In Vitro Structure–Activity Relationship Study of a Novel Octapeptide Angiotensin-I Converting Enzyme (ACE) Inhibitor from the Freshwater Mussel *Lamellidens marginalis*

Madhushrita Das[1] · Ankhi Halder[2] · Roshni Chatterjee[3] · Aditi Gangopadhyay[1] · Tanmoy K. Dey[4] · Souvik Roy[5] · Pubali Dhar[3] · Jana Chakrabarti[6]

Abstract

Angiotensin-I Converting Enzyme (ACE) plays a pivotal role in regulating hypertension. Inhibition of ACE by potent inhibitors is an excellent approach to treating high blood pressure and related cardiac ailments. Dietary proteins are a good source of bioactive peptides with a broad spectrum of pharmacological activities. An attempt has been made in this investigation to study the structure–activity relationship of a novel octapeptide ACE inhibitor having the amino acid sequence: isoleucine-glycine-proline-glycine-proline-phenylalanine-serine-arginine (IGPGPFSR; molecular weight 830.953 Dalton), isolated and identified from *Lamellidens marginalis*, a freshwater edible Mollusca and still underexploited for its health benefit molecules. The low molecular weight ACE inhibitory peptide has been evaluated for enzyme kinetics, isothermal titration calorimetry (ITC), and site-specific docking simulation study. Lineweaver–Burk plot analysis and ITC reveal that peptide-IGPGPFSR is a non-competitive inhibitor. Molecular docking simulation further confirms that this antihypertensive peptide bound to ACE by hydrogen bond interaction between Asp410, Lys468, His470, Arg479, and Glu104 of the enzyme with Ile1, Pro3, Gly4, Ser7, and Arg8 of the peptide respectively. Interaction of Pro5 and Phe6 of the peptide displaces the zinc (Zn^{2+}) ion from the active site which results in the incapability of the substrate: Hippuryl-L-Histidyl-L-Leucine (HHL) to bind to the active site of the enzyme and ultimately leads to inhibition of ACE. Present observations suggest that this novel antihypertensive octapeptide-IGPGPFSR is a potent inhibitor of ACE and can be used as an auxiliary for nutraceutical therapy against hypertension. Moreover, the underlying mechanism of ACE inhibition will help in widening our knowledge of drug design against enzyme targets.

✉ Jana Chakrabarti
  chakrabartijana0311@gmail.com

1 Department of Chemical Technology, Rajabazar Science College, University of Calcutta, 92, A.P.C Road, Kolkata 700009, West Bengal, India

2 Department of Food and Nutrition, Maharani Kasiswari College, 20, Ramkanto Bose Road, Kolkata 700003, West Bengal, India

3 Department of Home Science, Food & Nutrition Division, University of Calcutta, 20B Judges Court Road, Kolkata 700027, West Bengal, India

4 Dept of Chemistry, School of Basic and Applied Sciences, Adamas University, Barasat-Barrackpore Road, North 24 Parganas, Jagannathpur, Kolkata 700126, West Bengal, India

