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Purification and Identification of an ACE-Inhibitory Peptide from *Gracilaria tenuistipitata* Protein Hydrolysates

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Abstract: Edible marine species are valuable sources of bioactive peptides. This study investigated the ACE-inhibitory activity of protein hydrolysates from the red algae Gracilaria tenuistipitata. Fifteen groups of protein hydrolysates *were prepared by* a two-step enzymatic hydrolysis *of G. tenuistipitata*: initial hydrolysis with several glycolytic enzymes, followed by three separate proteolytic reactions (Alcalase, Neutrase and Flavourzyme) for 2–10 h. Results showed that the hydrolysate GTN4H had the highest ACE-inhibitory activity *in vitro*. Furthermore, oral administration of GTN4H significantly reduced systolic blood pressure in spontaneously hypertensive rats. Fraction A derived from GTN4H displayed the highest ACE-inhibitory activity among fractions. Further purification of fraction A by RP-HPLC obtained a purified peptide (MW: 1776 Da) with 17 amino acids and 95.4% ACE-inhibitory activity.

Keywords: *Gracilaria tenuistipitata*; protein hydrolysate; ACE inhibitory activity; hypertension

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1. Introduction

High blood pressure has been identified as a risk factor for death from all causes and cardiovascular diseases [1,2]. Hypertension is usually accompanied by atherosclerosis, stroke, myocardial infarction, and end-stage renal disease [3]. Diet therapy and lifestyle changes are the most effective ways to lower the risk of high blood pressure. Angiotensin I converting enzyme (ACE-I) plays an important role in regulating blood pressure in the renin−angiotensin system [4,5]. ACE inhibitors are hypertensive drugs that prevent the activation of the renin-angiotensin system, but common blood pressure-lowering drugs have various undesirable side effects, such as loss of taste, chronic cough, renal impairment, and angioneurotic edema [6]. It is known that ACE inhibitors derived from natural foods are stable and have comparatively fewer side effects [7,8].

Seaweed species are rich in beneficial nutrients and have been widely used in human diets since ancient times, but they are rarely seen as part of the diet in Europe [9]. The global use and the importance of the red algae *Gracilaria* are well documented [10]. Macroalgae, a popular food used in many oriental countries, have been identified as a potential source for protein-based biofunctional ingredients [11]. *Gracilaria* has been cultivated in Taiwan since 1961. More than 30,000,000 kg of *Gracilaria* are produced annually in Taiwan [12]. The seaweed is inexpensive and also an important aquaculture species [13].

Seaweed is a sustainable protein source in the production of peptide-based drugs and functional foods to prevent disease, especially cardiovascular diseases and diabetes [14]. Protein content varies among seaweeds, with 3–15% in brown seaweeds, 9–26% in green seaweeds, and 10–47% in red seaweeds (dry weight), respectively [15]. However, the protein extraction can be hindered by the complex polysaccharide matrix of seaweed [16]. The most common water and solvent extraction methods have disadvantages, such as low selectivity, low extraction efficiency, solvent residues, and environmental pollution [17].

Alternatively, enzyme-assisted extraction offers many advantages, such as increased yield, high specificity, and protein retention [18]. It is also a fast, scalable, and environmentally friendly method [19]. Furthermore, certain physical and chemical parameters of the protein extraction rate can be optimized, such as hydrolysis time, pH, and temperature, by the appropriate selection of enzymes or their mixtures [20–22].

Although macroalgae have proven to be a rich source of compounds with ACE inhibition activity [23,24], no previous report has examined the ACE-inhibitory activity of peptides from *Gracilaria tenuistipitata*. Therefore, the objective of this study was to isolate ACE-inhibitory peptides from *G. tenuistipitata* protein hydrolysate. In addition, the antihypertensive action of the purified peptide with the most ACE-inhibitory activity *in vitro* was investigated *in vivo* by oral administration in spontaneously hypertensive rats (SHRs).

2. Materials and Methods

2.1. Algal Material

The red algae (Rhodophyta) *G. tenuistipitata* was collected in Kouhu, Yunlin County, Taiwan, in December 2016. Salt, sediment, organic debris, and epiphytes were removed by washing the algae in fresh water. Cleaned algal material was stored at 4 °C in plastic bags until further analysis. Finally, *G. tenuistipitata* was dried in a vacuum freeze-dryer (FD series, Panchum Scientific Corp., Kaohsiung, Taiwan), ground with a laboratory hammer mill to obtain a powder with a particle size below 1 mm, and stored at 4 °C in plastic bags for further analysis.

2.2. Chemicals

Hydrolysates were prepared using six commercial enzymes, including three carbohydrases (Viscozyme L, Celluclast 1.5 L FG, and Termamyl 120 L) and three proteases (Alcalase 2.4L FG, Neutrase 0.8 L, and Flavourzyme 500 MG), and Pepsin and Trypsin, all kindly provided by Sigma-Aldrich (St. Louis, MO, USA). The optimum hydrolysis conditions [25,26], characteristics, and origin of these enzymes are summarized in Table 1. HPLC-grade acetonitrile (ACN) and methanol as solvents, *N*-hippuryl-L-histidyl-L-leucine hydrate (HHL), and angiotensin ACE-I from rabbit lung were also obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

Table 1. Summary of the optimum hydrolysis conditions, characteristics, and sources of specific enzymes used in this study.

