***In vitro* and *in silico* analyses of the angiotensin-I converting enzyme inhibitory activity of peptides identified from *Bellamyia bengalensis* protein hydrolysates**

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Abbreviated Running title: **ACE-inhibitory peptide from foot muscle of *Bellamyia bengalensis***

22 Abstract:

23 The study focuses on identification of ACE-inhibitory peptides from the proteolytic digests of muscle
 24 protein of *Bellamya bengalensis* and its underlying mechanism. 120 min Alcalase-hydrolysates were
 25 ultrafiltered to isolate the small peptide fraction (<3kDa) and *in vitro* ACE-inhibitory activity was
 26 analyzed. The IC₅₀ value of the 120 min hydrolysate ultrafiltered fraction was found to be 86.74 ± 0.575
 27 $\mu\text{g/mL}$, while the IC₅₀ of Lisinopril is $0.31 \pm 0.07 \mu\text{g/mL}$. This fraction was assessed in MALDI-ToF
 28 mass-spectrometer and five peptides were sequenced via *de novo* sequencing. The ACE-inhibitory
 29 potential of the peptides have a positive correlation with the hydrophobicity of the amino acids.
 30 Synthetic analogue of the peptide (IC₅₀ value $8.52 \pm 0.779 \mu\text{g/mL}$) was used to understand the
 31 thermodynamics of the inhibition by checking the binding affinity of the peptide to ACE by Isothermal
 32 titration calorimetry compared with lisinopril, and further substantiated by *in silico* site specific
 33 molecular docking study.

34

35 **Keywords:** *Bellamya bengalensis* protein hydrolysate, Alcase, ACE-inhibitory activity, Lisinopril,
 36 Isothermal Titration Calorimetry, uncompetitive inhibition, co-operative ligand binding, site-specific
 37 molecular docking.

38 1. Introduction:

39 A number of naturally grown traditional food items are known to have precise therapeutic roles against
 40 specific disease aetiology (**Kwon, Apostolidis, Kim, & Shetty, 2007**). Current foodomic research has
 41 identified the effective ‘nutraceuticals’ and established their health-promoting activities of such
 42 traditional food items, but most of these traditional food items, present in the nature, still remain to be
 43 analyzed for their health promoting attributes. **Incorporation of natural nutraceuticals are much better**
 44 **alternatives than synthetic pharmaceuticals as the xenobiotics can’t exactly mimic natural behavior of**
 45 **the biomolecules within the physiological microenvironment (Bhattacharya et al., 2014)**. Hence the
 46 modern concept of diet and nutrition focuses on making our daily foodstuffs more enriched or more
 47 fortified with such natural nutraceuticals to make them more healthy and ‘functional’ in terms of
 48 boosting and maintaining health parameters.

49 Fresh water molluscs are one such traditional food item which serves as an extremely appreciated
 50 cuisine in European countries like Netherlands, France, Austria etc. since long time. In the Indian
 51 context, these fresh water molluscs, especially the gastropod snail *Bellamya bengalensis* provides a
 52 major source of animal protein, mostly among the tribal population of both India and Bangladesh
 53 (**Prabhakar & Roy 2009; Baby, Hasan, Kabir, & Naser, 2010**).

54 World-over, there is a promising trend towards better utilization of small ‘bioactive peptides’ for their
 55 important nutraceutical potential, i.e. antioxidative, antihypertensive, anti-cancer etc. (**Udenigwe, &**
 56 **Aluko, 2012**). Bioactive peptides with 2-10 amino acids, isolated from natural food components by
 57 enzymatic bioprocessing techniques, are conceptualized to function better within body due to **better**
 58 **digestibility** and higher bioavailability, although scientific ratification of the molecular mechanism is
 59 still inadequate. Among various bioactive peptides that have been studied extensively, antihypertensive

