Original Article

Enzymatic Protein Hydrolysates, and Ultrafiltered Peptide Fractions from Two Molluscs: *Tympanotonus fuscatus* var. *radula* (L.) and *Pachymelania aurita* (M.), with Angiotensin-I-Converting Enzyme Inhibitory and DPPH Radical Scavenging Activities

Abraham Abbey Paul, Queensley Adesuwa Eghianruwa[1], Olayemi Grace Oparinde, Adetunji Samuel Adesina, Omolaja Osoniyi

Department of Biochemistry and Molecular Biology, Obafemi Awolowo University, Ile-Ife, [1]Department of Biochemistry, University of Uyo, Uyo, Nigeria

Abstract

Context: Multifunctional food protein-derived peptides attract a great deal of research interest due to their health-promoting benefits. Particularly, peptides that have both antihypertensive and antioxidant properties are desired, since both effects can be synergistic in prevention of cardiovascular diseases. **Aim:** The aim of this study was to investigate the angiotensin-I-converting enzyme (ACE) inhibitory and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities of two species of the Nigerian periwinkles: *Pachymelania aurita* and *Tympanotonus fuscatus*. **Methods:** The ACE inhibitory and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities of simulated gastrointestinal digestion (SGID) hydrolysates and ultrafiltered (UF) fractions of *T. fuscatus* var. *radula* and *P. aurita* were determined. Human SGID of the protein extracts of *T. fuscatus* and *P. aurita* was carried out using pepsin, trypsin, and chymotrypsin, and the hydrolysates were fractionated into two by centrifugal ultrafiltration. The ACE inhibitory and DPPH radical scavenging activities of the crude hydrolysates and UF fractions were tested. The UF permeates were observed to have relatively higher activities and was subjected to gel filtration chromatography on Sephadex G-50. The chromatographic fractions showed absorbance at 215, 225, and 280 nm and were assayed for DPPH radical scavenging activity. **Results:** The inhibitory effect of the fractions on ACE activity was reported as the minimum concentration of extract that caused 50% of the inhibition (IC_{50}), where the IC_{50} values of *P. aurita* UF permeate and *P. aurita* UF retentate were 65.2 ± 6.4 and 301.9 ± 59.1 µg/ml, respectively, and that of *T. fuscatus* UF permeate (TFUFP) and *T. fuscatus* UF retentate were 54.93 ± 2.83 and 291.7 ± 8.6 µg/ml, respectively. **Conclusion:** This study suggests the potential health benefits of consuming *T. fuscatus* var. *radula* and *P. aurita* in health maintenance.

Keywords: *Angiotensin-I-converting enzyme inhibitory peptides, bioactive peptide, DPPH radical scavenging activity, hydrolysate, marine functional foods, Nigerian periwinkles, Pachymelania aurita, Tympanotonus fuscatus*

Introduction

Hypertension or high blood pressure is an important public health problem affecting more than 1.3 billion people worldwide.[1] It is a risk factor for cardiovascular diseases, stroke, and renal damage and reported to cause 17 million deaths annually.[2] The renin–angiotensin–aldosterone system (RAAS) is a major pathway that regulates the blood pressure. RAAS is an enzyme-catalyzed pathway that is capable of producing compounds that can regulate blood pressure. Renin and angiotensin-I-converting enzyme (ACE) are the key enzymes of RAAS. ACE is a multifunctional metalloenzyme that acts to increase the blood pressure by producing angiotensin II (a vasoconstrictor) and inactivating bradykinin (a vasodilator).[3] Thus, inhibition of ACE has been considered as a key target for the treatment of hypertension. Indeed, most antihypertensive drugs are inhibitors of ACE. Most of these drugs are well tolerated by patients, but their prolonged administration is reported to cause undesirable side effects.[4] There is, therefore, a continuing search for safer and milder ACE inhibitors from natural sources.

Furthermore, the significant role of oxidative stress in the initiation and progression of cardiovascular diseases is

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Address for correspondence:
Dr. Omolaja Osoniyi, Department of Biochemistry and Molecular Biology, P.M.B. 013, Obafemi Awolowo University Ile-Ife, Nigeria.
E-mail: rosoniyi@oauife.edu.ng

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being highlighted.[5] Particularly, angiotensin-II-induced oxidative stress is seen as both a cause and consequence of hypertension.[5] Thus, the role of antioxidant therapy in the management of hypertension is garnering research attention.