Enzyme	Optimum Conditions [a]		Characteristics	Source
	pH	Temperature (°C)		
Alcalase 2.4 L FG	8.0	50	Endo-peptidase	*Bacillus licheniformis*
Neutrase 0.8 L	6.0	50	Metallo-endoprotease	*Bacillus amyloliquefaciens*
Flavourzyme 500 MG	7.0	50	Endo-protease and exo-peptidase	*Aspergillus oryzae*
Viscozyme L	4.5	50	A multi-enzyme complex (containing arabanase, cellulase, β-glucanase, hemicellulose, and xylanase)	*Aspergillus aculeatus*
Celluclast 1.5 L FG	4.5	50	Cellulase	*Trichoderma reesei* ATCC 26921
Termamyl 120 L	6.0	60	Heat-stable α-amylase	*Bacillus licheniformis*

[a] Park et al. [25]; Sato et al. [26].

2.3. Preparation of the Gracilaria tenuistipitata Protein Hydrolysate

Samples were preheated at 90 °C for 10 min before enzyme addition. Hydrolysis conditions were a substrate concentration of 1 g/100 g ultrapure water and an enzyme-to-substrate ratio of 1:100 (w/w). The first step involved a sequential pre-digestion with two carbohydrase enzymes (Celluclast and Termamyl) at 50 °C, pH 4.5 for 4 h, then incubation with the third carbohydrase enzyme (Viscozyme) at 60 °C, pH 6.0 for 4 h. In the second step, three separate hydrolysis reactions were carried out with Alcalase, Neutrase, and Flavourzyme, respectively, at the optimal pH value at 50 °C for 2, 4, 6, 8, and 10 h. Afterward, the Pepsin was added at 37 °C, pH 2.0 for 2 h, and Trypsin was added at 37 °C, pH 7.5 for 4 h. At the end of all hydrolysis reactions, the reaction mixture was heated at 95 °C for 10 min to inactivate the enzymes. Once the solution had cooled to room temperature, it was centrifuged at 10,000× g (CF 15RX, Hitachi, Tokyo, Japan) for 20 min. The resultant supernatant was collected and dried in a vacuum freeze-dryer (FD series, Panchurm scientific corp., Kaohsiung, Taiwan). Hydrolysates generated using Alcalase, Neutrase, and Flavourzyme were named Crude *Gracilaria tenuistipitata* Alcalase (CGTA), Crude *Gracilaria tenuistipitata* Neutrase (CGTN) and Crude *Gracilaria tenuistipitata* Flavourzyme (CGTF), respectively.

Crude hydrolysates (CGTA, CGTN, and CGTF) were precipitated in three sequential steps at 0–30%, 30–60%, and 60–90% ammonium sulfate from an initial solution of 1 g of sample in 100 mL of pure water. At each step, the ammonium sulfate was completely dissolved, then the solution was left to equilibrate for 30 min and centrifuged at 8000× g at 4 °C for 30 min to obtain the salted-out sediments. The three separate sediments were ultimately combined.

The salted-out precipitate was dissolved in 100 mL of distilled water and filtered through a membrane with a 5 kDa MW cutoff (Millipore, Bedford, MA, USA). The filtrate was collected, lyophilized, and then purified and separated by fast protein liquid chromatography (ÄKTA, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using a gel filtration column (C16/900 16 × 900 mm, GE Biotech Bio-Sciences AB, Uppsala, Sweden). Before analysis, the lyophilizate was dissolved in deionized water and filtered through a 0.22 μm filter membrane. The injection volume was 2 mL, the flow rate was 0.5 mL/min, the collection volume per tube was 5 mL, and the mobile phase was deionized water with 0.02% NaN_3. The collected liquid was vacuum freeze-dried, and the powders obtained from CGTA, CGTN, and CGTF were called GTA, GTN, and GTF, respectively.

2.4. In vitro ACE-Inhibitory Activity

The ACE-inhibitory activity of the sample was measured as described by Wu and Ding [27] with some slight modifications. Briefly, 20 μL 5 mM HHL as the substrate was added to 10 μL of hydrolysate in 200 mM borate buffer containing 300 mM NaCl (adjusted to pH 8.3), and the mixture was pre-incubated at 37 °C for 10 min. The reaction was initiated by adding 20 μL of ACE (0.1 U/mL) in 200 mM borate buffer. The reaction was incubated statically at 37 °C for 30 min. The reaction was stopped by adding 20 μL 1 M HCl. HHL and its hydrolysis product, hippuric acid, were analyzed using an HPLC system (L-2130 pump, Hitachi, Tokyo, Japan) equipped with an ACE® 5μ 100Å C_{18} column (4.6 × 250 mm, Advanced Chromatography Technologies, Scottish, UK). The mixtures were separated by isocratic elution with a mobile phase composed of 50% methanol in 0.1% trifluoroacetic acid (TFA) at a constant flow rate of 0.8 mL/min for 20 min. Hippuric acid was detected by the UV detector at 228 nm (UV/Visible detector 2420, Hitachi, Tokyo, Japan). The ACE inhibition (%) was determined according to the following formula:

$$\text{ACE Inhibition (\%)} = [(\Delta A_{\text{Control}} - \Delta A_{\text{Sample}})/(\Delta A_{\text{Control}} - \Delta A_{\text{Blank}})] \times 100$$

$\Delta A_{\text{Control}}$ is the peak area of the buffer, ΔA_{Sample} is the peak area of the reaction mixture, and ΔA_{Blank} is the peak area when the stop solution was added before the reaction occurred.