60 peptides need special mention. Enzymatic hydrolysis of numerous sources like fish proteins, seed
 61 proteins especially from legumes and oilseeds, milk proteins etc. have been studied for short peptides
 62 with potent Angiotensin I converting enzyme (ACE) inhibitory activity (Udenigwe, & Aluko, 2012;
 63 Aluko, 2015). The ACE inhibitors play a pivotal role in the regulation of blood pressure. It acts
 64 through the renin–angiotensin–aldosterone system (RAAS) to control blood pressure of the body. ACE
 65 (E.C. 3.4.15.1.), a dipeptidylcarboxypeptidase plays a key role in the RAAS system by activating
 66 vasoconstrictor angiotensin-II by cleaving His-Leu dipeptide from the angiotensin I (Asp-Arg-Val-Tyr-
 67 Ile-His-Pro-Phe-His-Leu) (Natesh, Schwger, Sturrock, & Acharya, 2003) and inactivating
 68 vasodilator bradykinin (Raghavan & Kristinsson, 2009), thereby reducing blood pressure and
 69 maintaining electrolyte equilibrium in blood. Successful inhibition of ACE can thus regulate
 70 hypertension. Synthetic ACE inhibitors like captopril, lisinopril, enalapril etc. are being used as effective
 71 measure for treating hypertension but their utilization causes countless side effects like allergy,
 72 dermatitis, cough, etc. (Chen, Wang, Ye, Wu, & Xia, 2013). Herein lays the importance of
 73 nutraceuticals as natural alternative ACE inhibitors with no such adverse effects.

74 The objective of the present work was to explore the bioactive short peptides from enzymatic digests of
 75 *Bellamyia bengalensis* protein isolate for both nutritional and therapeutic benefits with targeted action.
 76 Further emphasis was laid on understanding the mechanism of the biochemical interactions between
 77 the bioactive short peptides and ACE. The findings of the work would reveal the translational potential
 78 of such low cost highly abundant, non-conventional food resource for biomedical application.

79 **2. Materials and Methods:**

80 **2.1. Collection of materials:**

81 *Bellamya bengalensis* f. *typica* (Lamarck 1822) samples were collected from freshwater water bodies of
 82 Kakdweep, South 24 Parganas, West Bengal, eastern part of India and as identified by Zoological
 83 Survey of India, Prani Vigyan Bhaban, M Block, New Alipore, Kolkata-53. [F. No. 229–10/98-
 84 Mal./7606]. After removing the shells, the foot muscle (edible snail meat) was separated from viscera,
 85 washed in clean water and minced before storing in -80°C.

86 **2.2. Preparation of *B. bengalensis* protein concentrates (BBPC):**

87 *B. bengalensis* meat was homogenized (Ultra Turrax T18, IKA®Werke GmbH & Co., KG, Stufen,
 88 Germany) in phosphate buffer (pH 7.0) at a ratio of 1:10 for 60 min at 12000 rpm. The homogenate
 89 was centrifuged and the protein-rich supernatant was collected by partitioning over chloroform. The
 90 protein-rich fraction was lyophilized under partial vacuum upto dry powder form and stored at -
 91 80°C until further use.

92 **2.5. Preparation of *B. bengalensis* protein hydrolysate (BBPH):**

93 15 g/L of BBPC was hydrolyzed by Alcalase® 2.4 L (from *Bacillus licheniformis*, Subtilisin A, P4860,
 94 Sigma Aldrich, MO, USA) with different enzyme concentration (0.1% v/v to 0.5% v/v) at conditions as
 95 optimized by Ahn, Jeon, Kim, & Je, 2012. The reaction was stopped at time intervals i.e. 0 min, 10
 96 min, 30 min, 60 min, 90 min and 120 min, by inactivation of alcalase (Mohammad, Kumar, &
 97 Basha, 2015) and the hydrolysates were stored at -20°C until further use (Chatterjee, Dey, Ghosh, &
 98 Dhar, 2015).

99 Degree of hydrolysis (DH; %) was determined by Ninhydrin method (Molnar-Perl & Pinter-
 100 Szakacs, 1989). DH was defined as the percentage of peptide bonds hydrolyzed was calculated by the
 101 determination of free amino groups reacting with Ninhydrin. The degree of hydrolysis was determined
 102 from the following equation:

$$103 \quad \text{DH (\%)} = h \frac{h}{h_{\text{tot}}} \times 100 \dots\dots\dots \text{equation 1}$$

104 where, h = concentration of peptide bond hydrolysed (meq/gm) and h_{tot} = total amount of peptide bond,
 105 taken as 8 amino meq/gm.