This dual activity (ACE inhibitory and antioxidant) is exhibited by certain intracellular antioxidants, peptides derived from egg-white proteins, and caseins.[6] The tendency of having such multifunctional activity in a single agent seems to be higher in food than in synthetic drugs, and this has fuelled research interest in functional foods, which can be taken as drugs.

Functional food is broadly defined as any food that enhances health upon digestion. Among naturally occurring compounds in functional foods, bioactive peptides (peptides having between 2 and 20 amino acid residues with amino acid sequence[s] that could exert physiological effect in the body when they are released) are taking center stage.

Marine-derived bioactive peptides have been subject of extensive research in functional food due to their broad spectrum of bioactivities such as ACE inhibition and antioxidant activity[7] among other activities. Two gastropods of the phylum Mollusca, *Pachymelania aurita* and *Tympanotonus fuscatus* var. *radula* (commonly called Nigerian periwinkles), belong to a group of soft-bodied molluscs commonly found in the coastal regions of West Africa. They are frequently consumed in significant quantity as a protein staple in the South-South region of Nigeria. Recently, the antimicrobial[8] and antiproliferative[9] potentials of peptide fractions from these two species of Nigerian periwinkles were reported. However, there is no available data on the ACE inhibitory and radical scavenging activities of peptides derived from proteins of *T. fuscatus* and *P. aurita*. The current study, therefore, investigated the ACE inhibitory and radical scavenging activities of the simulated human gastrointestinal digestion hydrolysates and fractions of the two species of Nigerian periwinkles: *T. fuscatus* and *P. aurita*.

Materials

ACE (0.1 UN, ≥2 units/mg), hippuryl-histidyl-leucine (HHL), pepsin from porcine gastric mucosa (≥500 U/mg), α-chymotrypsin from bovine pancreas (≥40 units/mg protein), porcine trypsin (1000–2000 BAEE units/mg solid), and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (USA), and other reagents were of analytical grade.

Methods

Sample collection

Live *T. fuscatus* var. *radula* and *P. aurita* were purchased from the Oron Beach Market, Oron, Akwa Ibom State, Nigeria (GPS coordinates: 4°49′37.6′′ N 8°14′04.4′′ E).

Sample preparation and protein extraction

Flesh and hemolymph of *P. aurita* and *T. fuscatus* (500 g) were separately homogenized in 0.1 M phosphate-buffered saline (pH 7.2) in a ratio of 1:3 (w/v), using an electric blender. The homogenates were swirled and left at 4°C to extract for 24 h with intermittent swirling (typically at 2-h interval) after which the homogenates were centrifuged using a tabletop centrifuge (Microfield Instruments, England, model 800D) at 3500 rpm for 15 min. The supernatants were collected and the pellets were re-extracted in the same manner as described. The supernatants of each mollusc were pooled and lyophilized to give the crude protein extract of *P. aurita* and crude extract of *T. fuscatus*.

Simulated gastrointestinal digestion

To determine the ability of the peptides to survive gastrointestinal degradation, simulated gastrointestinal digestion (SGID) was carried out according to a method described by Mune Mune *et al*.[10] Crude protein extracts were reconstituted in 0.01N HCl (25 mg/ml of solid) and the pH was adjusted to 2.1 and pepsin was added at an enzyme-substrate ratio (ESR) of 8% (w/w) and incubated in a water bath at 37°C for 60 min. The reaction medium was then adjusted to pH 7.5 after which trypsin and chymotrypsin were added at the ESR of 4 and 2% (w/w), respectively, and incubated for another 90 min and then boiled at 98°C for 10 min. The hydrolysates were centrifuged and the supernatant was collected as the crude hydrolysate of *P. aurita* and crude hydrolysate of *T. fuscatus*. Samples were kept at −20°C until required.

Fractionation of angiotensin-I-converting enzyme inhibitory peptides from the simulated gastrointestinal digestion hydrolysates

The SGID hydrolysate was subjected to ultrafiltration through a hydrophilic 3 kDa cutoff membrane centrifugal filter according to the manufacturer's instruction. The permeates (peptides ≤3 kDa) were collected and hereafter referred to as *P. aurita* ultrafiltered (UF) permeate (PAUFP) and *T. fuscatus* UF permeate (TFUFP). The retentates (peptides >3 kDa) were referred to as *P. aurita* UF retentate and *T. fuscatus* UF retentate and were kept at −20°C until required. One unit of ACE will produce 1.0 μmol of hippuric acid from HHL per min in 100 mM Tris HCl, 300 mM NaCl, and 10 μM $ZnCl_2$ at pH 8.3 at 37°C, where the specific activity of ACE is 2 unit/mg.