2.5. Purification of ACE-Inhibitory Peptides

2.5.1. Gel Filtration Chromatography

The most active fractions among the ultrafiltered hydrolysates were applied to a C16/900 column (16 × 900 mm; Amersham Pharmacia Biotech AB, Uppsala, Sweden) packed with Sephadex G-25 resin, pre-equilibrated with distilled water containing 0.02% NaN_3. The flow rate was 0.5 mL/min and the elution peak was monitored at 280 nm. Fractions were collected by the fraction collector at 10 min intervals. Those showing ACE inhibitory activity were pooled and lyophilized.

2.5.2. Reversed-Phase HPLC Analysis

The fraction with the highest ACE-inhibitory activity was dissolved in distilled water and separated by reversed-phase HPLC (RP-HPLC) on a Discovery®BIO Wide Pore C18 column (10 mm × 250 mm; particle size, 5 µm; Sigma-Aldrich, St. Louis, MO, USA). The RP-HPLC analysis was performed in gradient elution mode with the mobile phase consisting of 0.1% TFA in distilled water (v/v) as eluent A and 0.1% TFA in ACN as eluent B at A: B ratios of 100:0 from 0 to 20 min, and 20:80 from 20 min to 80 min at a flow rate of 1.0 mL/min. The detector wavelength was 215 nm. The UV absorbance of the eluent was monitored at 280 nm. Fractions with ACE-inhibitory activity were collected and lyophilized, followed by identification of the amino acid sequences.

2.6. Peptide Sequencing Analysis

The fractions separated by RP-HPLC were entrusted to the Biotechnology Center, National Chung-Hsing University (NCHU, Taichung, Taiwan) for peptide sequence identification and molecular weight (MW) estimation by liquid chromatography (UltiMate 3000 RSLCnano LC Systems, Thermo Fisher Scientific, Middlesex, MA, USA) coupled with TripleTOF 6600 Q-TOF mass spectrometry (Applied Biosystems Sciex, Taipei, Taiwan) (LC-MS/MS). The column used was an Acclaim PepMap C18 (75 µm I.D.×25 cm nanoViper, 2 µm, 100 Å, Thermo Fisher Scientific, Middlesex, MA, USA). The RP-HPLC analysis was performed in gradient elution mode with the mobile phase consisting of 0.1% formic acid in distilled water (v/v) as eluent A and 0.1% formic acid in ACN as eluent B at A: B ratios of 95:5 from 0 to 4.5 min, and 65:35 from 4.5 min to 31 min, and 10:90 from 32 to 52 min, and 95:5 from 53 to 70 min at a flow rate of 300 nL/min.

2.7. Animal Experiment

2.7.1. Source and Breeding Conditions of Experimental Animals

Male spontaneously hypertensive rats (SHR) were used as experimental animals. Thirty male SHRs (100 ± 12 g) were purchased from Lasco Biotechnology Co., Ltd., Taipei, Taiwan and housed in a laboratory cage under controlled conditions: 25 ± 2 °C, 40−60% relative humidity, 12-h light/dark cycle from 07:00 to 19:00 h.

2.7.2. Animal Grouping

The GTN4H hydrolysate was freeze-dried, dissolved in distilled water, and tube-fed to SHRs. For the first 6 weeks, the experimental animals (n = 30) were given normal adult rat feed (Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water (deionized water). After acclimatization, the animals were randomly divided into 5 groups (n = 6 per group): normal diet group (ND) (1 mL/rat), high-dose group (200 mg/kg body weight (BW)) (1 mL/rat), medium-dose group (50 mg/kg BW) (1 mL/rat), low-dose group (10 mg/kg BW) (1 mL/rat), and positive control group (quinapril, 5 mg/kg BW) (1 mL/rat). During the experiment, the rats were fed by oral tube, and the blood pressure changes at 0 h, 2 h, 4 h, 6 h, 8 h, and 24 h after tube feeding were measured (as described in the next paragraph). The ND group was given water by oral gavage.

The blood pressure of rats was measured using the tail-cuff method with a blood pressure monitor (MK-1030, Muromachi Kikai Co., Tokyo, Japan). Before the measurement, the rat was fixed in an animal holder, placed in an insulation blanket at 40 °C for 3 min, and the tail was fitted into a sensor to measure blood pressure (cuff-pulse). Heart rate, systolic blood pressure (SBP), diastolic blood pressure, and mean blood pressure were obtained.

2.8. Statistical Analysis

Data were analyzed using the SPSS 20.0 (Statistical Product and Service Solutions, IBM, Armonk, NY, USA) statistical package software. Statistical significance was determined by one-way analysis of variance (ANOVA), followed by Duncan's test at $p < 0.05$.

3. Results and Discussion

3.1. Yield Content of the Enzymatic Extracts

The dried *G. tenuistipitata* sample contained approximately 16.58% ± 0.47% (data not shown) crude protein. Seaweeds have high protein content, especially red algae, which has 8–47 g/100 g on a dry basis [15], consistent with the present results. Furthermore, *G. tenuistipitata* was treated with cell wall polysaccharide-degrading enzymes to improve the protein yield, which was 48.25% ± 0.55% (data not shown) dry weight of the hydrolysates. Enzymatic hydrolysis increased the protein content 2.91 times.

