flow column. **(C)** G1, G2 and G3: fractionated from P2 by Sephadex G-25. **(D)** G1H1-G1H4: fractionated from G1 by Phenyl Sepharose CL-4B hydrophobic chromatography. **(E)** G1H2-1 and G1H2-2: separated from G1H2 by Sephadex G-25. Both proteins were combined in one fraction, G1H2G, because of their identical electrophoretograms. **(F)** G1H2GC1: separated from G1H2G by RP-HPLC. **(G)** G1H2GC2: separated from G1H2GC1 by RP-HPLC.

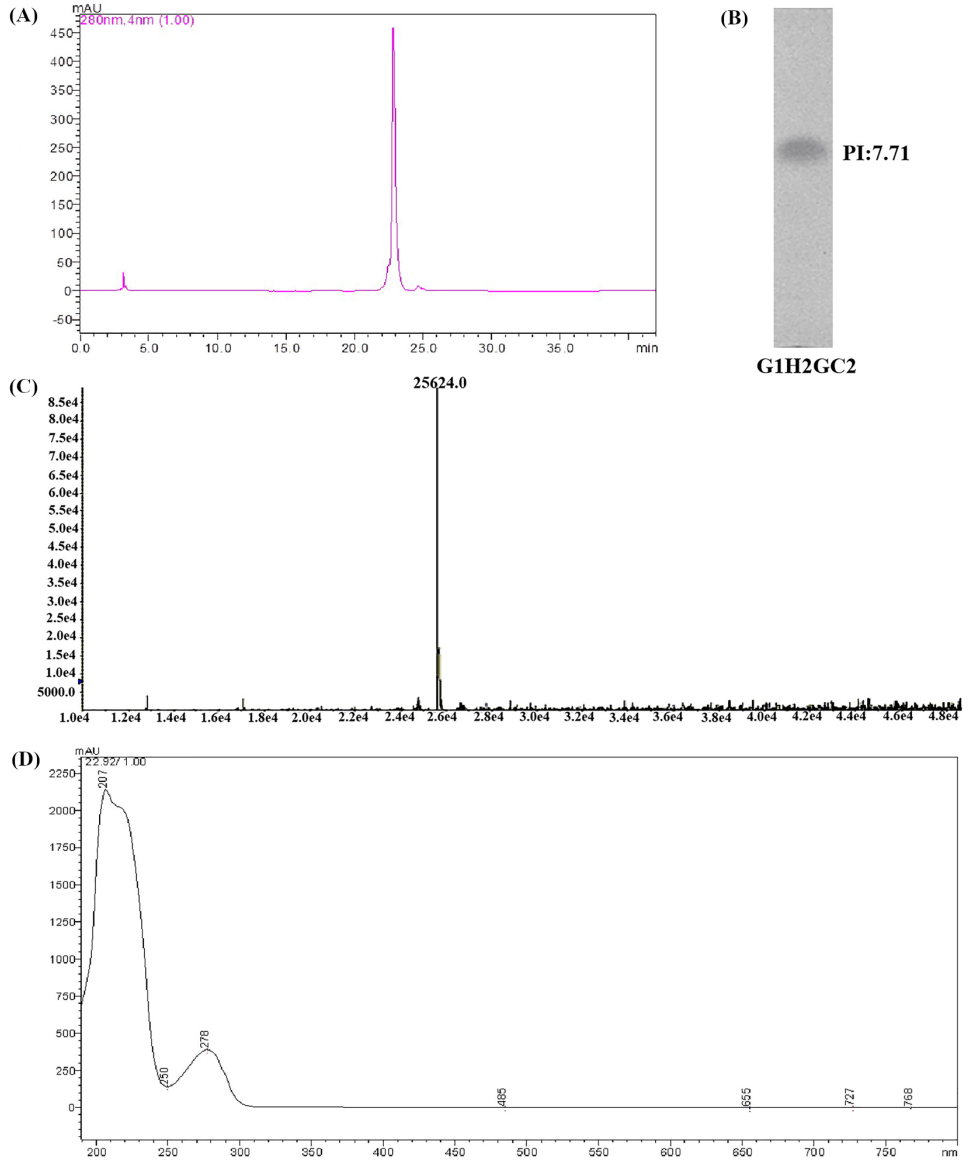

Figure 3. Purity, isoelectric point analysis and mass spectrum analysis of G1H2GC2. (A) Reversed-phase high-performance liquid chromatography (RP-HPLC) profile of G1H2GC2. **(B)** Isoelectric point determination of G1H2GC2 by IEF-PAGE. **(C)** Mass spectrum of G1H2GC2. **(D)** UV-wavelength scanning of G1H2GC2.

