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ACE Inhibitory Peptides from *Bellamya bengalensis* Protein Hydrolysates: In Vitro and In Silico Molecular Assessment

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Abstract: *Bellamya bengalensis* muscle meat is known for ethnopharmacological benefits. The present study focuses on the identification of ACE inhibitory peptides from the proteolytic digests of muscle protein of *Bellamya bengalensis* and its underlying mechanism. After ultrafiltration of 120 min alcalase hydrolysates ($BBPH_{A120}$) to isolate the small peptide fraction (<3 kDa), in vitro ACE inhibitory activity was analyzed. The IC_{50} value of the 120 min hydrolysate ultrafiltered fraction was 86.74 ± 0.575 µg/mL, while the IC_{50} of lisinopril was 0.31 ± 0.07 µg/mL. This fraction was assessed in a MALDI-ToF mass spectrometer and five peptides were identified from the mass spectrum based on their intensity (>1 × 10^4 A.U.). These peptides were sequenced via *de novo* sequencing. Based on the apparent hydrophobicity (%), the IIAPTPVPAAH peptide was selected for further analysis. The sequence was commercially synthesized by solid-phase standard Fmoc chemistry (purity 95–99.9%; by HPLC). The synthetic peptide (IC_{50} value 8.52 ± 0.779 µg/mL) was used to understand the thermodynamics of the inhibition by checking the binding affinity of the peptide to ACE by isothermal titration calorimetry compared with lisinopril, and the results were further substantiated by in silico site-specific molecular docking analysis. The results demonstrate that this peptide sequence (IIAPTPVPAAH) can be used as a nutraceutical with potent ACE inhibition.

Keywords: gastropod snail; angiotensin-converting enzyme-inhibitory activity; alcalase; lisinopril; isothermal titration calorimetry; uncompetitive inhibition; cooperative ligand binding; site-specific molecular docking

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1. Introduction

Several naturally grown traditional foods are known to have precise therapeutic roles against the etiology of a specific disease figure [1]. Recent foodomics research has identified a number of health-promoting nutraceuticals, but there is ample scope for further research with traditional food items for novel nutraceuticals. The incorporation of natural

nutraceuticals is a much better alternative than the use of synthetic pharmaceuticals as the xenobiotics cannot exactly mimic the natural behavior of the biomolecules within the physiological microenvironment [2]. Hence, the modern concept of diet and nutrition focuses on making our daily foodstuffs more enriched or more fortified with such natural nutraceuticals to make them more healthy and 'functional' in terms of boosting and maintaining health parameters.

Freshwater mollusks are traditional foods that have long served as an extremely appreciated cuisine in European countries such as the Netherlands, France, and Austria. In the Indian context, these freshwater mollusks, especially the gastropod snail *Bellamya bengalensis*, provide a cheap and sustainable source of animal protein, mostly among the tribal population of both India and Bangladesh [3,4]. This snail meat (foot muscle) is nutritious and an enriched source of valuable amino acids, low in cholesterol and fat. This meat is also quite popular for its culinary delicacy and appetizing properties [5]. This snail meat has found traditional application in anti-inflammatory, immune booster, antimicrobial, antioxidative, antihypertensive roles [6,7]. Bioactive peptides with nutraceutical applications are produced using cheap protein sources. *Bellamya bengalensis* snail meat was used in the current research for production and characterization of bioactive peptides using enzyme technology [3,5].

Bioactive peptides are short chains of amino acids obtained from protein hydrolysates. Worldwide, there is a promising trend towards better utilization of small 'bioactive peptides' for their important nutraceutical potential, e.g., antioxidative, antihypertensive, and anticancer [5,8]. Among various bioactive peptides that have been studied extensively, antihypertensive peptides need special mention. Enzymatic hydrolysis of numerous sources such as fish proteins; seed proteins, especially those from legumes and oilseeds; and milk proteins have been studied for short peptides with potent angiotensin I-converting enzyme (ACE) inhibitory activity [8,9]. The ACE inhibitors play a pivotal role in the regulation of blood pressure. They act through the renin–angiotensin–aldosterone system (RAAS) to control the blood pressure of the body. In this regard, ACE (E.C. 3.4.15.1.), a dipeptidyl carboxypeptidase, plays a key role in the RAAS system by activating vasoconstrictor angiotensin II by cleaving His-Leu dipeptide from the angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) and inactivating vasodilator bradykinin [10,11]. There by, it reduces blood pressure and maintains electrolyte equilibrium in the blood. Therefore, a successful inhibition of ACE can regulate hypertension. Synthetic ACE inhibitors such as captopril, lisinopril, and enalapril are being used as effective measures for treating hypertension, but their utilization causes countless side effects such as allergy, dermatitis, and cough [12]. Therein lies the importance of nutraceuticals as natural alternative ACE inhibitors with no such adverse effects.

The objective of the present work was to explore the bioactive short peptides from enzymatic digests of *Bellamya bengalensis* protein isolate for both nutritional and therapeutic benefits with targeted action. Further emphasis was laid on understanding the mechanism of the biochemical interactions between the bioactive short peptides and ACE. The findings of the work reveal the translational potential of such a low-cost, highly abundant, nonconventional food resource for biomedical applications.

