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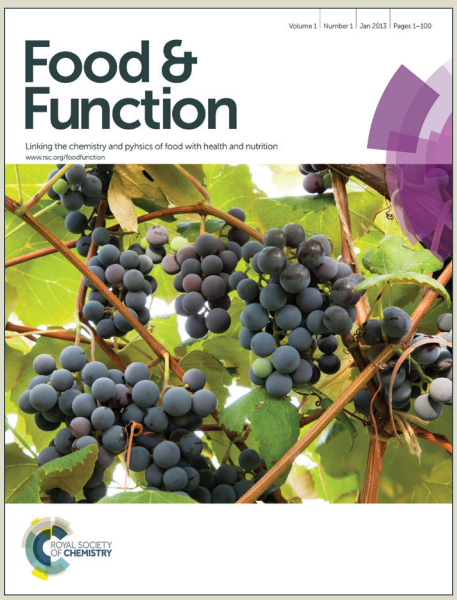

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Anti-proliferative effect of *Dendrobium catenatum* Lindley polypeptides on human liver, gastric and breast cancer cell lines

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Running title: Anti-proliferation polypeptide separation

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Abstract

Dendrobium catenatum Lindley is a precious plant with both dietary and medicinal applications. However, the anti-proliferation activity of *D.catenatum*-derived peptides has not been investigated. In this study, the whole proteins of *D.catenatum* were extracted, hydrolysis with three proteases (alcalase 2.4L, alcalase 37017 and trypsin) was performed, and gel filtration chromatography was employed to obtain 9 fractions. Among them, A3 possessed the best anti-proliferation activity in vitro, with the inhibitory percentages 73.38%, 78.91% and 86.8% on cancer cells HepG-2, SGC-7901 and MCF-7, respectively, as well as 5.52% only on normal liver cells L-O2, at 500 μg/mL. Subsequently, mass spectrometry analysis revealed the existence of 10 alcalase-derived peptides in fraction A3, and the sequences of top 3 peptides were determined by de novo sequencing: RHPFDGPLLPPGD, RCGVNAFLPKSYLVHFGWKLLFHFD and KPEEVGGAGDRWTC. Moreover, these peptides were synthesized and their anti-proliferation activities in vitro were also confirmed. This suggests that fraction A3 may be promising ingredients in food and pharmaceutical applications.

Key words: *Dendrobium catenatum*; hydrolysis; polypeptide; separation; anti-proliferation

1 Introduction

Although the development of many strategies in fighting against cancer such as chemotherapy, surgery, and radiation, cancer is still a major concern in relation to human morbidity and mortality. Importantly, the intrinsic or acquired resistance generated by antitumor drug is considered to be the widespread cause for tumor recurrence.[1] This encourages researchers worldwide to seek novel anticancer agents with specific mechanism of action. Due to the properties of simple structure, low molecular masses, fewer side effects and easy absorption,

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bioactive peptides are considered to be promising, which have been reported to possess anticancer or anti-proliferative activities.[2,3] Many bioactive peptides and depsipeptides with anticancer potential have achieved clinical trials. Aplidine, a cyclodepsipeptide isolated from the tunicate Aplidium albicans, has shown antitumor activity in phase I trials,[4,5] and has already undergone active phase II studies in solid tumors.[6] Kahalalides is a family of peptides isolated from the sacoglossan mollusk Elysia rufescens. Kahalalide F exhibited clinical benefits and low toxicity in treated patients in phase I clinical trials, and is undergoing phase II clinical trials for the treatment of lung and prostate cancers, and melanoma.[7]

Dendrobium catenatum Lindley, *Orchidaceae*, called Tie Pi Shi Hu in Chinese, is a dietary and medicinal plant.[8,9] In traditional chinese medicine, it has been used for treatment of hepatitis, asthma and immunological disorders for thousands of years.[10] More than fifty *Dendrodium*-based health food products have been approved by the State Food and Drug Administration of China.[11] Such as, the green stem of *D. catenatum* can be directly used as a high quality agricultural vegetable in diets,[12] or can be processed into drink products, functional capsules and powders.[13] In recent years, polysaccharides derived from *D. catenatum* have been reported to possess some bioactivities. For example, oral administration of *D. catenatum* polysaccharides was found to significantly enhance cellular immunity and nonspecific immunity in mice.[14] Three polysaccharides from *Dendrobium huoshanense*, *D. catenatum* and *D. Nobile* exhibited hypoglycemic and antioxidative activities in alloxan-induced diabetic mice after oral administration.[15] However, the bioactivity of *D. catenatum*-derived peptides has not been elucidated. The purpose of this study was to perform hydrolysis of the whole proteins extracted from *D. catenatum*, to separate and identify peptides with anti-proliferation activity.