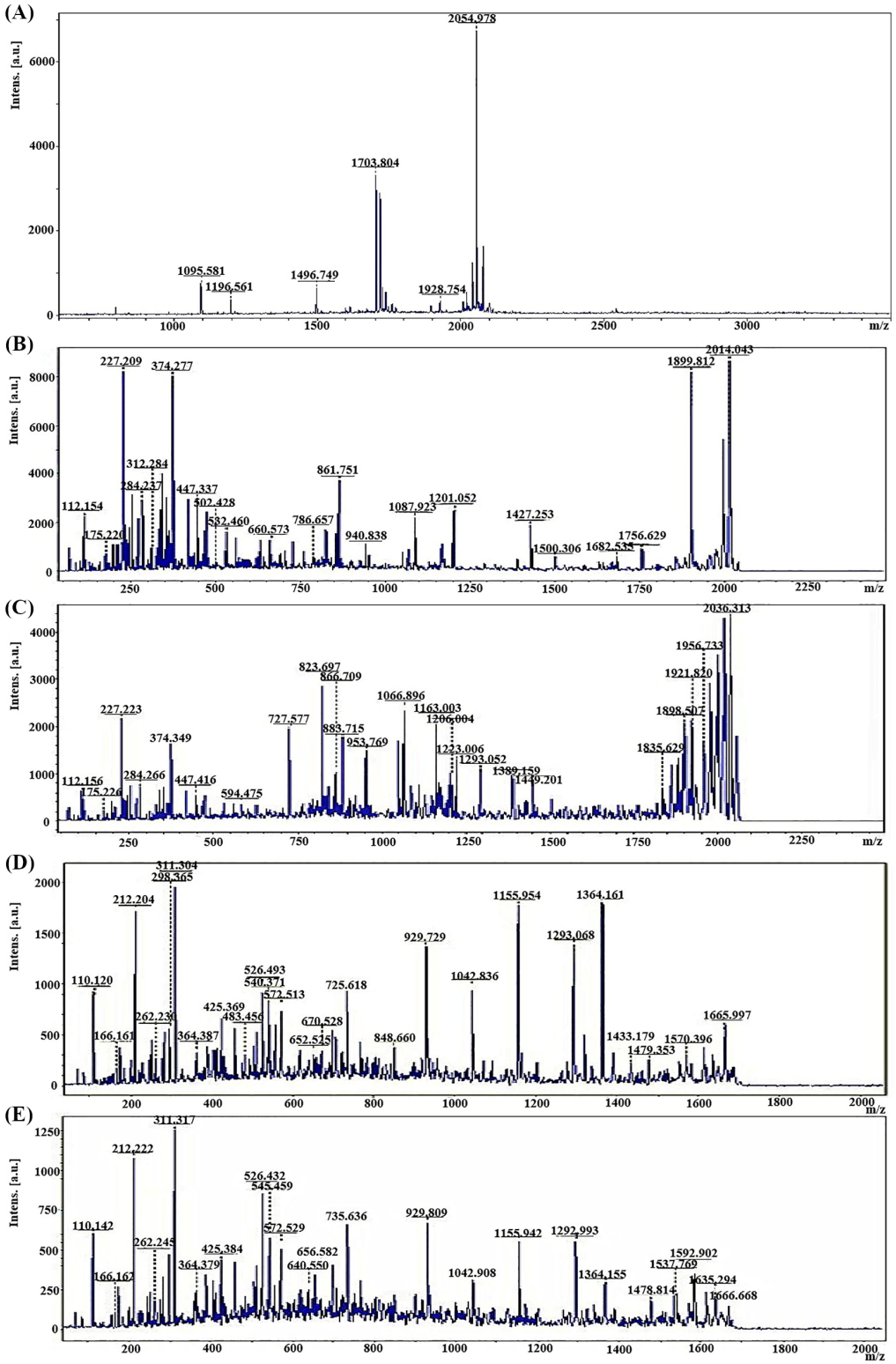

Figure 4. Mass spectrograms of G1H2GC2. (A) Primary mass spectrogram of G1H2GC2. **(B-E)** Secondary mass spectrogram of G1H2GC2.