2. Materials and Methods

2.1. Collection of Materials

Bellamya bengalensis f. *typica* (*Lamark 1822*) samples were collected from freshwater water bodies of Kakdweep, South 24 Parganas, West Bengal, eastern part of India, and identified by Zoological Survey of India, Prani Vigyan Bhaban, M Block, New Alipore, Kolkata 700053 (F. No. 229-10/98- Mal./7606). After removing the shells, the foot muscle (edible snail meat) was separated from the viscera, washed in clean water, and minced before storing at −80 °C.

2.2. Preparation of B. bengalensis Protein Concentrates (BBPCs)

B. bengalensis meat was homogenized (Ultra Turrax T18, IKA, Werke GmbH & Co., KG, Stufen, Germany) in phosphate buffer (pH 7.0) at a ratio of 1:10 for 30 min (10 min cycles with 5 min of rest in between) at 12,000 rpm, in an ice bath. The homogenate was centrifuged for 5 min at 6000 rpm, and the protein-rich supernatant was collected by partitioning over 10 mL chloroform. The protein-rich fraction was lyophilized under partial vacuum to dry powder form and stored at −80 °C until further use.

2.3. Preparation of B. bengalensis Protein Hydrolysates(BBPHs)

BBPC (15 g/L in phosphate buffer, pH 7.0) was hydrolyzed using alcalase 2.4 L (from *Bacillus licheniformis*, Subtilisin A, P4860, Sigma Aldrich, MO, USA) at different concentrations (0.1–0.5%, *v*/*v*) [13]. The reaction was stopped at time intervals (10 min, $BBPH_{A10}$; 30 min, $BBPH_{A30}$; 60 min, $BBPH_{A60}$; 90 min, $BBPH_{A90}$; 120 min, $BBPH_{A120}$) by inactivating alcalase, and the hydrolysates were stored at −20 °C until further use [14].

The degree of hydrolysis (DH; %) was determined following the ninhydrin method [15]. DH was defined as the percentage of peptide bonds hydrolyzed was calculated by the determination of free amino groups reacting with ninhydrin. The degree of hydrolysis was determined by the following equation:

$$\text{DH } (\%) = \text{h}\frac{\text{h}}{\text{h}_{\text{tot}}} \times 100, \quad (1)$$

where, h = concentration of peptide bond hydrolyzed (meq/g) and h_{tot} = total amount of peptide bond, taken as 8 amino meq/g [13].

B. bengalensis protein hydrolysates (BBPHs) were subjected to centrifugal ultrafiltration using 3 kDa cut-off polyethersulfone (PES) membrane filtration unit (Vivaspin20, VS2091, Sartorius AG, Goettingen, Germany) to separate the low-molecular-weight peptide fraction (<3 kDa). A 15 mL peptide solution was loaded onto an individual filtration unit and centrifuged according to the manufacturer's protocol. The ultrafiltered samples were lyophilized under partial vacuum until dry and stored as fine powder at −80 °C until they were further used for bioactivity assays.

2.4. In Vitro ACE Inhibitory Assay

In vitro ACE inhibitory activity assay was performed on the basis of a standard method [16] with slight modifications. ACE (200 µL of 20 mU/mL; from rabbit lung, A67778, Sigma-Aldrich, St. Louis, MO, USA) was added to 2.17 mM hippuryl-L-histidyl-L-leucine (HHL, H1635, Sigma-Aldrich, MO, USA) in 100 µL sodium borate buffer (100 mM, pH 8.3) containing 300 mM NaCl to start the reaction by incubating at 37 °C for 30 min. Then, 50 µL of 1 mg/mL (*w*/*v*) short peptides (<3 kDa, ultrafiltered) in sodium borate buffer (100 mM, pH 8.3), isolated from the BBPHs, were added to the reaction mix before adding the enzyme to assess the inhibition potential of the peptides, and the results were compared with those obtained for a standard ACE inhibitor, lisinopril (L2777, Sigma-Aldrich, St. Louis, MO, USA), used as positive control (50 µL of 2 µg/mL). The reaction was stopped by 125 µL of 1M HCl and, 30 s later the reaction was neutralized by adding 0.5 mL of 1M NaOH. Then, 2 mL of diluent buffer containing 0.2M KH_2PO_4 and 1.5 mL of the color reagent containing 3 mg/mL cyanuric chloride in 1,4-dioxane was added, vortexed, and centrifuged at 3000 rpm for 10 min to remove any particulate matter. In order to evaluate the in vitro activity of ACE, the chromogen developed by the reaction of cyanuric chloride with the released hippurate was measured spectrophotometrically at 382 nm against the corresponding reagent blank. The ACE inhibition activity was calculated using the following equation:

$$\text{ACE inhibitory activity } (\%) = \frac{\text{C} - \text{S}}{\text{C} - \text{B}} \times 100, \quad (2)$$

where, C is the optical density of the control reaction mixture consisting of ACE and substrate HHL, S is the optical density of the reaction mixture in presence of the peptide sample, and B is the optical density of blank.

For every sample, a gradient of concentrations of the sample was added to the reaction for effective inhibition of ACE. The ACE inhibitory potential of each sample was expressed in terms of its IC_{50} value, defined as the concentration of the sample required to inhibit 50% of the ACE activity.