106 *B. bengalensis* protein hydrolysates (BBPH) were subjected to centrifugal ultrafiltration using 3kDa
 107 cut-off membrane filtration unit (Vivaspin20, VS2091, Sartorius AG, Goettingen, Germany) to
 108 separate the low molecular peptide fraction (< 3kDa). The ultrafiltered samples were lyophilized
 109 under partial vacuum until **dry and stored as fine powder** in -80°C until they were further used
 110 for bioactivity assays.

111 **2.6. *In vitro* ACE inhibitory assay:**

112 *In vitro* ACE inhibitory activity assay was performed on the basis of the method as described by **Li,**
 113 **Liu, Shi, & Le, 2005** with slight modifications. 200 µl of ACE (20 mU/mL; from Rabbit lung,
 114 A67778, Sigma-Aldrich, MO, USA) was added to 2.17mM hippuryl-L-histidyl-L-leucine (HHL,
 115 H1635, Sigma-Aldrich, MO, USA) in **100mM sodium borate buffer (pH 8.3)** containing 300mM NaCl
 116 to start the reaction by incubating at 37°C for 30 min. 50 µL of 1 mg/mL (w/v) short peptides (< 3kDa,
 117 ultrafiltered) in sodium borate buffer, isolated from the BBPHs were added to the reaction mix before
 118 adding the enzyme to assess the inhibition potential of the peptides and the results have been compared
 119 with a standard ACE inhibitor Lisinopril (L2777, Sigma-Aldrich, MO, USA), used as positive control
 120 **(50 µL of 2 µg/mL).**

121 The reaction was stopped by 125 µL of 1M HCl and 30 sec later the reaction was neutralized by
 122 adding 0.5 mL of 1M NaOH. 2 mL of diluent buffer containing 0.2M KH_2PO_4 and 1.5 mL of the
 123 colour reagent containing cyanuric chloride in 1, 4-dioxane was added, vortexed and centrifuged to
 124 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 corresponding reagent blank. The ACE inhibition activity was calculated using the following equation:

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

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 concentration of the sample were added to the reaction for effective inhibition of ACE. The ACE inhibitory potential of each sample were expressed in terms of their IC₅₀ value, defined as the concentration of the sample required to inhibit 50% of the ACE activity.

2.7. Identification and preparation of ACE inhibitory peptide:

Based on best ACE-inhibitory activity, the sub-3kDa fraction of the 120 minute hydrolysate (BBPH_{A120}) was analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry (Chatterjee et al., 2015) for identifying peptides probably responsible for the inhibitory activity, using Ultraflexxtreme ToF/ToF mass spectrometer (Bruker Daltonics, 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, to *de novo* was sequencing to identify the amino acid sequence of the peptides.

These peptide sequences were cross-matched with the sequences enlisted in the AHTPDB database (<http://crdd.osdd.net/raghava/ahtpdb/>) of the established ACE inhibitory peptides isolated from

edible sources reported till date. The peptide sequence (henceforth referred as Belpap) with highest match (mostly with previously reported peptides isolated from milk protein, soy protein and pea protein; especially with low IC_{50} values), was chosen for synthesized by solid-phase method, using standard Fmoc-chemistry, at 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 μ L of Belpap (2 μ g/mL) in sodium borate buffer as mentioned above. This activity is further mechanistically validated for enzyme-substrate binding via isothermal calorimetric (ITC) analysis and the *in silico* modelling, using lisinopril as positive control.

2.8. Determination of the kinetics of ACE inhibitory activity of the peptide:

The kinetics of the reaction catalyzed by ACE where HHL was hydrolysed to release the hippuric acid, was studied both in presence and absence of inhibitor, i.e. belpap as described in the present study. ACE activity was measured at various substrate (HHL) concentrations i.e. 0.5mM, 1.0mM, 2.0mM and 4.0mM, while the enzyme concentration was maintained at 20 mU/mL. The concentration of the inhibitor belpap 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 belpap mediated inhibition, whether competitive, noncompetitive or uncompetitive, was also interpreted from the Lineweaver-Burk plot (Ahn et al., 2012).