The extraction of seaweed protein is often difficult due to the structural complexity and rigidity of the seaweed matrix, as well as the cross-linking of polysaccharides in the cell wall through disulfide bonds [19]. In a previous study of various brown seaweeds, the yield of the enzymatic extracts of seaweeds ranged from 30.3% ± 1.3% to 92.9% ± 2.2% compared with 18.7 % ± 0.3% to 50.8% ± 1.3% for water extracts [28]. Seaweed cell wall composition can vary among phyla and species and, therefore, the right choice of carbohydrase(s), together with the optimization of operating conditions (enzyme: substrate ratio (E: S), temperature, pH) of individual enzymes or cocktails must be proved prior to large-scale extraction to maximize protein recovery [29]. Thus, the optimization strategies in enzyme-assisted extraction are crucial.

3.2. In Vitro Analysis of ACE-Inhibitory Activity of Gracilaria tenuistipitata Protein Hydrolysate

The *G. tenuistipitata* protein hydrolysate generated using different enzymes was prepared at 10 mg/mL and reacted with HHL and ACE enzyme (Figure 1). All hydrolysates had potential to inhibit ACE. Results showed that the *in vitro* ACE-inhibitory activity of the hydrolysates in the CGTF, CGTN, and CGTA groups was 64.7~68.0%, 78.4~82.7%, and 45.9~51.8%, respectively; GTN4H had comparatively better ACE-inhibitory activity, so GTN4H was used for subsequent separation and purification.

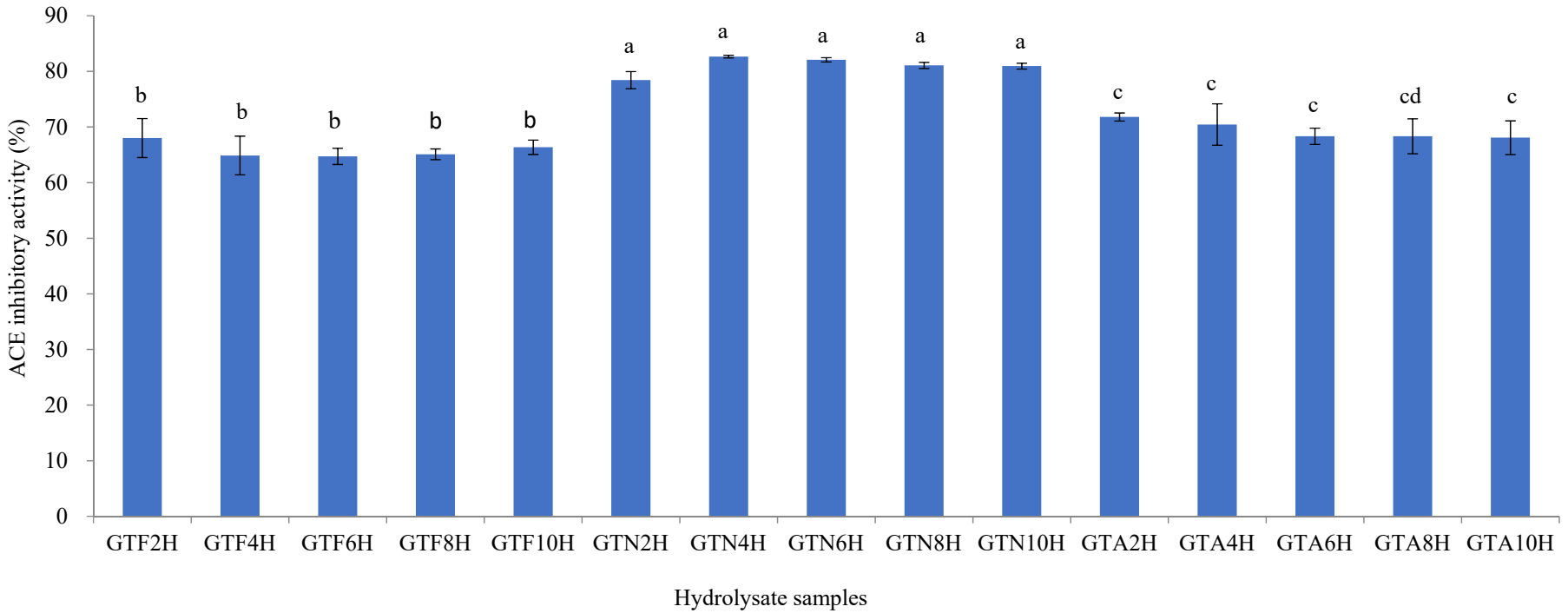

Figure 1. *In vitro* ACE-inhibitory activity of 10 mg/mL of protein hydrolysates from groups GTF, GTN, and GTA. Data represent mean ± SD (n =3). The different lower-case letters are significant differences ($p < 0.05$).

3.3. Animal Experiments

The GTN4H hydrolysate was freeze-dried, dissolved in distilled water, and tube-fed to SHRs at a low dose (10 mg/kg BW), medium dose (50 mg/kg BW), and high dose (200 mg/kg BW) to explore the effect of GTN4H hydrolysate on blood pressure regulation. The control group was tube-fed with distilled water, and the drug group was administered 5 mg/kg quinapril.