2.5. Identification and Preparation of ACE Inhibitory Peptides

Based on best ACE-inhibitory activity, the sub-3 kDa fraction of the 120 min hydrolysate ($BBPHA_{120}$) was analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry for identifying peptides probably responsible for the inhibitory activity [15], using Ultraflextreme ToF/ToF mass spectrometer (Bruker Daltonics GmbH & Co. KG, Fahrenheitstr. 4, Bremen, Germany). The mass spectra (600–3000 m/z) were acquired in reflector mode, and from the mass spectrum, the signals with particularly high intensity (>1 a.u.) were further analyzed as their MS-MS spectra were acquired in LIFT mode using Flex Control (version 3.4) software, with 2000 shots added per sample. From the MS-MS spectra of the selected peptides, de novo sequencing was conducted to identify the amino acid sequence of the peptides. These de novo sequenced peptides were also screened for cross-matching with the peptide sequences with very low IC_{50} values, as reported in the AHTPDB database of the established ACE inhibitory peptides isolated from edible sources reported to date [17].

2.6. Molecular Docking Studies

The de novo sequences of the short bioactive peptides were translated into energy-minimized pdb files with the help of I-TASSER online server [18] and Avogadro (version 1.XX) molecular builder and visualization tool [19]. The apparent hydrophobicity of the sequenced peptides was calculated using the online server Peptide 2.0 Inc. (https://www.peptide2.com; accessed on 20 August 2019, Chantilly, VA, USA). The model for ACE used in this study was imported from the Protein Data Bank (1O86.pdb) and represented the crystal structure of the human testicular angiotensin-converting enzyme (*t*-ACE) with the standard inhibitor lisinopril bound to the critical angiotensin-converting site at the C-terminal domain at 2 Å resolution [10,20]. Before the docking of the peptide sequences to the ACE, the cofactors such as zinc and chloride atoms were retained in the active site, fixed to their crystal positions in the ACE–lisinopril complex (1O86.pdb), whereas the lisinopril and the water molecules were removed. The automated molecular docking of the peptide with human angiotensin-converting enzyme (ACE) was performed through GalaxyPepDock web server [21] using all default parameters. The binding site was selected as described by Jimsheena and Gowda [22] so that the binding site of lisinopril within the crystal structure of ACE was well sampled with a grid resolution of 0.3 Å. The three-dimensional structures of the resulting docked structures were visualized and analyzed in PyMol software. The best docking pose of each peptide in the active site of ACE was obtained on the basis of the lowest binding energy value and further analyzed to identify the hydrogen bonds and other hydrophobic or hydrophilic interactions between the amino acid residues at the ACE active site. The docked complexes were compared for their binding efficiency in terms of their binding affinity (pKd) and free energy states (ΔG, kCal/mol). The shortest peptide sequence (henceforth referred to as Belpep) with the best combination of these two factors along with the most common docked interaction with lisinopril was selected for further biochemical binding assays.

The Belpep sequence was commercially synthesized by solid-phase method, using standard Fmoc chemistry (Pepmic Solutions, Suzhou, China). The purity (95–99.9%) of the synthesized peptide was confirmed by high-performance liquid chromatography (HPLC), and the molecular weight was verified by mass spectrometry (MS) study. The pure peptide was utilized to validate its ACE inhibitory activity by assessing the hippuric acid release

adding 50 µL of Belpep (2 µg/mL) in sodium borate buffer as mentioned above. This activity was further mechanistically validated for enzyme–substrate binding via isothermal calorimetric (ITC) analysis and the in silico modeling, using lisinopril as positive control.

2.7. Determination of Kinetics of ACE Inhibitory Activity of the Peptide

The kinetics of the reaction catalyzed by ACE, where HHL was hydrolyzed to release the hippuric acid, was studied both in presence and absence of inhibitor. ACE activity was measured at various substrate (HHL) concentrations (0.5, 1.0, 2.0, and 4.0 mM), while the enzyme concentration was maintained at 20 mU/mL. The concentration of the inhibitor Belpep used in the study was 1.0 mg/mL. The kinetic parameters (V_{max}, K_m) of the enzyme kinetics were established through the Lineweaver–Burk plot, in presence or absence of inhibitor. The type of the mechanism of Belpep-mediated inhibition, whether competitive, noncompetitive, or uncompetitive, was also interpreted from the Lineweaver–Burk plot [13].

2.8. Isothermal Titration Calorimetry (ITC)

ITC experiments were performed using an isothermal titration calorimeter (MicroCal iTC200, GE Healthcare Bioscience limited). Enzyme–substrate reactions were carried out within the reaction cell at 37 °C, with a sodium borate buffer (pH 8.3) background. In a control experiment, the reaction cell was loaded with 350 µL ACE (0.357 µmol/L) which was titrated with 20 identical injections of 2 µL of HHL (1.0 mmol/L). The time interval between two injections was 120 s. The heat change in the reaction cell due to the enzyme–substrate reaction was measured against a thermally stabilized reference cell. The thermogram peaks corresponding to the heat change in the reaction cell were integrated using ORIGIN 6 software (MicroCal) supplied with the instrument [23]. In the negative control experiment, only the buffer was injected into the reaction cell, without the enzyme. In the reactions involving inhibitors, the ITC cell was filled with 350 µL of ACE (0.357 µmol/L sodium borate buffer, pH 8.3) and 50 µL of pure Belpep (0.15 mg/mL in sodium borate buffer, pH 8.3), which was then titrated with HHL (1.0 mmol/L) as described earlier. The thermograms were compared to assess the binding mechanism of the Belpep.