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2 Experimental procedures

2.1 Materials and chemicals

Dendrobium catenatum Lindley (10.7% of total protein contents) was from Crops Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, China. Bio-Rad Protein Assay Kit (500-0002) was purchased from Bio-Rad Laboratories. Trypsin (1:250 U/g) was from Guangzhou Qiyun Biotech, China. Alcalase 2.4 L (2.4 U/g, Sigma, USA) and Alcalase 37017 (4 U/g, Novozymes, Denmark) were from Bacillus licheniformis. Sephadex G-25 was from Pharmacia. Other reagents were of analytical grade and commercially available.

2.2 Protein extraction

Dendrobium catenatum Lindley powder (100g) was dissolved in 2 L of PBS (0.1M, pH 7.4, 25℃) for 24 h. Ammonium sulfate (20%) was introduced into the extraction solution overnight. After removing precipitation by centrifugation (4500×g), the concentration of ammonium sulfate was adjusted to 80% overnight, centrifuged (4500×g) to obtain protein paste. Subsequently, the protein paste was dissolved in PBS and subjected to dialysis (6000-8000 Da., 25℃) for three days, freeze-dried, and the protein powder was obtained. The protein content was assessed by Bio-Rad Protein Assay kit (No. 5000001, Yezhou Biotech Co., Shanghai, China) and the extraction ratio was calculated as the ratio of protein contents in the extracts to total protein contents in plant cells.

2.3 Protein hydrolysis

Dendrobium catenatum Lindley protein powder was diluted to 2% of protein solutions and subjected to hydrolysis with three proteases (alcalase 2.4L, alcalase 37017 and trypsin) under controlled conditions. No specific purpose for the selection of these enzymes, just depending on the availability in our lab, Alcalase and trypsin are the frequently used enzymes in protein

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hydrolysis, alcalase 2.4L and alcalase 37017 have different activity. For alcalase 2.4L, the ratio of enzyme to substrate (E/S) 6% w/w, temperature 50 ℃, pH 8.5 and reaction time 8 h. For alcalase 37017, the ratio of enzyme to substrate (E/S) 3% w/w, temperature 50 ℃, pH 8.5 and reaction time 8 h. For trypsin, the ratio of enzyme to substrate (E/S) 6% w/w, temperature 42 ℃, pH 8 and reaction time 8 h. After hydrolysis, enzyme was inactivated by placing the samples in boiling water for 10 min. The cooled hydrolysates were then subjected to centrifugation at 4500×g for 10 min. The supernatants were used to measure the degree of hydrolysis (DH) by formaldehyde tiltration method: DH = (free amino acid nitrogen in the hydrolysate (g/100 mL) – free amino acid nitrogen before hydrolysis (g/100 mL))/total protein nitrogen (g/100 mL).

2.4 Gel filtration chromatography

Three milliliters of lyophilized enzymatic hydrolysates dissolved in distilled water at a concentration of 170 mg/mL were loaded onto a Sephadex G-25. The column was eluted with distilled water at a flow rate of 0.5 mL/min. The eluates were collected (3 mL/tube) and detected at 280 nm. The eluates at the same peak were combined and freeze-dried, which were used for further anti-proliferation activity assay. The peptide content was measured by Bicinchoninic Acid Kit (BCA-1, Sigma-Aldrich, Shanghai, China).

2.5 Anti-proliferation activity assay

Human liver cancer cells (HepG-2), gastric cancer cells (SGC-7901), breast cancer cells (MCF-7) and normal liver cells (L-O2), purchased from Animal Experimental Center of Sun Yat-Sen University, Guangzhou, China, were cultured in a 37 ℃ humidified atmosphere with 5% CO_2. DMEM (Gibco, USA), supplemented with 10% fetal bovine serum (FBS).

The anti-proliferation activity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)

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22,5-diphenyl- tetrazolium bromide (MTT, Amresco Co., USA). Cells were plated at a density of 5×10^4 cells/well in a 96-well microtiter plate overnight, then treated with varying concentrations of chromatographic fractions dissolved in distilled water (50–500 μg/mL on a total weight basis). No drug was used as negative control and the standard drug 5-FU (5-flurouracil, a standard chemotherapeutic drug to treat cancers) dissolved in DMSO solution was used as a positive control. All treatments were added in culture medium. After incubation for 48 h, about 20 μL of MTT solutions (5 mg/mL) were added to each well, and incubated for additional 4 h at 37 ℃. The supernatant was aspirated and the MTT-formazan crystals formed by metabolically viable cells were dissolved in 100 μL of DMSO for 15 min. Finally, the absorbance was read at 490 nm with a microplate reader (Model 550, Bio-Rad, USA). The percentage of inhibition was determined by the formula: Inhibition (%) = (1-[the optical density values for experimental groups/the optical density values for control group]) ×100%.