Figure 2 shows the changes in SBP due to GTN4H hydrolysate and quinapril in SHRs. The SBP of the high-dose GTN4H hydrolysate (200 mg/kg BW) group increased with time, and the blood pressure decreased slowly. It was found that the blood pressure reduction was positively correlated with the dose, but this effect differed among the doses, and the SBP of each group decreased the most at 8 h. Only that of the drug group dropped the most at 6 h and then returned to the original SBP range at 24 h.

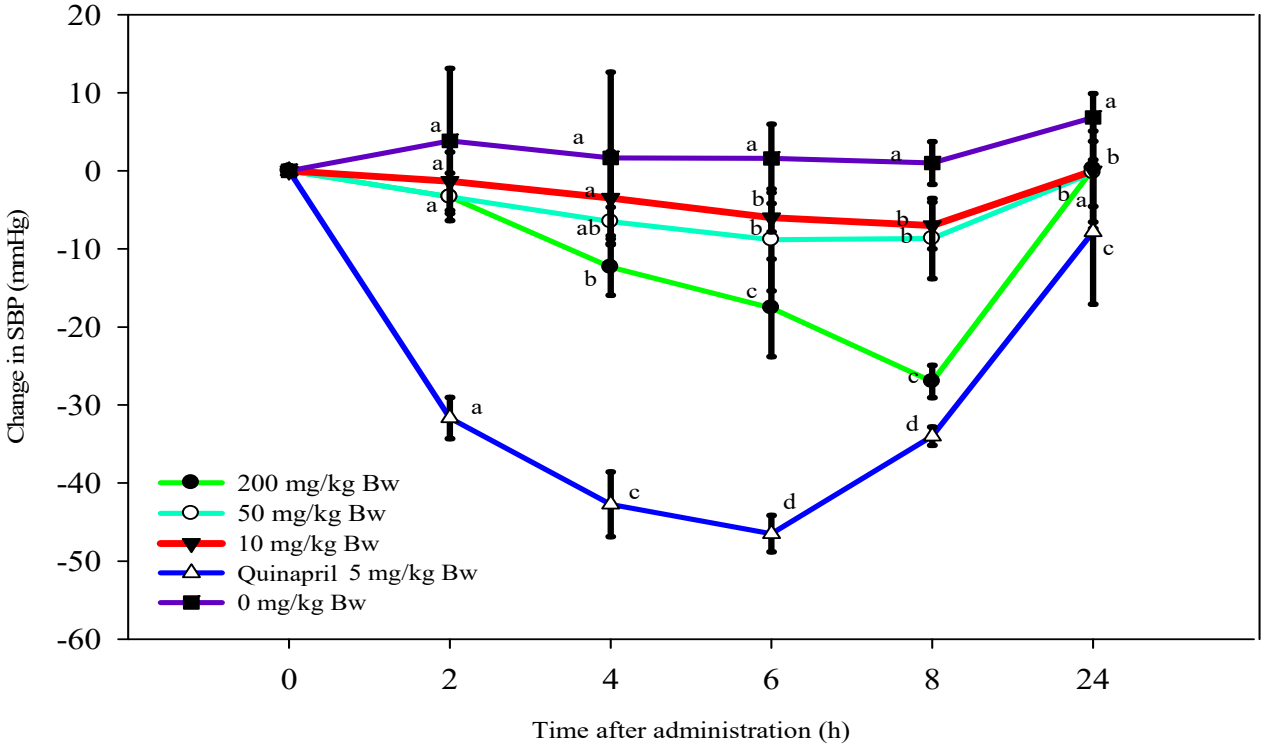

Figure 2. *In vivo* antihypertensive effect of GTN4H and quinapril in SHRs. Systolic blood pressure for 24 h after administration (■ 0, ▼10, ○ 50, ● 200 mg/kg BW) and compared with Quinapril (△ 5 mg/kg BW). Values are mean ± SD, $n = 6$. Different lowercase letters within the same time after administration indicate significant differences ($p < 0.05$).

It was found that the blood pressure reduction was positively correlated with the dose, but this effect differed among the doses. For the drug group, SBP decreased from 0 to 6 h and then increased, returning to the original SBP (0 h) range at 24 h.

According to the animal experiment results of the Taiwan Ministry of Health and Welfare for the "Evaluation method of auxiliary blood pressure regulation of healthy food" [30], the test substance decreased SBP by 20 mmHg ($p < 0.05$). The maximum dose of 200 mg/kg BW in this experiment decreased SBP by about 25 mmHg, so it was preliminarily determined that 200 mg/kg BW of GTN4H is a potential source of ACE-inhibitory/antihypertensive peptides.

In a previous experiment, the antihypertensive effect of the purified peptide was also evaluated in SHRs by measuring the change in SBP at 0 h, 2 h, 4 h, 6 h and 8 h after single oral administration at a dose of 10 mg/kg BW. The peptide reduced SBP significantly, and the activity was maintained for 6 h. The maximum decrements in SBP attributed

to the peptides and quinapril (positive control) were 22.8 mmHg and 35.4 mmHg at 4 h, respectively [31], suggesting that the *G. tenuistipitata*-derived peptides could be used as alternatives to synthetic ACE inhibitors.