2.9. Isothermal Titration Calorimetry:

The Isothermal Titration Calorimetry (ITC) experiments were performed using 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 μ L ACE (0.357 μ mol/L) which was titrated with 20 identical injections of 2 μ L of HHL (1.0 mmol/L). The time interval between two injections was 120 sec. 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 (Ni, Li, Liu, & Hu, 2012). In the negative control experiment, only the buffer was injected in the reaction cell, without the enzyme. In the reactions involving inhibitors, the ITC cell was filled with 350 μ L of ACE (0.357 μ mol/L sodium borate buffer, pH 8.3) and 50 μ L 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.

2.10. Molecular Docking studies:

The model for ACE used in this study was imported from the Protein Data Bank (1O86.pdb) which represented the crystal structure of the human angiotensin converting enzyme-lisinopril complex at 2 Å resolution (Natesh et al., 2003). The co-crystal structure as the basic mold was chosen because lisinopril is known to be one of the competitive inhibitor of the enzyme; thus localization of lisinopril within the ACE structure would identify its main catalytic site. ChemOffice 2004 software (CambridgeSoft Co., MA, USA) was used to construct the initial structure of the purified peptide. Before the docking between the belpep sequence and ACE, the cofactors like 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 with through GalaxyPepDock web server (Lee, Heo, Lee, & Seok, 2015) using all default parameters. The binding site was selected as described by Jimsheena & Gowda, 2010, 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 two- and three-dimensional structures of the resulted docked structures were visualized and analyzed in PyMol software. The best docking pose of the belpep in the active site of ACE was obtained on the basis of least 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 and the belpep.

2.11. Data analysis:

All results were presented as means $\pm$ standard deviation (SD) of a minimum of 3–5 replicates of data. Data comparisons and analyses were done using the software Origin 8.1 (OriginLab Corporation, MA, USA). Significance of differences between two samples was determined by the Student t-test. Significance limit was set at $p < 0.05$.

3. Results and Discussion:

3.1. Degree of hydrolysis of *B. bengalensis* protein concentrate by Alcalase and their ACE inhibitory activities:

The alcalase mediated hydrolysis curve of *Bellamya bengalensis* protein concentrate (BBPC) along a time gradient of 120 min was shown in **Supplementary Figure 1**. It is evident that with the increase in enzyme concentration (% v/v), degree of hydrolysis were increasing until it reached an optimum concentration (0.3% v/v). A highest degree of hydrolysis of 67.43 % was achieved at 120 min with 0.3% (v/v) enzyme concentration at a substrate concentration as high as 15 g/L. **Demirhan, Apar, & Özbek, 2011** also reported an optimum hydrolytic activity at similar conditions. Further increase in the enzyme concentration didn't showed significant better hydrolytic activity. Also extending the reaction beyond 120 min, the increase in degree of hydrolysis was found to be insignificant. Similar results were

215 reported by **Bhaskar, Benila, Radha, & Lalitha, 2008**, where the *Catla catla* visceral protein was
 216 utilized as substrate. The increase in degree of hydrolysis was inferred as the increase in the number of
 217 cleaved peptide bonds and amplification in the number of smaller peptides.

218 The pertinent literature suggests that smaller peptides with low molecular weight have shown better
 219 ACE-inhibitory potential (**Chalé, Ruiz, Fernández, Ancona, & Campos, 2014; Wu, Jia, Yan, Du, &**
 220 **Gui, 2015; Wua, Du, Jia, & Kang, 2016**). Hence, peptide fractions <3kDa were fractionated using an
 221 ultrafiltration membrane (Vivaspin[®]20, Sartorius, India). The filtrates were lyophilized and further
 222 solubilized in sterile buffered medium to assess their ACE-inhibitory activity and compared with
 223 Lisinopril (**Figure 1**). Being the standard inhibitor, Lisinopril reduced hippuric acid release ($0.65 \pm$
 224 $0.03 \mu\text{g/mL}$) significantly ($p = 0.00154$) compared to the uninhibited ACE activity (12.34 ± 0.774
 225 $\mu\text{g/mL}$). Among all the alcalase hydrolysate groups, hydrolysates at 120 min (BBPH_{A120}, *Bellamya*
 226 *bengalensis* protein hydrolysates by Alcalase for 120 min) had shown the maximum inhibitory effect,
 227 with the least hippuric acid release ($6.97 \pm 0.274 \mu\text{g/mL}$). Alcalase, being a serine endopeptidase, had
 228 extensively hydrolysed the BBPH thus liberated higher concentration of smaller peptides which were
 229 otherwise buried deep the native protein. This rationale can justify the increasingly better ACE
 230 inhibitory activity of the time-gradient BBPH hydrolysates. The IC₅₀ values of the BBPHs were also
 231 mentioned in the **figure 1**. BBPH_{A120} has shown the least IC₅₀ value, which again substantiated the
 232 ACE inhibitory activity of BBPH_{A120} as inferred from the inhibition of hippuric acid release.