Determination of protein concentration of hydrolysate and fractions

The protein content was determined by the protein-binding assay method of Bradford as described by Mæhre *et al*.[11] using bovine serum albumin (BSA) as standard protein (containing 0–10 μg/ml of BSA prepared in 0.02 M phosphate buffer, pH 7.2) and Coomassie brilliant blue as dye.

Gel filtration chromatography of 3 kDa ultrafilter permeate

PAUFP and TFAFP were purified using size-exclusion chromatography according to a method described by Moayedi *et al.*[12] The permeate (1.8 ml) was loaded onto a Sephadex G-50 chromatography column (7.0 cm × 40 cm) and eluted with 0.01 N HCl at a flow rate of 30 ml/h. 2.5 ml fractions were collected (total of 150 fractions) and the elution was monitored at 215, 225, and 280 nm and the fractions showing absorbance at these wavelengths were pooled and screened for DPPH radical scavenging activity.

Angiotensin-I-converting enzyme inhibitory activity

The ACE inhibitory activity was measured according to the method of Cushman and Cheung described by Wu *et al.*[13] 10 µL of samples (either crude hydrolysate or UF fractions) of *P. aurita* and *T. fuscatus* (of different concentrations prepared in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3) and 45 µl HHL (6.5 mM HHL in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3) was preincubated at 37°C for 5 min and then incubated with 15 µl of ACE in 0.1 M borate buffer (containing the 0.3 M NaCl, pH 8.3) at 37°C for 30 min. The reaction was stopped by adding 85 µl of 1 M HCl to the reaction mixture. The blank and control were conducted in the absence of enzyme and inhibitor, respectively. The hippuric acid released was extracted into ethyl acetate and centrifuged. The ethyl acetate layer was then collected and evaporated at 100°C. The residue was dissolved in distilled water and its absorbance was read at 228 nm. The inhibition was calculated as follows:

$$\text{ACE inhibition activity (\%)} = [(C - S)/(C - B)] \times 100$$

where C, S, and B are the absorbance of control (without sample), the absorbance in the presence of different concentrations of sample, and the absorbance of blank, respectively.

DPPH radical scavenging activity

The radical scavenging ability of the crude extracts and their Hydrolysates was determined using the stable radical 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) as described by Brand-Williams *et al.*[14]

The percent of inhibition was calculated in following way:

$$\text{Inhibition (\%)} = ([A_{blank} - A_{sample})/A_{blank}) \times 100$$

where A_{blank} is the absorbance of the blank reaction and A_{sample} is the absorbance in the presence of the test compound. Sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extract concentration.

Results

Characterization of angiotensin-I-converting enzyme inhibitory capacity of the hydrolysates

Captopril, a standard, gave an inhibitory activity which was significantly different ($P < 0.05$) from other inhibitory effects exhibited by the crude, hydrolysate, and fractions of the two samples of molluscs, as shown in Figure 1a. Within the same species, PAUFP and TFUFP displayed the highest inhibitory effects in *P. aurita* and *T. fuscatus,* respectively.

Table 1: DPPH radical scavenging activity of crude, simulated gastrointestinal digestion hydrolysates, and ultrafiltration fractions of *Tympanotonus fuscatus* and *Pachymelania aurita*

Treatment	IC_{50} (µg/ml)	
	Pachymelania aurita	***Tympanotonus fuscatus***
Crude	120.55	97.11
SGID hydrolysate	103.25	342.38
Ultrafiltration fraction (≤3 kDa)	34.83	24.69
Ultrafiltration fraction (>3 kDa)	205.74	53.85
Standard (ascorbic acid)	5.30	