2.9. Data Analysis

All results are presented as mean ± standard deviation (SD) of a minimum of three replicates of data [15]. Data comparisons and analyses were done using the software Origin 8.1 (OriginLab Corporation, Northampton, MA, USA). The significant differences were determined by the Student *t*-test ($p < 0.05$).

3. Results and Discussion

3.1. Degree of Hydrolysis of BBPCs and Their ACE Inhibitory Activities

The alcalase-mediated hydrolysis curve of *Bellamya bengalensis* protein concentrate (BBPC) along a time gradient of 120 min is shown in Supplementary Figure S1. It is evident that with the increase in enzyme concentration (%, *v*/*v*), the degree of hydrolysis increased until it reached an optimum concentration (0.3%, *v*/*v*). The highest degree of hydrolysis, 67.43%, was achieved at 120 min with an enzyme concentration of 0.3% (*v*/*v*) and a substrate concentration as high as 15 g/L. Demirhan et al. [24] also reported an optimum hydrolytic activity in similar conditions. Further increase in the enzyme concentration did not show significantly better hydrolytic activity. When extending the reaction beyond 120 min, the increase in the degree of hydrolysis was found to be insignificant. Similar results were reported by Bhaskar et al. [25], where the *Catla catla* visceral protein was utilized as substrate. The increase in the degree of hydrolysis was inferred as the increase in the number of cleaved peptide bonds and the amplification in the number of smaller peptides.

The pertinent literature suggests that smaller peptides with low molecular weight have shown better ACE inhibitory potential [26,27]. Hence, peptide fractions of <3 kDa

were fractionated using an ultrafiltration membrane (Vivaspin20, Sartorius, Mumbai, Maharashtra India). The filtrates were lyophilized and further solubilized in sterile buffered medium to assess their ACE inhibitory activity and compared with lisinopril (Figure 1).

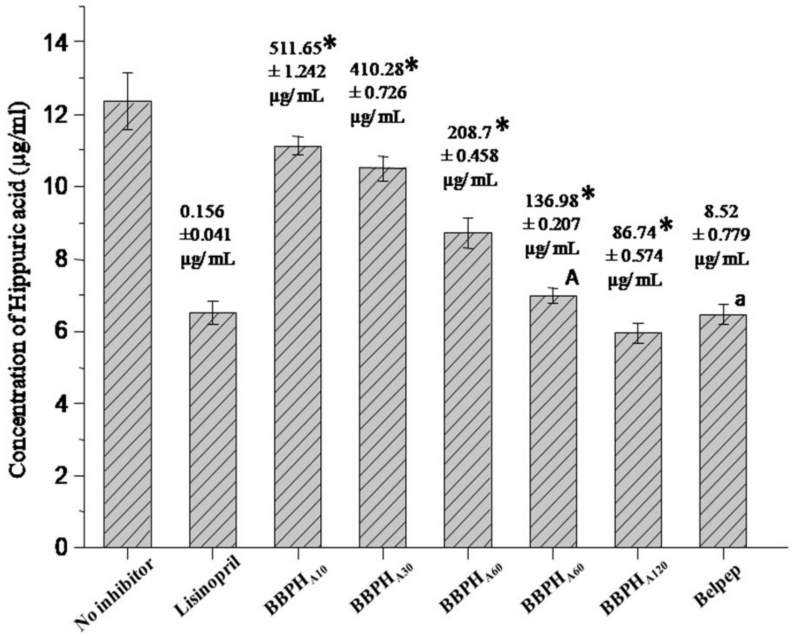

Figure 1. ACE inhibitory activity of *B. bengalensis* protein hydrolysates at different gradient times (10 min, $BBPH_{A10}$; 30 min, $BBPH_{A30}$; 60 min, $BBPH_{A60}$; 90 min, $BBPH_{A90}$; 120 min, $BBPH_{A120}$) and the synthesized peptide (Belpep). IC_{50} values of each reaction set are shown over each bar as mean ± SD value, n = 3. The capital letter indicates the significant reduction inhippurate release ($p < 0.05$), as affected by $BBPH_{A90}$, in comparison to $BBPH_{A60}$ ($p = 0.01987$). The asterisk (*) indicatesthe significant difference between the IC_{50} values of hydrolysates of two subsequent time points ($BBPH_{A10}$ vs. $BBPH_{A30}$ ($p = 0.0000139$); $BBPH_{A30}$ vs. $BBPH_{A60}$ ($p = 0.0000029$); $BBPH_{A60}$ vs. $BBPH_{A90}$ ($p = 0.0000075$); $BBPH_{A90}$ vs. $BBPH_{A120}$ ($p = 0.0000211$)). The lowercase letter signifies the significant difference in the IC_{50} values of Belpep and $BBPH_{A120}$ ($p = 0.0000992$).