2.6 Mass spectrometry analysis and peptide identification

Alpha-cyano-4-hydroxycinnamic acid (5 mg/mL) dissolved in acetonitrile/water 60:40 (v/v) with 0.1% trifluoroacetic acid was used as matrix. One μL of sample solutions (1 mg/mL) and 1 μL of matrix solutions were spotted onto AnchorChip target plate. MS and MS/MS experiments were performed on a matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) (UltraFleXtreme, Bruker, Germany) in the positive ion reflectron mode. A protein molecular mass range of 800–3500 Da and a mass tolerance of 100 ppm were used for the internal calibration. For peptide identification, two methods were used: database search and de novo sequencing. The data obtained from MALDI-TOF-MS measurements were firstly analyzed by SEQUEST database search using Mascot software with following settings: MS/MS tol. ±

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0.5Da; Pebtide tol. ±0.5Da ; database (NCBInr, SwissProt, cRAP and EST(Expressed Sequence Tags,http://www.ncbi.nlm.nih.gov/genbank/dbest)) accessed on April 28, 2014; fixed modifications and variable modifications (none selected); one missed cleavage. The expectation value (chance of misidentification) is less than 0.05. Then, the composition-based de novo sequencing approach was applied using the computer program PEAKS.[16] Calculations of amino acid compositions of peptides were performed using accurately measured mass spectra with a tolerance of 2 ppm for precursor ions and fragment ions (The isobaric peptides leucine and isoleucine could not be differentiated by exact mass measurements due to their identical elemental composition).

2.7 Peptide synthesis and anti-proliferation activity

After sequence determination, peptides were custom-synthesized by Aite Biotechnol Ltd. (Nanjing, China) using standard Fmoc method. The synthesized peptides with a purity of over 98% (see supplemental materials) were subjected to anti-proliferation activity assay as described above.

2.8 Statistical analysis

All of the tests were conducted in triplicate. The experimental data were expressed as the mean±standard deviation. Student's t tests were used for all statistical analysis between different groups. P values below 0.05 and 0.01 were considered significant and very significant, respectively.

3 Results and discussion

After simple phosphate buffered saline extraction and ammonium sulfate fraction, about 55.7% of *D.catenatum* Lindley whole proteins were harvested. The extracted proteins were

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157 subjected to hydrolysis with three proteases (alcalase 2.4L, alcalase 37017 and trypsin) under
158 controlled conditions. The final degree of hydrolysis (DH) was determined as: 28.4%, 22.3% and
159 17.2% for alcalase 2.4L, alcalase 37017 and trypsin, respectively. Subsequently, the hydrolysates
160 from three enzymes were separated by Sephadex G-25 column chromatography. After water
161 elution, totally 9 fractions were obtained (Fig.1), three fractions A1, A2 and A3 for alcalase 2.4L,
162 three fractions S1, S2 and S3 for alcalase 37017, and three fractions Y1, Y2 and Y3 for trypsin.
163 Their peptide contents were measured by bicinchoninic acid method: 39.6%, 47.2% and 99.2% for
164 A1, A2 and A3, respectively; 33.8%, 40.6% and 86.4% for S1, S2 and S3, respectively; 33.7, 54%
165 and 58.2% for Y1,Y2 and Y3, respectively.

166 The inhibitory activities of 9 fractions on human gastric cancer SGC-7901 cells were
167 measured by MTT method at 200 μg/mL and 400 μg/mL. The results showed that the hydrolysates
168 digested by alcalase exhibited strong cancer-inhibition activity (17~67.5%), compared with the
169 hydrolysates digested by trypsin (4~18%) (Fig.1). The inhibitory activities of the fractions
170 A1,A2,A3 and S2 were higher than the inhibitory activities of other fractions. Especially, A3
171 displayed the highest activity 67.5% at 400 μg/mL. On the other hand, the peptide content of A3
172 was also the highest (99.2%), so A3 was used for further investigation.

173 Table 1 showed that A3 exhibited dose-dependent anti-proliferation activities on 3 cancer
174 cells within the concentrations 50~500 μg/mL: 35~73% for liver cancer cells HepG-2; 26~79%
175 for gastric cancer SGC-7901; 36~87% for breast cancer MCF-7. The inhibitory percentages of
176 5-fluorouracil on HepG2, SGC-7901, MCF-7 and L-O2 cells were 86.1%, 80.1%, 84.87% and
177 78.88% at 500 μg/mL, respectively.

178 Thus, it is interesting to determine the composition of fraction A3. MALDI-TOF-MS analysis

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indicated that A3 was composed of 10 alcalase-derived peptides (Fig.2), their details were summarized in Table 2. It can be seen that top three peptides with m/z = 1417.840 (P1), 2994.743 (P2) and 1504.81 (P3) possess the peak area 33.7%, 15.2% and 12.6%, respectively, totally 61.5%. Subsequently, the three peptides were further fragmented to obtain MS/MS spectra (Fig.2). For sequence identification of these peptides, database searching and de novo sequencing were applied in the present study. Unfortunately, no satisfactory match was found by database searching. By de novo sequencing, the amino acids sequences of peptides P1, P2 and P3 were derived (Table 3).