5 Bose Institute, College More, EN Block, Sector-V, Kolkata 700091, West Bengal, India

6 Department of Zoology, A.P.C Roy Government College, Himachal Vihar, Matigara, Siliguri 734010, West Bengal, India

Graphical Abstract
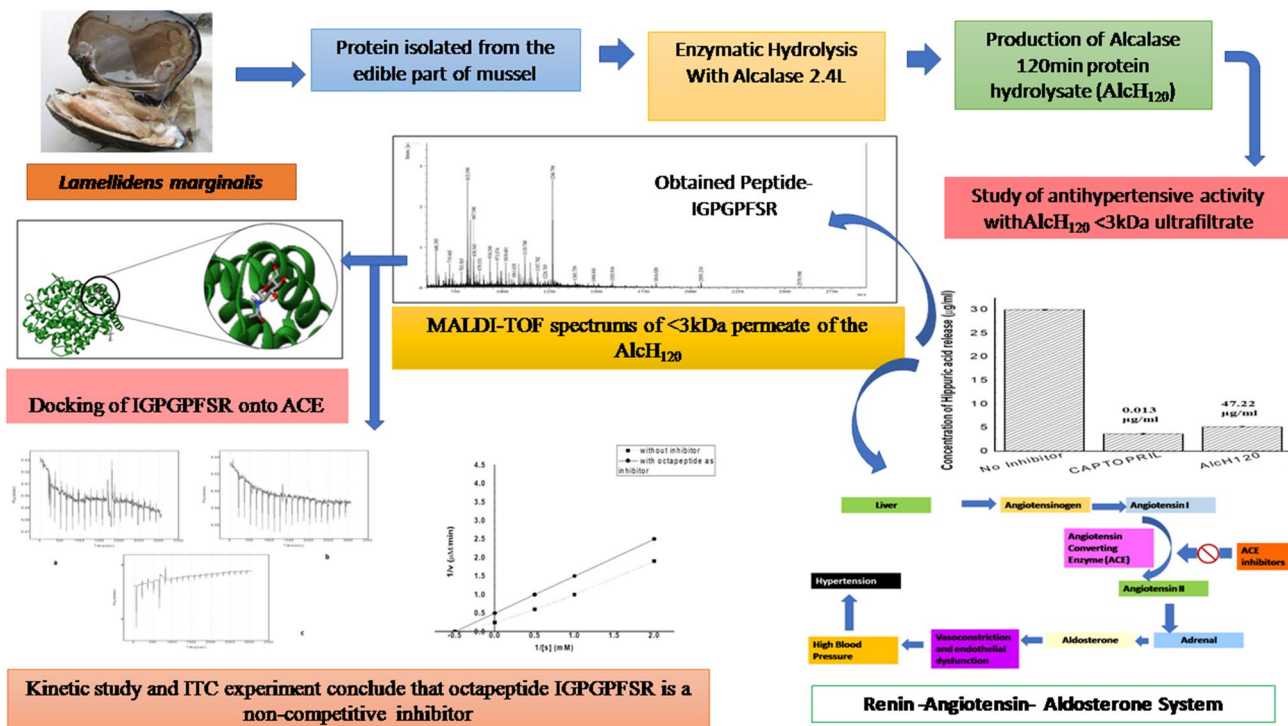
Keywords Angiotensin-I converting enzyme · Isothermal titration calorimetry · *Lamellidens marginalis* · Molecular docking simulation · Non-competitive inhibition · Thermal curve

Introduction

According to the World Health Organization (WHO), cardiovascular diseases (CVD) are the highest-ranked ailments causing global mortality in the twenty-first century (Yusuf et al. 2001). High blood pressure is a multifactorial quantitative trait, controlled by physiological, genetic, and as well as by environmental factors (Jacob 1999). Blood pressure is regulated by the renin–angiotensin–aldosterone system (RAAS). Angiotensin II (Ang II) is formed from the precursor molecule, angiotensinogen which is primarily cleaved into Angiotensin I (Ang I) by renin. Again, Ang I is transformed into Ang II by Angiotensin-I Converting Enzyme (ACE). Ang II contributes to hypertension by inducing vascular smooth muscle constrictions and renal tubule sodium reabsorption (Corvol and Williams 1998). It also imparts a turn-down effect by degrading bradykinin, a vasodilator (Murray and FitzGerald 2007). Angiotensin-I converting enzyme is a membrane-bound zinc metalloproteinase. Two isoforms, namely somatic ACE (sACE) and testis ACE (tACE) have been documented in mammals.

Both the isoforms are very much identical in their C-domain orientation, but sACE contains 36 unique residues in the N-terminal end (Li et al. 2007). C-Domain is important for blood pressure regulation as it has higher catalytic efficiency for both Ang I and the substrate: Hippuryl-L-Histidyl-L-Leucine (HHL) (Wei et al. 1992). Thus, ACE is considered a potential target to treat hypertension. Despite the success of synthetic inhibitors, their long-term consumption may cause serious side effects like loss of taste, dry cough, angioedema, etc. Thus, the need of the hour is to search for natural ACE inhibitors with the least side effects and virtually no risk of overdose compared to synthetic ones. Dietary proteins have long been recognized for their nutritional and pharmacological properties. Several food-derived ACE-inhibitory peptides have been demonstrated as potent antihypertensive agents. Peptides obtained from plant sources through solvent extraction, fermentation, or enzymatic hydrolysis (Dikmen et al. 2017) act as excellent inhibitors of ACE. He et al. (2013) reviewed that marine organisms like shrimps, oysters, and seashells are excellent sources of ACE inhibitory peptides because they adapted themselves to extreme conditions like high and low temperatures, high salt concentration, high pressure, etc. Because of this diverse environmental condition's amino acid sequence of these proteins are quite different from other animal proteins. Thus, peptides from marine sources are excellent sources of ACE inhibitory peptides.