3.4. $ABTS^{\bullet+}$ scavenging activity

The $ABTS^{\bullet+}$ assay is based on the generation of the blue/green radical cation species $ABTS^{\bullet+}$, which can be reduced by antioxidants; the resulting decrease in absorbance can be monitored at 734 nm. The ability of G1H2GC2 to scavenge $ABTS^{\bullet+}$ was measured to evaluate its antioxidant activities. As shown in Figure 6, G1H2GC2 was found to eliminate $ABTS^{\bullet+}$ *in vitro* in a dose-dependent manner, in which the radical-scavenging activity increased as the protein concentrations in the samples increased. The percentage scavenging activities

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  1 MFSRRSHGDV KKSTQKVLDP KKDVLTRLKH LRALLDNVDA SDLKQFFETN
 51 YSQIYFIFYE NFITLENSLK LKGNNKSQRE ELDSILFLFE KILQFLPERI
101 FFRWHYQSIG STLKKLLHTG NSIKIRCEGI RLFLLWLQAL QTNCAEEQVL
151 IFACLVPGFP AVLSSRGPCT LETLINPSPS VVDAKIYPEE ITPLLPAVSG
201 EKIAEDQTCF FLQILLKYMV IQAASLEWKN KENQDTGFKF LFTLFRKYYL
251 PHLFPSFTKL TNIYKPVLEI PHLRPKPLYV TVTRDNETIY STKIPYMAAR
301 VVFIKWIVTF FLEKKYLTAT QNTKNGVDVL PKIIQTVGGG AIQEKVPELD
351 GAGATEQDKS HSNSSTLSDR RLSNSSLCSI EEEHRTVYEM VQRILLSTRG
401 YVNFVNEVFR QAFLLPSCEI SITRKVVQVY RKWILQNKPV FMEEPDKKDV
451 AEEDADKLGL SETDSKEVSS ESSGHKRSSS WGRTYSFTSA MSRGCVTEED
501 NTNVKAGAQA MLQVFLTNAA NVFLLEPCVE VPMLLREQVD ACKAVLIIFR
551 RMIMELTMNQ KTWEQMLQIL LRITEAVMQK PKDKLVQDSF ARSLAGLLFR
601 TLIVAWIRAN LCVYISRELW DDFLRVLSSL TEWEELITEW SNIMDSLTAV
651 LARTVYGVEM TNLPLDKLSE QKEKKQRGKG CVLEPQKGTA VGRSFSLSWR
701 SHPDVTEPMR FRSATTSGAP GVEKARNTVR QKATEVEEFQ QAESTAAADC
751 DYLVVGQQPV PRSSSTSDIT ERLYSDSSQG QKVENSQNLS SSEPKSVQES
801 KGHVTHEHEG VTILVRRSSS PAELDLKEES QQTHGRCRER QKSESTGSDM
851 AVGYSNEAEL PVSPWQTCEE DPELSTPTDA VADSDARHWL QLSPTDASNL
901 TESRECLADD CSIIAGGNLT GWHPDSAAVL WRRVLGILGD VNNIQSPKIH
951 AKVFSYLYEL WYKLAKIRDN LAISLDNQSS PSPPLLIPPL RMFASWLFKA
1001 TTLPNEYKEG KLQAYKLICA MMTRRQDVLP NSDFLVHFYL VMHLGLTSED
1051 QDVLNTIIKN CSPRFFSLGL PGFSMLVGDF ITAAARVLST DMLAAPRSEA
1101 LTLLGSLVCF PNTYQEIPLL QSVPEVSDVV TGAEDVKHYL INILLKNATE
1151 EPNECARCIA ICSLGVWICE ELAQCASHPQ VKDAINVIGV TLKFPNKIVA
1201 QVACDVLQLL VSYWEKLQMF ETALPRKMAE ILVATIAFLL PSAEYSSVET
1251 DKKFIVSLLL CLLDWCMALP VSALLHPVST AVIEELHPSR APLLDYIYRV
1301 LHCCVCGSST YTQQSHYTLT LADLSSTDYD PFLPLANVRN SEPVQYHSSA
1351 DLGNLLTVEE EKKRRSVELI PLTARMVMAH LVNHLGHYPL SGGPAVLHSL
1401 VSENHDNAHV EGTELSSEVF RSPNLQLFVF NDSTLISYLQ TPAEGPAGGT
1451 SGGSLSDVRV IVRDISGKYS WDGKVLYGPL EGRLAPSGRN PSFQISGWHH
1501 HTCGPQSNLF HGEEGDDVLD KLLENIGHTS PECLLPSQLN LNEPSPTPSA
1551 MNCDQEKEII EVILRQSTQE DEYVQRCHSN SAVKVTSQGQ PSPVEPRGPF
1601 YFCRLLLDDL GMNSWDRRKN FHLLKKNSKL LRELKNLDSR QCRETHKIAV
1651 FYIAEGQEDK CSILANERGS QAYEDFVAGL GWEVDLSTHC GFMGGLQRNG
1701 STGQTAPYYA TSTVEVIFHV STRMPSDSDD SLTKKLRHLG NDEVHIVWSE
1751 HSRDYRRGII PTAFGDVSII IYPMKNHMFF ITITKKPEVP FFGPLFDGAI
1801 VSGKLLPSLI CATCINASRA VKCLIPLYQS FYEERALYLE AIIQNHREVM
1851 TFEDFAAQVF SPSPSYSLSG TD
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Figure 5. Protein identification of G1H2GC2: Text in red showed the parts of the sequence that could be matched.