Then, P1, P2 and P3 were synthesized, and their purity and molecular weights were assayed by HPLC-MS analysis (see supplemental materials). In consideration of the fact that the proportions of P1, P2 and P3 in fraction A3 are 33.7%, 15.2% and 12.6%, respectively, the same proportion of contributions to cancer inhibition of A3 could be expected for P1, P2 and P3. For example, at 500 μg/mL, the inhibitory activity of A3 on HepG-2, SGC-7901 and MCF-7 was 73.78%, 78.91 and 86.8%, respectively, the expected inhibitory activity of P1~P3 should be 9.3%~24.86% for HepG-2, 9.94%~26.59% for SGC-7901, and 10.94%~29.25% for MCF-7. Indeed, Table 4 showed that at 500 μg/mL the inhibitory activities of the synthetic peptides P1~P3 on three cancer cells were 21.7%~33.4% for HepG-2, 27.8~33.9% for SGC-7901, and 30%~41.8% for MCF-7. Nevertheless, the three peptides displayed no synergistic effects except for P2 and P3 (42.1% for P2+P3, 39.3% for P2 and 30% for P3) (Table 4). Notably, not too many sequences of anticancer peptides obtained by hydrolysis were identified, such as, a pentapeptide isolated from rice bran, Glu-Gln-Arg-Pro-Arg, possessed cancer growth inhibitory properties on colon, breast, lung and liver cancer cells, >80% at 600-700 μg/mL;[17] An anticancer peptide from enzymatic hydrolysate of Mytilus coruscus, Ala-Phe-Asn-Ile-His- Asn-Arg-Asn-Leu-Leu, had the

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LC50 values 0.94, 1.41, and 1.22 mg/mL on prostate cancer (PC-3), lung cancer (A549), and breast cancer (MDA-MB-231) cells, respectively.[18]

Antitumor peptides have been proposed to be promising agents for antitumor therapy due to their numerous advantages over other chemical drugs, including simple structure, low molecular masses, fewer side effects, easy absorption.[19,20] A large quantity of antitumor peptides from plants have been studied for cancer treatment.[21] For instance, the peptide lunasin,isolated from soy beans and other seeds, was reported to suppress chemical carcinogen-induced tumorigenesis.[22] The peptides StAP1 and StAP3, separated from the potato *Solanum tuberosum*, were capable of inducing apoptosis in Jurkat T leukemia cells.[23] The isolated peptides Cr-ACP from *Cycas revoluta* was shown to repress the cell proliferation of human epidermoid cancer (Hep2) and colon carcinoma.[24] Recently, Wang and Zhang [25] separated a polypeptide *Chlorella pyrenoidosa* anti-proliferation polypeptide (CPAP) from the unicellular green algae *Chlorella pyrenoidosa*, which showed the highest inhibitory activity on human liver HepG2 cancer cells (49%). Another study from the same team reported that an anti-proliferation polypeptide Y2 from trypsin digest of multicellular edible blue-green alga *Spirulina platensis* proteins was obtained, which exhibted potent inhibitiory activity on MCF-7 and HepG2 cells.[26]

However, no reports are available on anti-proliferation peptides derived from *D. catenatum* Lindley. In this study, by enzymatic hydrolysis and gel filtration chromatography, we separated a fraction A3, which is a alcalase 2.4L-digested hydrolysate of *D. catenatum* proteins. A3 has been shown to possess dose-dependent anti-proliferation activities on 3 cancer cells (HepG2, SGC-7901 and MCF-7). The maximum inhibitory activity was amounted to 86.8% on breast cancer cells at 500 μg/mL. On the other hand, at 500 μg/mL, almost no inhibitory activity on normal cells L-O2

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was observed for A3 (5.5%), while the inhibitory activity of 5-fluorouracil was up to 78.8%. This suggests that the fraction A3 may be promising ingredients in food, nutraceutical, and pharmaceutical applications. Of course, additional studies are also highly required, such as in vivo evaluation, further identification of sub-peptide sequences in fraction A3 (only top 3 peptides were identified now), mechanism of action, large-scale preparation, etc.

4 Conclusion

For the first time, this study separated a fraction A3 containing 10 sub-peptides from *Dendrobium catenatum* Lindley. A3 exhibited anti-proliferation activity on HepG2, SGC-7901 and MCF-7 cells, but less cytoxicity on normal liver cells L-O2. This suggests that A3 should be promising in food and medicinal applications and warrant further study.

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Declarations of interest

The authors report no declarations of interest.