233 The results were in agreement with recent studies that mentioned that alcalase mediated hydrolysis of
 234 food proteins had produced bioactive peptides with higher ACE inhibitory potentials (**Chen, Wang,**
 235 **Zhong, Wu, & Xia, 2012; Forghani et al., 2016**), which was inferred to be due to the endopeptidase
 236 activity of alcalase.

237 3.2. Identification of the peptide sequence:

238 Based on the degree of hydrolysis and ACE inhibition activity, <3kDa fraction of BBPH_{A120} was
 239 analyzed by MALDI-ToF mass spectrometry to identify the resultant peptides due to the alcalase
 240 hydrolysis. From the mass-spectrum, five peptides (molecular weights ranging from 914.608 Da to
 241 1653.991 Da) had been identified with significantly high intensity ($> 1 \times 10^4$ a.u.) compared to other
 242 peptides (**figure 2**). *De novo* sequencing of these small peptides revealed high concentration of proline,
 243 along with other hydrophobic amino acids (**supplementary table 1**). Current research on ACE
 244 inhibitory peptides accommodates quantitative structure-activity relationship (QSAR) approach for
 245 initial screening of the peptide sequences (**Majumder & Wu, 2010; Nongonierma & FitzGerald,**
 246 **2016**). Previous reports showed that presence of hydrophobic amino acids in the C-terminal was the
 247 **key feature that influenced** the ACE inhibitory action of the peptides and also their binding to the
 248 inhibitor binding site in the close proximity of the zinc molecule within the quaternary structure of
 249 human ACE (**Nongonierma & FitzGerald, 2016; Castellano et al., 2013**). Based on these
 250 observations, the hydrophobic amino acid rich peptide sequences were checked for overlapping
 251 sequences from AHTPDB (<http://crdd.osdd.net/raghava/ahtpdb/>), a comprehensive database of
 252 experimentally validated antihypertensive peptides. The peptide sequences identified from the mass
 253 spectrum of **ultrafiltrate fraction** of BBPH_{A120}, had shown significant sequence overlap with many
 254 previously reported ACE inhibitory peptide sequences that had shown significantly high *in vitro* ACE
 255 inhibitory activity, as demonstrated by the low IC₅₀ value (**Supplementary table 1**).
 256 Interestingly Belpep which was to be the most concentrated peptide in the BBPH_{A120} mass spectrum
 257 (3.25×10^4 a.u.) was also found to have highest sequence overlap with the previously mentioned
 258 AHTPDB database. Based on these observations, pure Belpep sequence (**IIAPTPVPAAH**) was
 259 synthetically prepared in order to analyze the molecular mode of inhibition. The purified Belpep had
 260 shown a IC₅₀ value of **$8.52 \pm 0.779 \mu\text{g/mL}$** which reflects strong inhibition on proteolytic activity of

ACE that release the hippuric acid from hippuric acid-histidine-lysine (HHL) (**figure 1**). The C-terminal sequence of Belpap was highly hydrophobic that might have facilitated its entry within the inhibitor binding pocket and resulted into its efficient ACE-inhibitory activity (**Castellano et al., 2013**).