of G1H2GC2 for $ABTS^{\bullet+}$ were 52.84% at a concentration of 15 mg/mL and 43.81% at a concentration of 10 mg/mL. The EC_{50} value of G1H2GC2 against $ABTS^{\bullet+}$ was 17.67 mg/mL.

4. Discussion

Proteins, as a crucial material foundation for human survival, participate in all forms of life activities within biological organisms. Isolating proteins from animals and plants and conducting in-depth research on their structures and functions is of great significance for understanding the primary roles of proteins in human life activities. Most natural proteins are mixtures, and many important proteins exist in low concentrations in animals and plants. The separation of proteins without affecting their structure and function has always been a challenging aspect of protein research. There are numerous methods for protein extraction, including alkali solubilization, ultrasound extraction, organic solvent extraction, and enzymatic extraction. Research indicates that ultrasound-assisted extraction achieves a higher protein extraction rate (*35-37*). Therefore, we used ultrasound-assisted extraction to extract crude proteins from *A. subcrenata* Lischke.

There are numerous methods for protein separation and purification, including precipitation, chromatography,

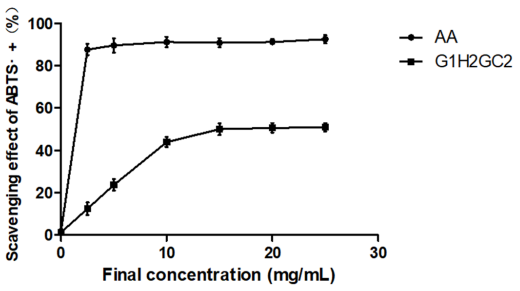

Figure 6. $ABTS^{\bullet+}$ scavenging activity of G1H2GC2.

and ultrafiltration (*38*). Different separation and purification methods operate on distinct principles, allowing the combination of multiple approaches for effective purification. To purify the protein G1H2GC2 from *A. subcrenata* Lischke, we employed a multi-step strategy. Initially, the target protein was precipitated using ammonium sulfate salting-out. Subsequently, guided by protein purity tracking, we applied a series of chromatographic techniques, including DEAE Sepharose Fast Flow anion exchange, gel filtration chromatography (Sephadex G-25), Phenyl Sepharose CL-4B hydrophobic chromatography and RP-HPLC. A protein from *Yarrowia lipolytica* was isolated and purified by ultrasonic assisted extraction, ammonium sulfate precipitation, anion exchange chromatography and gel filtration chromatography (*39*).

Many shellfish species have been identified as possessing potential pharmaceutical or food additive value. Bioactive proteins and their derivatives isolated from various shellfish have demonstrated multiple biological functions, including antioxidant, antitumor, antibacterial, and anti-inflammatory activities (*40-42*). A mucus protein derived from pearl oyster (*Pinctada martensii*) was shown to significantly accelerate wound healing and stimulate collagen regeneration in rats (*43*). Additionally, another water-soluble matrix protein isolated from pacific oysters (*Crassostrea gigs*) exhibits anti-osteoporosis activity (*44*). Consequently, shellfish represent a valuable natural resource with potential applications in the nutritional supplement and pharmaceutical industries. Therefore, it is essential to investigate the meat of *A. subcrenata* Lischke and reveal its latent functional components.

In this study, a novel protein, G1H2GC2, with molecular weight 25.6 kDa and isoelectric point 7.71, was purified from *A. subcrenata* Lischke by several column chromatography techniques including anion exchange, gel filtration chromatography, hydrophobic chromatography and RP-HPLC. In addition, the physicochemical and structural properties of G1H2GC2 were characterized by two electrophoresis techniques as well as Bradford assay, the phenol-sulfuric acid method, ESI-MS and MALDI-TOF MS. The antioxidant activity of G1H2GC2 was investigated by measuring its $ABTS^{\bullet+}$-

scavenging ability. The results showed that G1H2GC2 might be developed as a potential food additive agent.

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