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312 **Legends**

313 Figure 1 Gel filtration chromatography of enzymatic hydrolysates and their inhibitory activities on
314 human gastric cancer SGC-7901 cells: (A) alcalase 2.4L, (B) alcalase 37017, (C) trypsin, and (D)
315 inhibition percentages of 9 fractions, a, $p<0.05$, b, $p<0.01$, compared with A3.

316 Figure 2 Peptide mass fingerprinting of fraction A3 (A) and MS/MS spectra of its top three
317 peptides P1,P2 and P3 (B-D).

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320 **Table 1 Inhibitory effects of A3 on cancer and normal cells in vitro(±s, n= 5) (%)**

Concentration (μg/mL)	Negative Control	50	100	200	300	400	500	5-fluorouracil (500μg/mL)
HepG-2	0	35.04±0.011	38.87±0.003	46.11±0.002	58.04±0.004	64.00±0.016	73.38±0.006*	86.10±0.041
SGC-7901	0	26.09±0.006	42.28±0.003	53.35±0.007	61.24±0.024	72.31±0.010	78.91±0.005	80.10±0.002
MCF-7	0	36.10±0.003	48.88±0.012	59.53±0.011	65.28±0.009	77.22±0.019	86.80±0.006	84.87±0.015
L-O2	0	Not tested	Not tested	Not tested	Not tested	Not tested	5.52±0.004**	78.88±0.024

321 *p<0.05, **p<0.01, compared with 5-fluorouracil.

322 **Table 2 Peptide mass fingerprinting of fraction A3**

m/z	S/N	Area	Percentage
1417.84	113.3	273	33.7%
2994.743	29.8	123	15.2%
1504.81	26	102	12.6%
1792.062	36.6	80	9.9%
1806.072	21.4	71	8.8%
2400.218	14.2	41	5.1%
2289.222	17.6	32	3.9%
1400.813	15.2	31	3.8%
1678.978	11.9	29	3.6%
1692.99	12.3	29	3.6%

323

324 **Table 3 Information for top three peptides (P1,P2 and P3) in fraction A3**

	Sequence C → N	Measured m/z	Theoretical molecular mass	Measured molecular mass	ΔM
P1	RHPFDGPLLPPGD	1417.84	1416.7150	1416.8370	0.1220
P2	RCGVNAFLPKSYLVHFGWKLLFHFD	2994.74	2993.5526	2993.7427	0.1901
P3	KPEEVGGAGDRWTC	1504.81	1503.6776	1503.8099	0.1323

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Table 4 Inhibitory effects of synthetic peptides (P1,P2 and P3) on cancer and normal cells in vitro(±s, n= 5) (%)

Concentration (μg/mL)	Negative Control	P1 (500μg/mL）	P2 (500μg/mL）	P3 (500μg/mL）	P1+P2 (500μg/mL）	P2+P3 (500μg/mL）	P1+P3 (500μg/mL）	P1+P2+P3 (500μg/mL）
HepG-2	0	21.72±0.027	33.42±0.030	23.40±0.014				
SGC-7901	0	30.40±0.002	27.81±0.040	33.94±0.009				
MCF-7	0	41.80±0.013	30.02±0.001	39.31±0.012	34.34±0.009 a*b*	42.10±0.081 b**c*	38.72±0.003 a*	39.10±0.023 b*
L-O2	0	20.36±0.012	24.22±0.008	18.27±0.032	22.21±0.018	28.31±0.012 b*c**	26.23±0.002 a*c**	25.22±0.001 a*c**

*p<0.05, **p<0.01, a, b and c, compared with P1, P2 and P3, respectively.

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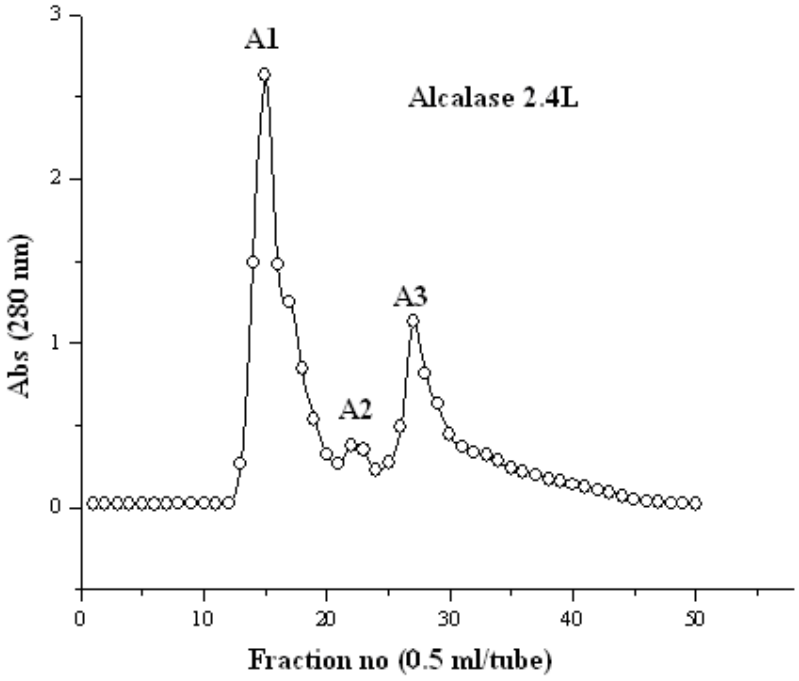
Graphical abstract