3.3. *Inhibitory Kinetics Study:*

These observations lead to the mechanistic assessment of the mode of belpap mediated inhibition of the ACE, which were elucidated by the Lineweaver-Burk plot as depicted in **figure 3**, which showed the kinetics of the Enzyme-substrate (ES) reaction in presence or absence of Belpap. From the Lineweaver-Burk plot (**figure 3**), it is evident that the mode of inhibition by the Belpap was of typical uncompetitive type. In uncompetitive inhibition, the inhibitor doesn't 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 gets increased, as suggested by obvious decrease in K_m value in comparison to the same from the uninhibited ES reaction (**Table 1**). Also, the V_0 and V_{max} of the belpap inhibited reaction were found to be decreased compared to the control uninhibited ES reaction. In fact the Lineweaver-Burk plots of the control and the belpap inhibited ES reactions showed no sign of convergence, neither at the y-intercept nor at the x-intercept (**figure 3**), which was characteristic of the uncompetitive inhibition of ES reaction (**Jao, Huang, & Hsu, 2016; Forghani et al., 2016**).

3.4. *Isothermal Titration Calorimetry:*

ITC experiments were conducted to confirm the mode of inhibition of ACE by Belpap based on the thermodynamic parameters of its binding to the ACE and HHL. The thermograms and binding isotherms of the Enzyme-Substrate titration at optimal conditions in absence and presence of the belpap were presented in **figure 4**.

284 **Figure 4A** showed a typical titration curve for the binding of HHL to ACE under optimal condition as
 285 ACE hydrolyze hippuric acid from HHL via an exothermic reaction (enthalpy change -5.66×10^6
 286 kJ/mol) (Ni et al., 2012). As the ACE get titrated with substrate (HHL), the catalytic sites were
 287 progressively get saturated and as a result the reaction tended towards equilibrium, net heat release
 288 diminished and only background heat of dilution peaks were remained as shown in the thermogram. At
 289 the end of reaction, it tended towards achieving saturation and it caused the enthalpy change due to the
 290 addition of moles of ligand to the enzyme solution at isothermal condition.

291 But in presence of an inhibitor belpep, the exothermic nature of the ACE-HHL reaction was changed to
 292 an endothermic pattern (**figure 4B**). Previous reports also had shown similar endothermic pattern of
 293 thermogram when standard inhibitor like lisinopril was used to inhibit the ACE-HHL reaction
 294 (Andújar-Sánchez, Cámara-Artigas, & Jara-Pérez, 2004). The area under each small injection
 295 peaks (heat absorbed per injection) in belpep inhibited ES reaction signified very small rate of heat
 296 change (0 to 0.20 μ cal/sec) in comparison to the uninhibited ES reaction, which was evident from the
 297 corresponding binding isotherms as well.

298 Interestingly, the binding isotherm curvature of the belpep inhibited ES reaction indicated towards a 2
 299 site co-operative ligand binding to the ACE molecules (Freiburger, Auclair, & Mittermaier, 2009;
 300 Brautigam, 2015). This observation was coherent to the uncompetitive inhibitory nature of Belpep.
 301 Initial slow but gradual increase in the rate of heat change with increase in the molar concentration of
 302 the ligand indicated towards initial substrate binding to the ACE molecule, which in turn promoted the
 303 binding of belpep to a secondary binding site on the ACE as confirmed by a comparatively sharp
 304 increase in the rate of secondary heat change. Thus, **ITC experiments validated** the binding of the
 305 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 on to the ACE molecule.