Lisinopril reduced hippuric acid release (0.65 ± 0.03 µg/mL) significantly ($p = 0.00154$) compared to the uninhibited ACE activity (12.34 ± 0.774 µg/mL). Among all the alcalase hydrolysate groups, $BBPH_{A120}$ showed the maximum inhibitory effect, with the least hippuric acid release (6.97 ± 0.274 µg/mL). Alcalase, being a serine endopeptidase, extensively hydrolyzed the BBPH, liberating a higher concentration of smaller peptides that were otherwise buried deep in the native protein. This rationale can justify the increasingly better ACE inhibitory activity of the BBPH hydrolysates over time. The IC_{50} values of the BBPHs are also shown in Figure 1. $BBPH_{A120}$ showed the lowest IC_{50} value, which again substantiates the ACE inhibitory activity of $BBPH_{A120}$ as inferred from the inhibition of hippuric acid release. These results are in agreement with recent studies that mentioned that alcalase-mediated hydrolysis of food proteins produces bioactive peptides with higher ACE inhibitory potentials [28], which was inferred to be due to the endopeptidase activity of alcalase.

3.2. Identification of the Peptide Sequence

Based on the degree of hydrolysis and ACE inhibition activity, the <3 kDa fraction of $BBPH_{A120}$ was analyzed by MALDI-ToF mass spectrometry to identify the resulting peptides due to the alcalase hydrolysis. From the mass spectrum, five peptides (molecular weights ranging from 914.608 to 1653.991 Da) were identified with significantly high intensity ($>1 \times 10^4$a.u.) compared to other peptides (Figure 2). *De novo* sequencing of these

small peptides revealed a high concentration of proline, along with other hydrophobic amino acids (Table S1).

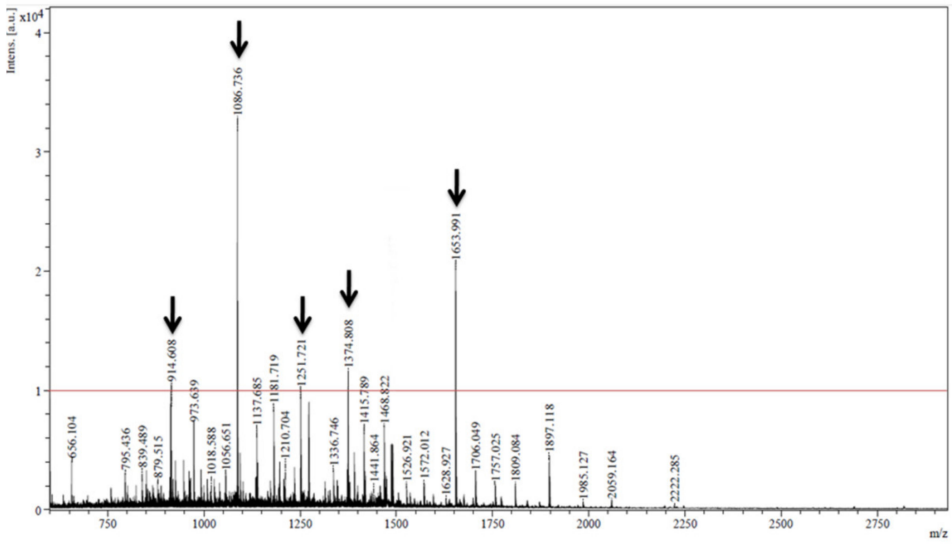

Figure 2. MALDI-ToF spectrum of the *B. bengalensis* protein hydrolysate of alcalase at 120 min. The arrows mark the peptides selected for the *de novo* sequencing. The red line across the spectrum marks the baseline intensity of 1×10^4 a.u.

Current research on ACE inhibitory peptides accommodates a quantitative structure-activity relationship (QSAR) approach for initial screening of the peptide sequences [29]. Previous reports showed that the presence of hydrophobic amino acids in the C-terminus was the key feature that influenced the ACE inhibitory action of the peptides and their binding to the inhibitor binding site in the close proximity of the zinc molecule within the quaternary structure of human ACE [29].

The five peptide sequences identified from the mass spectrum of the ultrafiltrate fraction of $BBPH_{A120}$ with significantly high concentration (>1.0×10^4 a.u.) showed significant sequence overlap with many previously reported ACE inhibitory peptide sequences with low IC_{50} values [17] (Table S1). Interestingly, the peptide with sequence IIAPTPVPAAH was found to be the most concentrated peptide in the $BBPH_{A120}$ mass spectrum (3.25×10^4 a.u.). Moreover, it was also found to have the highest sequence overlap with the previously mentioned AHTPDB database [17,30–32].

3.3. Molecular Docking

The binding affinities of the de novo sequenced peptides to the crystal structure of human *t*-ACE were calculated via KDEEP, a protein–ligand affinity predictor based on a deep convolutional neural network [33], and are presented in Table 1. The energy-minimized docked poses of the peptides in the binding domain of *t*-ACE are represented in Figure 3. Interestingly, the pKd and ΔG values indicate that three of the five sequences (i.e. IIAPTPVPAAH, LNPGAGLPRGPNGADTF, and TIGAPDGIPSAPR) have highly similar ACE-binding efficiency when compared to lisinopril (Table 1).

Table 1. The binding efficiency parameters of the de novo sequenced peptides to *t*-ACE.