This work separated a fraction A3 containing 10 sub-peptides from *Dendrobium catenatum* Lindley. A3 exhibited anti-proliferative activity on cancer cells.



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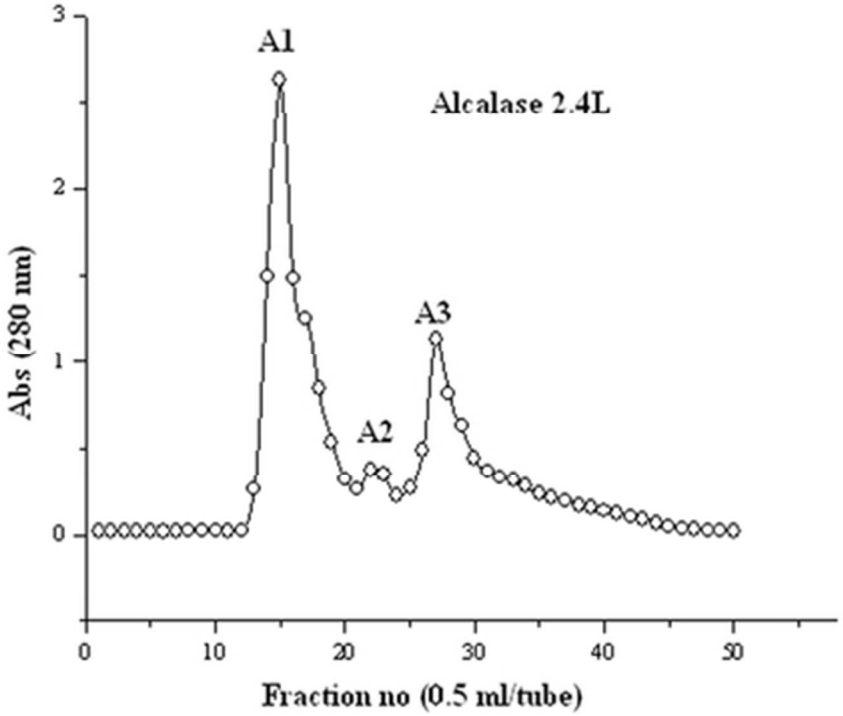

Fig.1A
Figure 1 Gel filtration chromatography of enzymatic hydrolysates and their inhibitory activities on human gastric cancer SGC-7901 cells: (A) alcalase 2.4L, (B) alcalase 37017, (C) trypsin, and (D) inhibition percentages of 9 fractions, a, $p<0.05$, b, $p<0.01$, compared with A3.
35x35mm (300 x 300 DPI)

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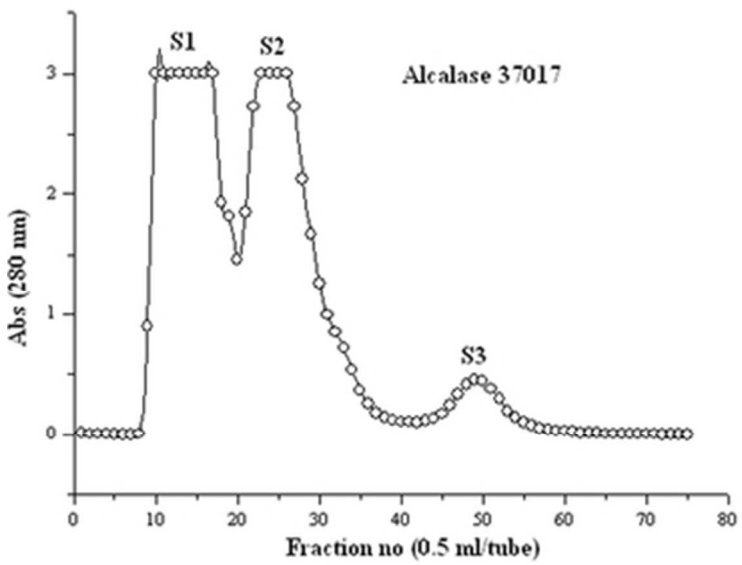