3.4. Gel Filtration Chromatography of GTN4H

Gel filtration chromatography is an effective separation technique based on its relative ability to penetrate into a suitable stationary phase or chromatographic resin. The hydrolysate GTN4H with high ACE activity was first filtered through a 5 kDa cutoff membrane by ultrafiltration. The filtrate was collected, lyophilized, and then purified and separated at 100 mg/mL by Sephadex G-25 gel filtration chromatography. As shown in Figure 3, GTN4H was separated into three subfractions-GTN4H-A (160–280 min), GTN4H-B (290–340 min), and GTN4H-C (350–440 min)-on the Sephadex G-25 column and after separate collection and lyophilization, the ACE-inhibitory activity of each purified fraction was investigated. ACE-inhibitory activities of 69.74% ± 0.80%, 24.04% ± 0.45%, and 59.95% ± 1.29% were recorded for GTN4H-A, GTN4H-B, and GTN4H-C, respectively (Table 2), with GTN4-A exhibiting the highest ACE activity. A Discovery®BIO Wide Pore C18 column (250 × 10 mm) was used for the second purification.

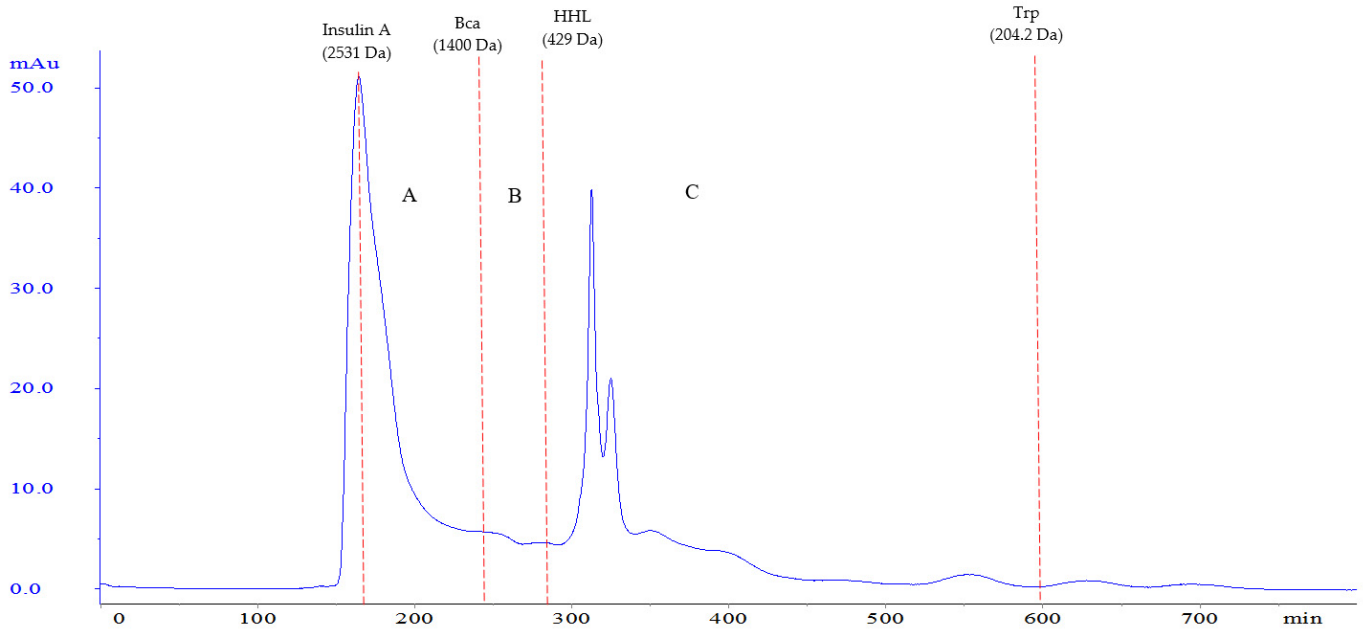

Figure 3. Sephadex G-25 gel filtration chromatography and molecular mass distribution of GTN4H. Insulin A (2531 Da), Bca: bacitracin zinc salt (1400 Da), HHL: hippuryl-histidyl-leucine (429 Da), Trp: tryptophan (204.2 Da). Time: A (160–280 min), B (290–340 min), C (350–440 min).

Table 2. *In vitro* ACE-inhibitory activity of GTN4H protein hydrolysate following fast protein liquid purification (fractions A, B, and C) and further purification of fraction A by RP-HPLC (fractions A1-A5).

Fraction	ACE Inhibition (%)
A	69.74 ± 0.80
B	24.04 ± 0.45
C	59.95 ± 1.29
A1	N.D.
A2	N.D.
A3	N.D.
A4	95.4 ± 0.6
A5	5.00 ± 1.8

N.D.: not detectable.

In a previous experiment, a hydrolysate of the green algae *Chlorella ellipsoidea* was ultrafiltered into three fractions of MW < 5, 5–10, and >10 kDa [30]. The <5 kDa fraction exhibited the strongest ACE-inhibitory activity and had an IC_{50} value of 0.883 mg/mL. This finding is in concurrence with Jung et al. [32] and our results showing that the lower MW fraction displays more potent ACE-inhibitory activity than the higher MW fraction.

Based on the molecular weight (log MW) and corresponding retention time of each standard fraction in the spectra (Figure 3), a regression curve of $y = -153.9x + 4849$ could be obtained, and fraction A (collection tubes #15–21) had an MW distribution of 1486–2531 Da. This size range covers the MW of the active A peptide (1776 Da).