SGID: Simulated gastrointestinal digestion

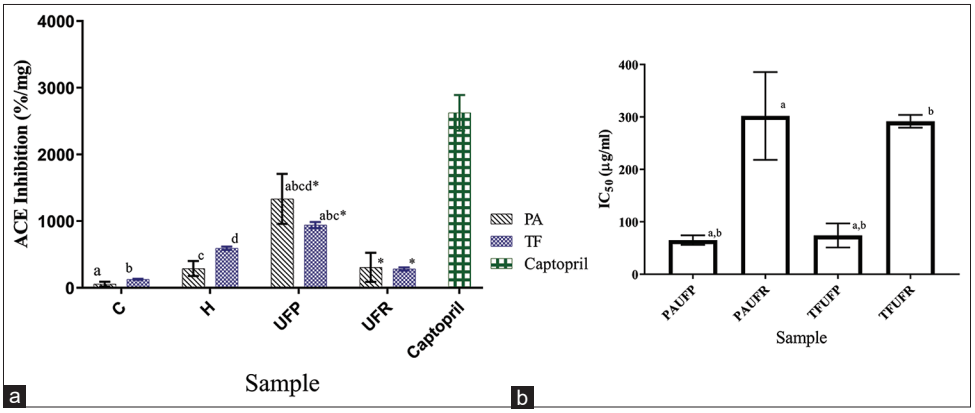

Figure 1: Angiotensin-I-converting enzyme inhibition of the crude extracts, hydrolysates, and fractions, (b) expressed as %/mg of *Pachymelania aurita* and *Tympanotonus fuscatus* and the ultrafiltered fractions, (a) expressed as IC_{50} values are expressed as mean ± standard deviation of duplicate determination. Bars with either same letters or asterisk (*) are significantly different from each other at $P < 0.05$. Where PA, TF, C, H, UFP, and UFR represent *Pachymelania aurita, Tympanotonus fuscatus*, crude, hydrolysate, ultrafiltered permeate, and ultrafiltered retentate, respectively

The concentrations of the fractions that caused 50% inhibition of ACE activity (IC_{50}) are presented in Figure 1b.

The elution profiles of *Pachymelania aurita* ultrafiltered permeate and *Tympanotonus fuscatus* ultrafiltered permeate on the gel filtration chromatography

The elution profile on size-exclusion chromatography using Sephadex G-50 of PAUFP and TFUFP is shown in Figures 2 and 3, respectively. The fractions showing absorbance at wavelengths 215, 225, and 280 nm were pooled together into 5 fractions for *P. aurita* and 4 fractions for *T. fuscatus*. The absorbance at 280 nm is a function of amino acid residue contained in the peptide, while absorbance at 215 and 225 nm is due to the presence of peptide bonds.

DPPH radical scavenging activity of the fractions of *P. aurita* and *T. fuscatus*

The DPPH radical scavenging activity of the pooled fractions obtained from gel filtration chromatography of PAUFP and TFUFP is presented in Figures 2 and 3, respectively.

Low molecular weight peptide fractions of *T. fuscatus* and *P. aurita* displayed a higher DPPH radical scavenging activity than the higher molecular weight counterparts and the crude hydrolysates as shown in [Table 1].

Table 2 summarizes the purification steps of the enzymatic hydrolysates of *P. aurita* and *T. fuscatus*

Discussion

The observed dramatic increase in ACE inhibitory effect of the hydrolysate and particularly the low molecular weight (PAUFP and TFUFP) could be due to the accessibility of the small peptides to the active site residues, thereby allowing for the inhibitory interactions. It is reported that low-molecular-weight peptides are better inhibitors of ACE.[15] More and more novel ACE inhibitory peptides are being isolated from marine sources,[16] most of which are low molecular weight (usually ≤5 kDa). ACE inhibitory peptides obtained from tilapia fish hydrolysate were demonstrated to be stable over a temperature range of 20°C–121°C.[17] This observation could be as a result of the fact that peptides (short amino acid sequences) do not possess secondary conformation, but the amino acids are held together by a strong covalent (peptide) bond. The role of the position of amino acid residue (amino acid sequence) in determining the extent and mechanism of ACE inhibition was aptly illustrated by Ono *et al.*[18]

The highest radical scavenging activity was displayed by low-molecular-weight UF fractions, and when these fractions were further subjected to the gel filtration chromatography, there was a dramatic increase in the activity. In general, small-molecular-weight bioactive peptides are desirable to elicit *in vivo* physiological effect because of their structural flexibility and high membrane permeability. Low-molecular-weight (≤3 kDa) DPPH radical scavenging peptide was characterized from duck