3.5. Molecular Docking:

The automated molecular docking pose involving the binding of the belpep to the ACE, with minimal binding energy was depicted in **figure 5A**. The hydrophobic nature of the belpep was evident from its sequence. Therefore the binding site of Belpep should be **logically** located within the core of the enzyme molecule, which was indeed what was found in the docked structure. Yet there were considerable differences between the amino acid residues that were found to be actively interacting with the Belpep, and the same that were involved in hydrogen bonding with lisinopril (**Natesh et al., 2003**). Belpep was found to be involved in hydrogen bonding with amino acid residues Asp376, His344, Tyr480, Glu123, Gln242, Lys 468 and Phe469, which were not part of the S_1 , S_1'' and S_2 substrate binding pockets of ACE that were occupied by lisinopril within the co-crystal (PDB no. 1O86) (**Natesh et al., 2003**) (**figure 5B**). Binding of substrate to this core groove of ACE within the close proximity of Zn^{2+} ion, probably rearranged the groove a little bit, so that a second ligand binding locus was formed about 4-5 Å away from the active site Zn^{2+} ion. This secondary binding locus was acquired by the Belpep to affect its uncompetitive inhibitory activity, which was coherent to the enzyme kinetic data. No direct interaction between the Belpep and the Zn^{2+} ion of ACE (**Figure 5A**) was observed in this simulation, although such direct interaction of the ligand with the Zn(II) atom of ACE for exerting strong inhibitory effect was a subject of controversy among the pertinent literature (**Wu et al., 2015; Rawendra, Chang, Chen, Huang, & Hsu, 2013; Jimsheena & Gowda, 2010; Guo, Pan, & Tanokura, 2009**).

4. Conclusion:

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

337 **5. Conflict of Interest:** The authors declare that there is no conflict of interest between them.

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Figures:

FIGURE 1: ACE INHIBITORY ACTIVITY (AS SHOWN BY HIPPURIC ACID RELEASE FROM HHL BY ACE) OF STANDARD ACE-INHIBITOR LISINOPRIL (AT A DOSE OF 50 µL OF 2 µG/ML SOLUTION) AND *B. BENGALENSIS* PROTEIN HYDROLYSATES (BBPHS, AT A DOSE OF 50 µL OF 1MG/ML SOLUTION) OF DIFFERENT TIME GRADIENT E.G. 10 MIN (BBPH_{A10}), 30 MIN (BBPH_{A30}), 60 MIN (BBPH_{A60}), 90 MIN (BBPH_{A90}) AND 120 MIN (BBPH_{A120}) RESPECTIVELY AND THE SYNTHESIZED PEPTIDE, MENTIONED AS BELPEP (AT A DOSE OF 50 µL OF 0.15 MG/ML SOLUTION). IC₅₀ VALUES OF EACH REACTION SET HAS BEEN SHOWN OVER EACH BAR AS MEAN ± SD VALUE, N=3.

THE CAPITAL LETTER SUPERScript LETTER INDICATE THE SIGNIFICANT REDUCTION OF HIPPURATE RELEASE ($P < 0.05$), AS AFFECTED BY BBPH_{A90}, IN COMPARISON TO BBPH_{A60} ($P = 0.01987$). THE * SIGNIFY THE SIGNIFICANT DIFFERENCE BETWEEN THE IC₅₀ 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 SMALL LETTER SUPERScript SIGNIFY THE SIGNIFICANT DIFFERENCE IN THE IC₅₀ VALUES OF BELPEP AND BBPH_{A120} ($P = 0.0000992$).

FIGURE 2: MALDI-TOF SPECTRUM OF THE *B. BENGALENSIS* PROTEIN HYDROLYSATE OF ALCALASE AT 120 MINUTES. THE ARROWS MARK THE PEPTIDES SELECTED FOR THE *DE NOVO* SEQUENCING. THE RED LINE ACROSS SPECTRUM MARKS THE BASELINE INTENSITY OF 1×10^4 A.U.

Figure 3: ACE inhibitory kinetics of the peptide isolated from ultrafiltered 3kDa permeates of *Bellamyia 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. $1/[S]$ and $1/V$ represent the reciprocal substrate concentration and velocity of the reaction, respectively.

Figure 4: The isothermal titration calorimetric analysis of the binding of angiotensin converting enzyme (ACE) at a concentration of 357nM to the substrate HHL at a concentration of 1mM and without any inhibitor (**Figure 4A**); or with belpap as inhibitor at a concentration of 0.5mM (**Figure 4B**). 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.

A.

B.

Figure 5: Docking of Belpap (IIAPTPVPAAH) with ACE shows that it binds at the core of the protein (**Figure 5A**). Superposition of docked structures with PDB 1O86 showed that it binds to similar locus compared to lisinopril (**Figure 5B**).

Tables:

Table 1: ACE inhibitory kinetics parameters of the peptide isolated from ultrafiltered BBPH_{A120} (*Bellamya bengalensis* alcalase hydrolysate at 120min).

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