3.5. Second Purification

The GTN4H-A fraction was injected at 1 mg/mL into a Discovery®BIO Wide Pore C18 semi-preparative column (250 × 10 mm) and eluted with a mobile phase of 0.1% TFA in deionized aqueous solution (0–20 min) and a linear gradient of 0–60% ACN solution containing 0.1% TFA (20–80 min). Five major peaks (A1 to A5) could be observed (Figure 4). The sharpest peaks (A3 and A4) eluted with about 30–40% ACN, indicating that the amino acid composition of these peptides might be relatively more hydrophobic. A4 exhibited the best ACE-inhibitory activity, showing an inhibition rate of 95.4% (Table 2). Hence, the A4 peptide with strong ACE-inhibitory activity and a high amine content was analyzed and sequenced.

3.6. Sequence Identification of A4

LC-MS/MS was used to identify the molecular mass and amino acid sequence of the peptide corresponding to peak A4. The total ion chromatogram is shown in Figure 5. A peptide of 17 amino acids in length with a primary sequence of MQDAITSVINAADVQGK and MW of 1776 Da was calculated (Mascot version 2.3.02, Matrix Science, Boston, MA, USA). Based on the number of tubes corresponding to the standard product in the figure, a regression curve of $y = -153.9x + 4849$ could be obtained, and the number of A partition was 15–21 tubes, and the MW distribution was 1486–2531 Da. This size range covered the MW of the active A4 peptide (1776 Da). Most peptides with effective ACE-inhibitory activity contain 2–20 amino acids [33], and the number of amino acids in peptide A4 was also in this range.

The biological activities of protein hydrolysates and peptides are directly affected by the composition of amino acids. Seaweed-derived proteins, peptides, and amino acids are promising ingredients in food, food supplements, feed and raw materials and in the development of new drugs because of their potential therapeutic effects [34]. Thus, the results of this study encourage further research on the mechanism of action, clinical efficacy, and potential toxicity of GTN4H-A and the other pharmacological benefits of seaweed components.

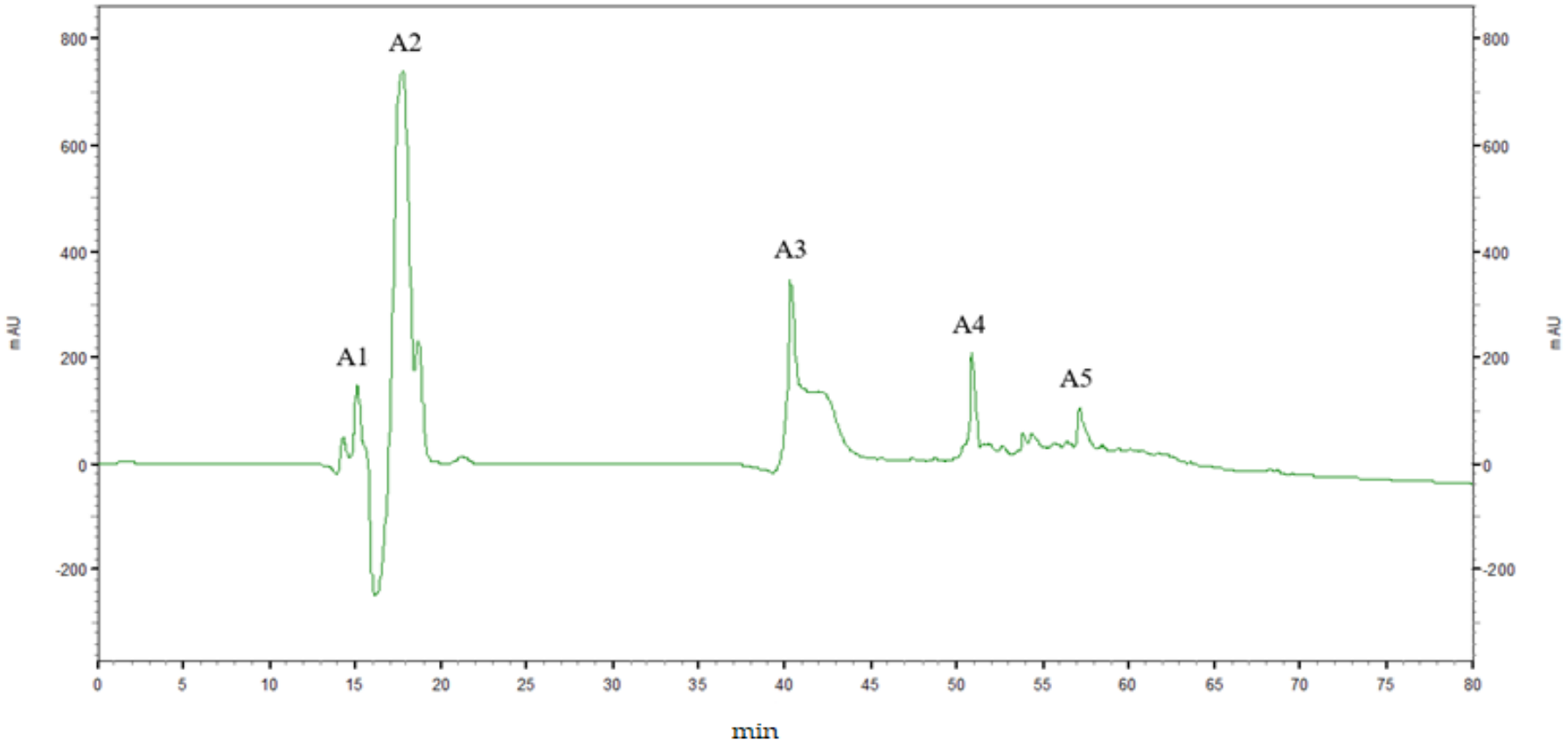

Figure 4. Elution profile of GTN4H-A by C18 reversed-phase HPLC. (Flow rate of 1 mL/min with a 0–80% linear gradient of acetonitrile in distilled water containing 0.1% TFA).