Sequence	Mol. wt. (g/mol)	Hydrophobicity (%)	pKd	ΔG (kcal/mol)
LTPVPGSPF	914.608	66.67	2.86	−3.86
IIAPTPVPAAH	1086.736	81.82	6.47	−8.49
TIGAPDGIPSAPR	1251.721	53.85	3.57	−5.87
HEFPGVVVGANDD	1374.808	46.15	3.64	−4.92
LNPGAGLPRGPNGADTF	1653.991	47.06	6.53	−8.82

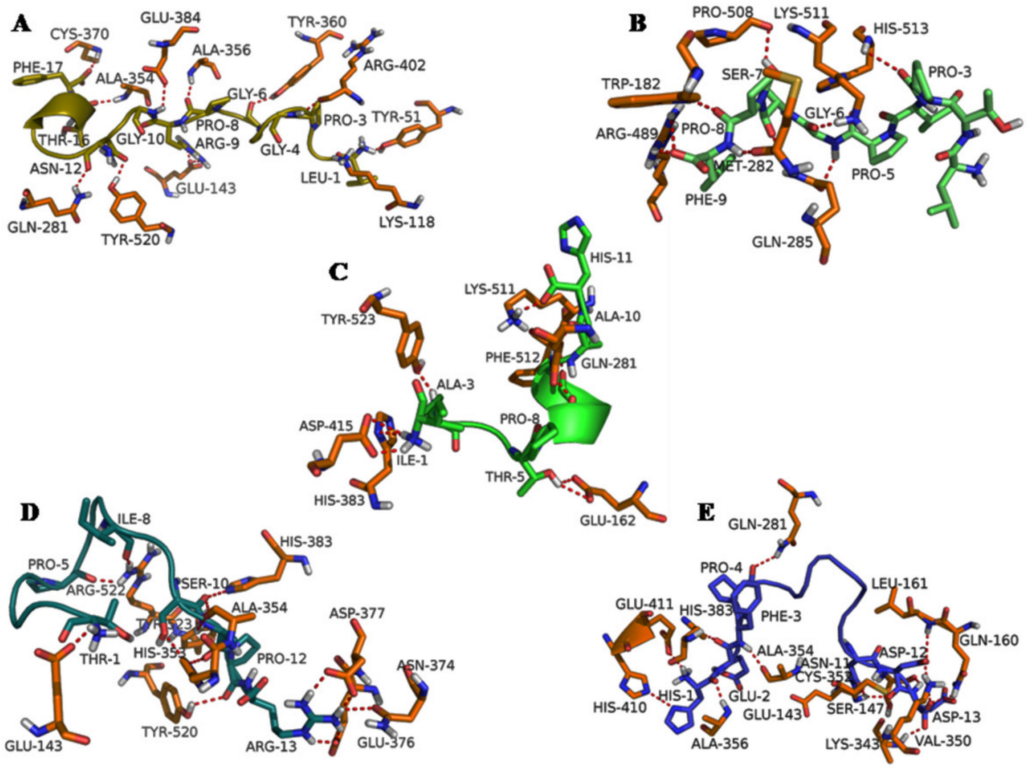

Figure 3. Docking poses of identified peptides ((**A**) LNPGAGLPRGPNGADTF; (**B**) LTPVPGSPF; (**C**) IIAPTPVPAAH; (**D**) TIGAPDGIPSAPR; (**E**) HEFPGVVVGANDD) with angiotensin-converting enzyme (PDB file 1O86.pdb) at the core of the enzyme.

Looking at the binding site of the lisinopril in the all-important carboxyl terminus of human *t*-ACE, it is very evident that the peptide ligands with higher hydrophobicity can diffuse into the catalytic site located in the hydrophobic core of the ACE crystal structure [34]. The peptide with IIAPTPVPAAH sequence showed the highest hydrophobicity (81.82%) compared to the other two peptides (Table 1), which might have facilitated its efficient ACE-inhibitory activity [29]. The non-constrained docking of the peptide ligands to the *t*-ACE molecules revealed that the peptide with sequence IIAPTPVPAAH (henceforth referred to as Belpep, as mentioned in Section 2) acquires the same 3D active site deep inside the enzyme molecule and interacts with the same amino acids (Glu162, Glu 281, Lyr511, Phe512, Tyr523) in the active site as lisinopril, a standard inhibitor (Figure 3), which clearly justifies the observed pKd and the ΔG values [20]. The smaller molecular volume of lisinopril likely prompted a comparatively favorable steric alignment in the binding pocket within the *t*-ACE molecule that enabled it to be the most effective inhibitor of ACE (pKd = 6.38; ΔG = −8.61). The data showed that Belpep (IIAPTPVPAAH) was a highly efficacious inhibitor among the isolated peptides from the enzymatic digests of the *B. bengalensis* muscle protein fraction.

Based on these observations, pure Belpep sequence (IIAPTPVPAAH) was synthetically prepared in order to analyze the molecular mode of inhibition. The pure Belpep showed a IC_{50} value of 8.52 ± 0.779 μg/mL, which reflects strong inhibition of the proteolytic activity of ACE that releases the hippuric acid from hippuric acid–histidine–lysine (HHL) (Figure 2).

3.4. Inhibitory Kinetics Study

These observations led to the mechanistic assessment of the mode of Belpep-mediated inhibition of the ACE, which was elucidated by the Lineweaver–Burk plot as depicted in Figure 4, which shows the kinetics of the enzyme–substrate (ES) reaction in the presence or absence of Belpep.