Fig.1B

Figure 1 Gel filtration chromatography of enzymatic hydrolysates and their inhibitory activities on human gastric cancer SGC-7901 cells: (A) alcalase 2.4L, (B) alcalase 37017, (C) trypsin, and (D) inhibition percentages of 9 fractions, a, $p<0.05$, b, $p<0.01$, compared with A3.
31x24mm (300 x 300 DPI)

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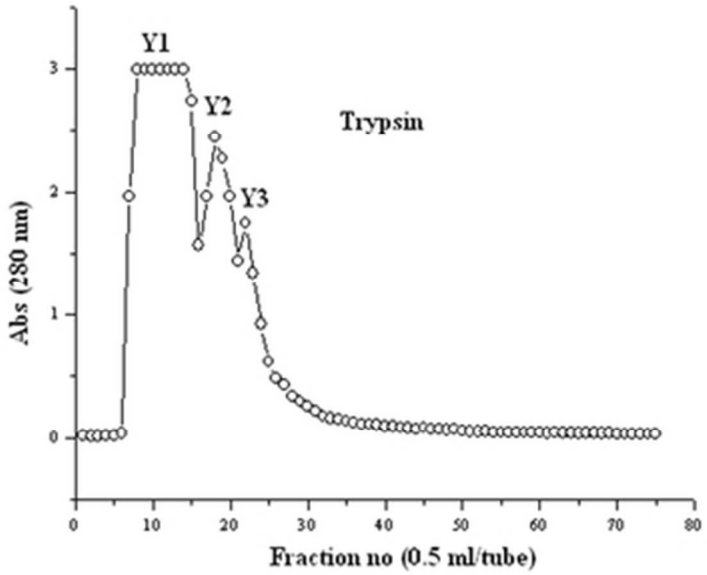

Fig.1C

Figure 1 Gel filtration chromatography of enzymatic hydrolysates and their inhibitory activities on human gastric cancer SGC-7901 cells: (A) alcalase 2.4L, (B) alcalase 37017, (C) trypsin, and (D) inhibition percentages of 9 fractions, a, $p<0.05$, b, $p<0.01$, compared with A3.
30x24mm (300 x 300 DPI)

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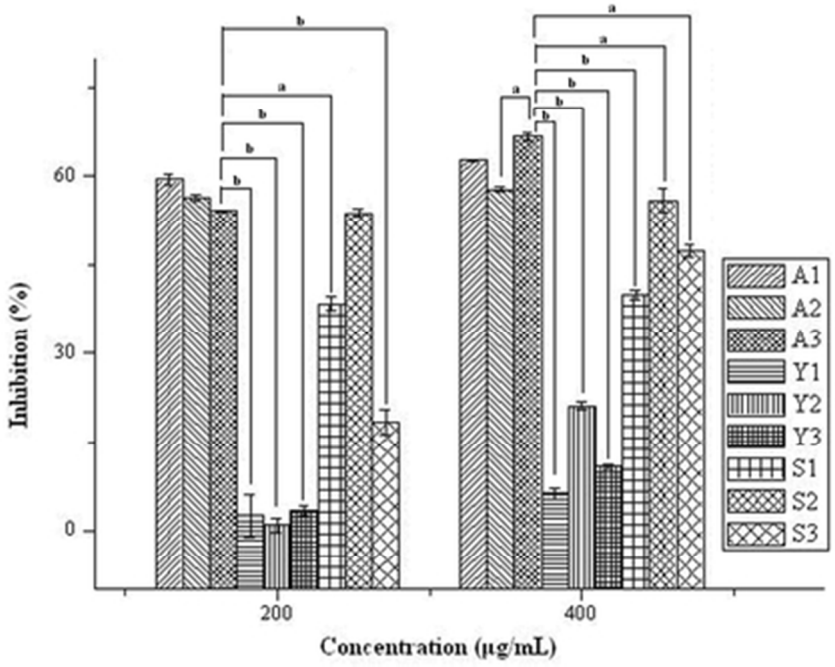

Fig.1D
Figure 1 Gel filtration chromatography of enzymatic hydrolysates and their inhibitory activities on human gastric cancer SGC-7901 cells: (A) alcalase 2.4L, (B) alcalase 37017, (C) trypsin, and (D) inhibition percentages of 9 fractions, a, $p<0.05$, b, $p<0.01$, compared with A3.
35x28mm (300 x 300 DPI)