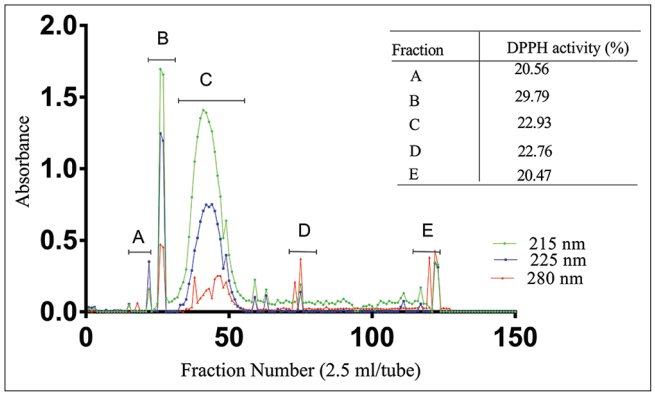

Figure 2: Gel filtration (Sephadex G-50) chromatogram of *Pachymelania aurita* ultrafiltered permeate. The flow rate was 30 ml/h and 2.5 ml was collected per tube in a chromatographic column having (7.0 cm × 40 cm) dimension

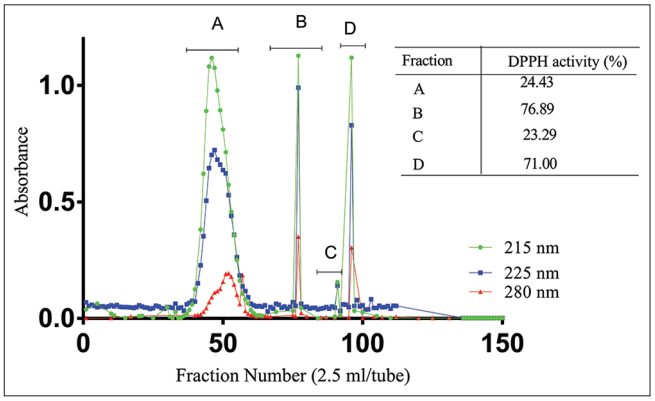

Figure 3: Gel filtration (Sephadex G-50) chromatogram of *Tympanotonus fuscatus* ultrafiltered permeate. The flow rate was 30 ml/h and 2.5 ml was collected per tube in a chromatographic column having (7.0 cm × 40 cm) dimension

Table 2: Purification table for *Tympanotonus fuscatus* and *Pachymelania aurita*

Purification step	Total protein (mg)		ACE inhibition (%)		Specific activity (%/mg)		Yield (%)	
	TF	PA	TF	PA	TF	PA	TF	PA
Crude	4.83	5.31	44.75	21.77	9.27	4.10	100	100
Hydrolysate	1.85	2.06	79.58	43.06	42.92	20.89	38.35	38.85
UF permeate	0.63	0.40	43.29	40.15	68.07	100.63	13.16	7.52
UF retentate	1.71	1.74	58.54	53.22	34.15	30.64	35.46	32.74

Where TF and PA are *Tympanotonus fuscatus* and *Pachymelania aurita*, respectively. ACE: Angiotensin-I-converting enzyme, UF: Ultrafiltered

breast protein hydrolysates.[19] However, the size of peptide is an important but not an entirely sufficient condition for biological activity. Other factors such as amino acid composition and sequence can also be influential in the determination of biological activity. The effect of amino acid sequence and composition might be partly responsible for the seemingly higher biological activity obtained from the hydrolysate and fractions of *T. fuscatus* than in the counterpart species, *P. aurita,* which was subjected to the same proteolytic and fractionation treatments.

The gel filtration chromatograms of the low-molecular-weight fractions of *T. fuscatus* and *P. aurita* show the existence of at least six groups of peptide. The marked increase in DPPH radical scavenging activity in the pooled fractions following the gel filtration chromatography suggests the fact that low-molecular-weight peptides are potent antioxidants. Typically, peptides with ACE-inhibitory activities are reported to have DPPH radical scavenging activity as well. This is because residues that act to scavenge DPPH radicals tend to have ACE inhibitory activity.[20]

Conclusion

The demonstrated pharmacological properties of these low-molecular-weight peptides from *T. fuscatus* and *P. aurita* are remarkable indeed. The shelled molluscs of the African continent are poorly studied in this regard. The findings signal the potential for even more significant pharmacological properties in these and similar molluscs.

Ethical clearance

Permission to carry out the study was granted by the university research committee (URC), Obafemi Awolowo University, Ile-Ife Nigeria.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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