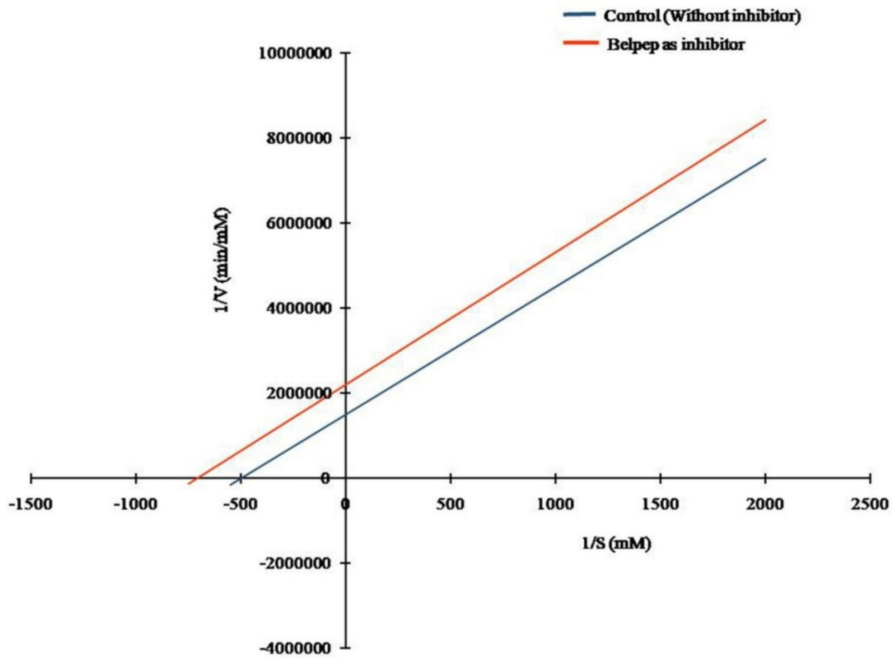

Figure 4. ACE inhibitory kinetics of the peptide isolated from ultrafiltered 3 kDa permeates of *Bellamya bengalensis* protein alcalase hydrolysate. Lineweaver–Burk plot of ACE inhibition by the peptide isolated from ultrafiltered $BBPH_{A120}$. The ACE inhibitory properties were evaluated both in presence and absence of inhibitor. Here, 1/[S] and 1/V represent the reciprocal substrate concentration and velocity of the reaction, respectively.

From the Lineweaver–Burk plot (Figure 4), it is evident that the mode of inhibition by the Belpep was of typical uncompetitive type. In uncompetitive inhibition, the inhibitor does not have any affinity towards the catalytic site of the ACE. Rather, the inhibitor preferentially binds to the ES complex, thereby hindering the release of the product and the enzyme from the ES complex. In absence of product, the substrate affinity of the ACE is increased, as suggested by an obvious decrease in K_m value in comparison to that of the uninhibited ES reaction (Table 2).

Table 2. ACE inhibitory kinetics parameters of the peptide identified from ultrafiltered $BBPHA_{120}$ (*Bellamya bengalensis* alcalase hydrolysate at 120 min).

	K_m	V_{max}
ACE+HHL+ (no inhibitor)	3.008 µM	0.001 µM/min
ACE+HHL+Belpep	1.55625 µM	0.0005 µM/min

The V_0 and V_{max} of the Belpep-inhibited reaction were found decreased compared to the control uninhibited ES reaction. In fact, the Lineweaver–Burk plots of the control and the Belpep-inhibited ES reactions show no sign of convergence, neither at the y-intercept nor at the x-intercept (Figure 4), which is characteristic of the uncompetitive inhibition of ES reaction [28,35].

3.5. Isothermal Titration Calorimetry

ITC experiments were conducted to confirm the mode of inhibition of ACE by Belpep based on the thermodynamic parameters of its binding to ACE and HHL. The thermograms and binding isotherms of the enzyme–substrate titration at optimal conditions in the absence and presence of the Belpep are presented in Figure 5.