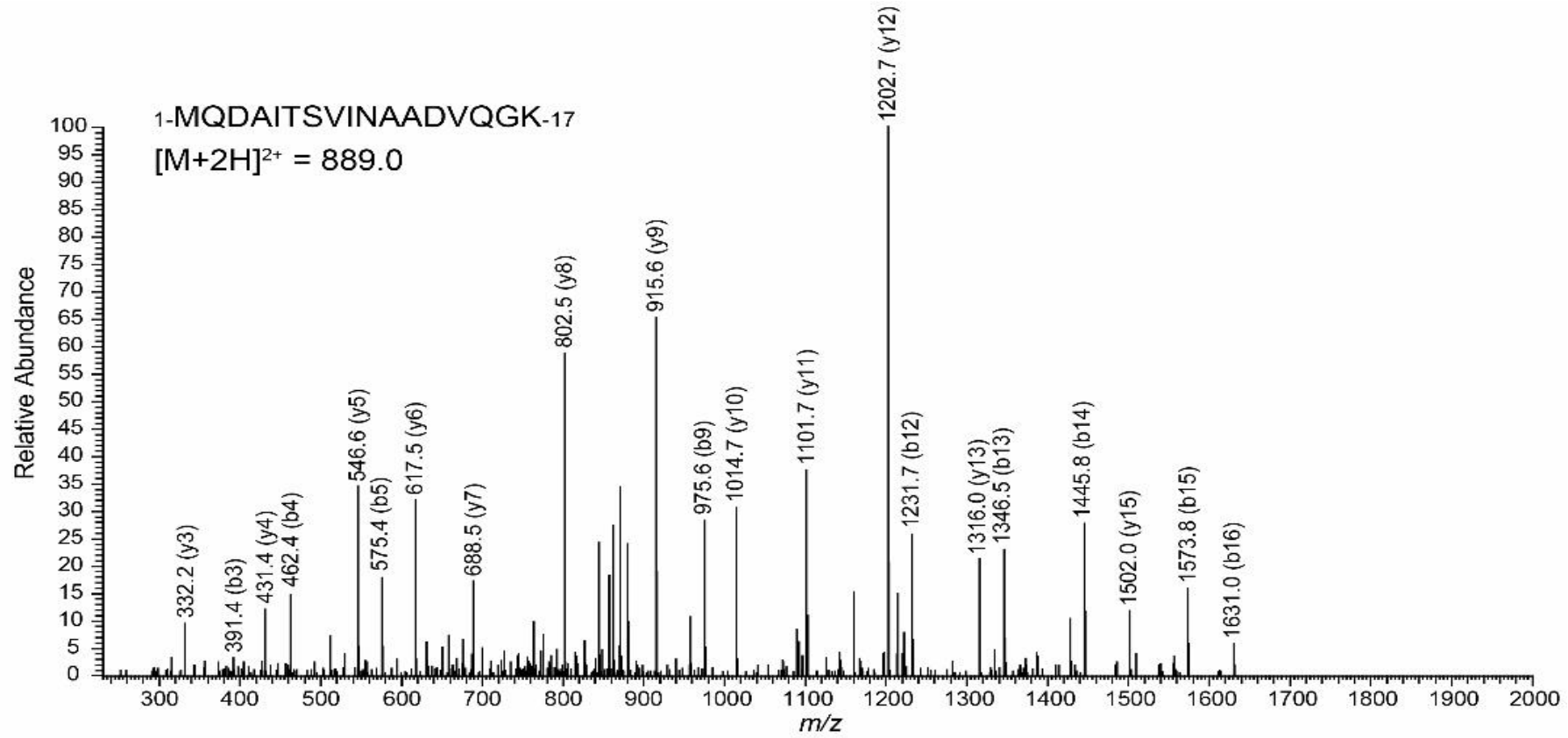

Figure 5. Identification of molecular mass and amino acid sequence of the purified peptide (fraction A4) by LC-MS/MS.

4. Conclusions

The ACE inhibition rate of GTN4H was 82.7%. In addition, it lowered the blood pressure in SHRs (8 h after feeding a single dose of 200 mg/kg BW, the blood pressure dropped by more than 20 mmHg). After purification by gel filtration chromatography, fraction A4 exhibited the better ACE inhibition effect and was further purified by C18 RP-HPLC. We isolated and characterized a novel ACE-inhibiting peptide (MQDAITSVINAADVQGK) from the hydrolysate of *G. tenuistipitata* protein with Neutrase. The MW was 1776 Da. In conclusion, our research helps increase the value of *G. tenuistipitata* protein utilization and provides a theoretical basis for the development and application of functional foods in the future.

Author Contributions: Y.-J.S.: Conceptualization, Investigation, Data curation, Validation, Writing-original draft, Methodology. H.-J.L.: Investigation, Project administration, Supervision, Validation. J.-I.Y.: Data curation, Project administration, Supervision, Validation, Writing-review and editing. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data used to support the findings of this study are included in the article.

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Conflicts of Interest: The authors declare no conflict of interest.

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