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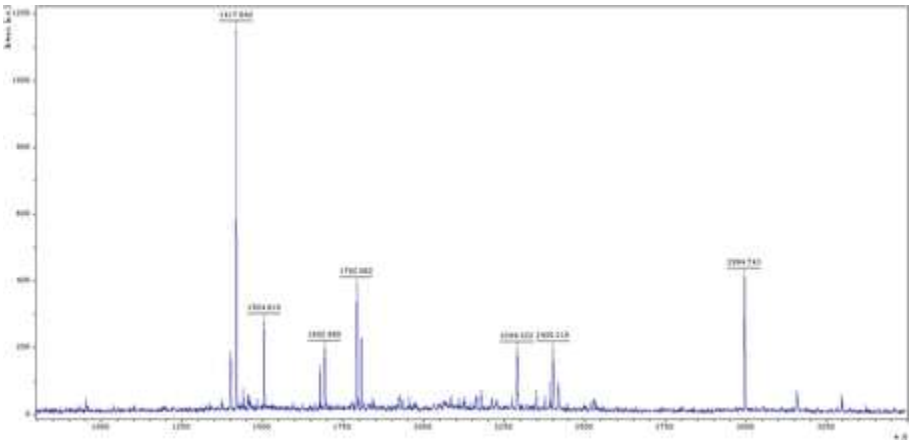

Fig.2A
Figure 2 Peptide mass fingerprinting of fraction A3 (A) and MS/MS spectra of its top three peptides P1,P2 and P3 (B-D).
38x18mm (300 x 300 DPI)

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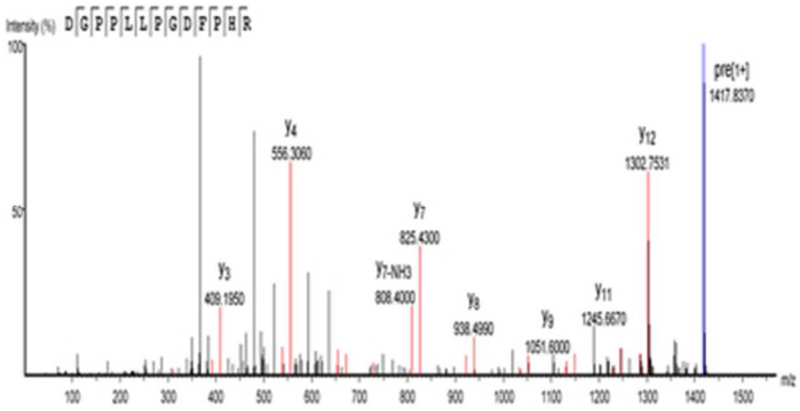

Fig.2B
Figure 2 Peptide mass fingerprinting of fraction A3 (A) and MS/MS spectra of its top three peptides P1,P2 and P3 (B-D).
33x17mm (300 x 300 DPI)

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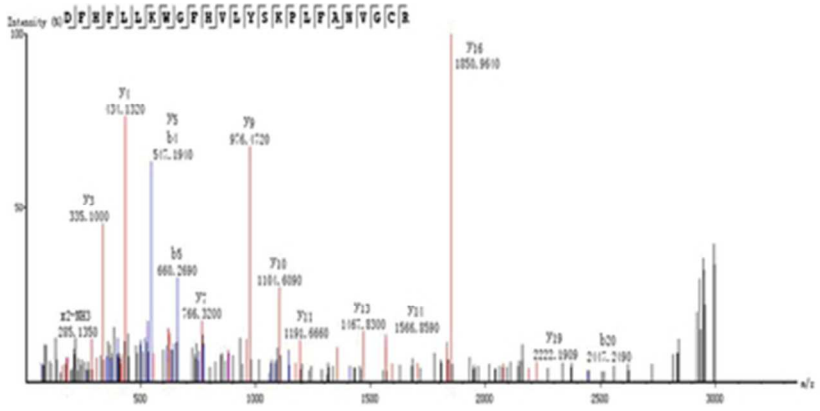

Fig.2C

Figure 2 Peptide mass fingerprinting of fraction A3 (A) and MS/MS spectra of its top three peptides P1,P2 and P3 (B-D).

34x17mm (300 x 300 DPI)

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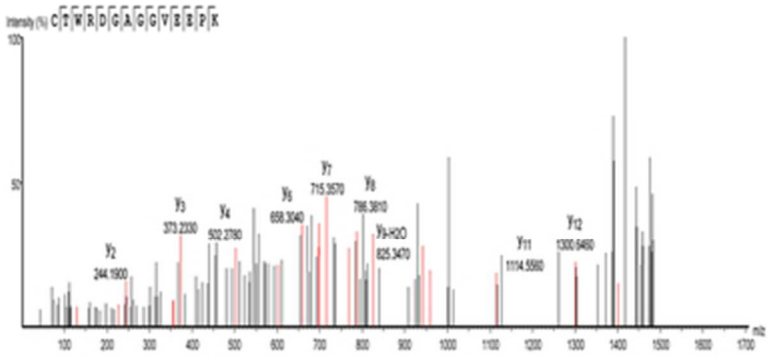

Fig.2D

Figure 2 Peptide mass fingerprinting of fraction A3 (A) and MS/MS spectra of its top three peptides P1,P2 and P3 (B-D).

32x14mm (300 x 300 DPI)

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