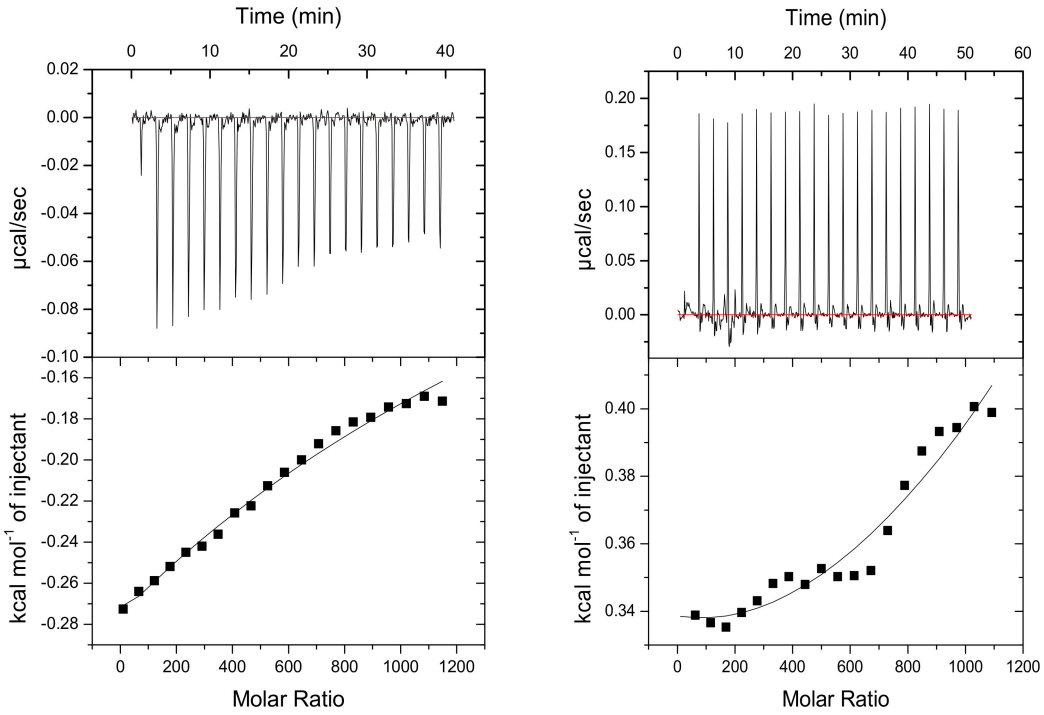

Figure 5. The isothermal titration calorimetric analysis of the binding of angiotensin-converting enzyme (ACE) at a concentration of 357 nm to the substrate HHL at a concentration of 1mM without any inhibitor (Figure **5a**) or with Belpep as inhibitor at a concentration of 0.5mM (Figure **5b**). The upper panel shows a typical ITC curve, showing the heat release as a function of time depicted in the thermogram for binding of the ligand and the enzyme at pH 8.3 and temperature 37 °C, whereas the lower panel of the figure shows the isotherm depicting the integrated heat evolved per mole of the inhibitor for addition of each injection with respect to the total molar ratio of the ligand over the enzyme concentration.

Figure 5a showed a typical titration curve for the binding of HHL to ACE under optimal conditions as ACE hydrolyzes hippuric acid from HHL via an exothermic reaction (enthalpy change −5.66×106 kJ/mol) [23]. As the ACE was titrated with the substrate (HHL), the catalytic sites became progressively saturated; as a result, the reaction tended towards equilibrium, net heat release diminished, and only background heat of dilution peaks remained, as shown in the thermogram. At the end of the reaction, it tended towards achieving saturation, and it caused the enthalpy change due to the addition of moles of ligand to the enzyme solution in isothermal condition.

In presence of Belpep as an inhibitor, the exothermic nature of the ACE–HHL reaction was changed to an endothermic pattern (Figure 5b). Previous reports also had shown a similar endothermic pattern of thermogram when a standard inhibitor such as lisinopril was used to inhibit the ACE–HHL reaction [36]. The area under each small injection peak (heat absorbed per injection) in the Belpep-inhibited ES reaction signified a very small rate of heat change (0–0.2 µcal/s) in comparison to the uninhibited ES reaction, which was evident from the corresponding binding isotherms as well.

Interestingly, the binding isotherm curvature of the Belpep-inhibited ES reaction indicated a two-site cooperative ligand binding to the ACE molecules [37,38]. The initial slow but gradual increase in the rate of heat change with the increase in the molar concentration of the ligand indicated initial substrate binding to the ACE molecule, which in turn promoted the binding of Belpep to a secondary binding site on the ACE, as confirmed by a comparatively sharp increase in the rate of secondary heat change. This was indeed the case, as the ACE molecule (A67778, Sigma-Aldrich, MO, USA) used in this experiment was of somatic type as it was isolated from rabbit lung. That means it has two inhibitor binding sites on both its N- and C-terminus. According to Fuchs et al. [39], the C-terminal inhibitor

binding site is at first acquired by the incoming inhibitor, which then facilitates further inhibitor binding to the designated site on the N-terminus of the somatic ACE. Thus, ITC experiments validated the binding of the Belpep to the ACE imparting inhibitory effect, coherent with the kinetics of enzyme inhibition. These observations were further validated by the molecular docking of the inhibitor Belpep onto the ACE molecule.

4. Conclusions

The present study confirmed the presence of ACE inhibitory peptides extracted from the hydrolysates of protein concentrate of *Bellamya bengalensis*. The enzymatic hydrolysis yields low-molecular-weight peptides with extraordinary properties such as lowering hypertension by means of ACE inhibition. This convenient mode of preparation of hydrolysate fraction produced sufficiently potent ACE inhibitors of commercial relevance that can act as a value-added substance in food formulations. The inhibitory mechanism observations with the combination of ITC and molecular docking represent a novel approach towards the in vitro inhibitory mechanism study. Bioactive peptide extraction from the meat of *Bellamya bengalensis* facilitates the opening up of innovative economic opportunities that can amplify the utilization of *Bellamya bengalensis* meat as functional foods.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pr9081316/s1, Figure S1: Degree of hydrolysis of *B. bengalensis* protein isolate by alcalase with respect to time, Table S1: Sequence-matching in relation to documented ACE inhibitory peptides.

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Institutional Review Board Statement: Not applicable as the study does not involve humans or animals. The sample for the present isn't the live mollusks, rather the edible foot muscles of *Bellamya bengalensis*.

Informed Consent Statement: Not applicable as the study does not involve human subjects.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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