Considerable amount of recent research attested to the potential of the bioactive peptides (2–20 amino acid residues), encrypted within the food proteins, for their utilization as functional food ingredients in nutraceutical therapy. Snake venom was the first from where ACE inhibitory peptide was derived (Ferreira et al. 1970). Many others have also been obtained from the enzymatic digestion of food proteins like fish (Curtis et al. 2002) whey (Ledesma et al. 2002), egg, porcine, and chicken muscle (Nakashima et al. 2002), etc. This scenario has prompted us to investigate the bioactive potential of a novel antihypertensive peptide isolated from a freshwater edible Mollusca *Lamellidens marginalis*; an unconventional resource of animal proteins but still an under-exploited source of health-benefit molecules. The oyster flesh, obtained as a by-product of pearl fisheries is a cheap protein source, and its ethnic medicinal use to treat hypertension has also been documented (Prabhakar and Roy 2009). Several in vivo studies reported that some ACE inhibitory peptides are resistant to degradation by digestive enzymes, capable of intestinal absorption, and remain active in circulation (Vermeirssen et al. 2004; Quiro's et al. 2008). Current studies on the structure–activity relationship have further demonstrated that the presence of amino acids like Trp, Met, Ala, Val, Gly, Ile, Pro, Phe, and Leu at the 3´C position of the peptide interacts with the active site of ACE i.e., S1, S1´ and S2´subsites (Nongonierma and FitzGerald 2015). The presence of tryptophan, phenylalanine, tyrosine, and proline at the C-terminal and branched aliphatic amino acids at the N-terminal end increase the ACE inhibitory activities while peptides containing dicarboxylic amino acids at the C-terminus have little inhibitory activity (Li et al. 2004).

Structure–activity relationship studies of natural ACE inhibitory peptides reveal that some are competitive inhibitors that occupy the active site of the enzyme and compete with the substrate: Hippuryl-L-Histidyl-L-Leucine (HHL) [Natesh et al. 2003; Watermeyer et al. 2008] while other ACE inhibitory peptides inhibit ACE in a non-competitive manner.

In our previous investigation (Haldar et al. 2022), *Lamellidens* muscle protein hydrolysates were prepared using commercially available food grade protease Alcalase2.4L and low molecular weight peptides(< 3 kDa), separated through ultrafiltration were used to study in vitro antihypertensive activity in terms of percentage of ACE inhibition. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed with the ultrafiltrate and six short peptides with high intensity and a molecular weight ranging from 815.445 to 1137.548 Dalton were identified. A novel ACE inhibitory peptide having the amino acid sequence: isoleucine-glycine-proline-glycine-proline-phenylalanine-serine-arginine (IGPGPFSR; molecular weight: 830.953 Dalton) of antihypertensive nature has been selected as this octapeptide consists of high concentration of proline and other hydrophobic residues. Moreover, it shows the highest number of overlaps with previously reported antihypertensive peptides such as GAHypGPAGPGGIHypGERG (Saiga et al. 2003), GPFPILV (Tan et al. 2013) with very low IC_{50} values of 23.42 μM and 42.40 μM respectively.

Thus, in the present investigation, an attempt has been made to explore the underlying mechanism of ACE inhibition by IGPGPFSR which not only help to understand the structure–activity relationship for its utilization as a functional food ingredient against hypertension but also widen our knowledge of drug designing against enzyme targets.

Materials and Methods

The bivalve *Lamellidens marginalis* were collected from the ponds of South 24parganas, West Bengal, India. Authentication of the species was done from the Zoological Survey of India, Prani Vigyan Bhavan, M-Block, and New Alipore. Alcalase2.4L (produced by *B. licheniformis,* Substilisin-A, Sigma Aldrich, P4860, MA, USA) a commercial edible protease was used for hydrolyzing the mussel protein. Hippuryl-L-histidyl-L-leucine (HHL; Sigma Aldrich: H1635), Angiotensin-I Converting Enzyme (ACE: from rabbit lung; Sigma Aldrich: A6778) and Captopril (Sigma Aldrich: C4042) were used for analysing the ACE inhibition activity. All other solvents and chemicals used were of analytical grade.

Preparation of Octapeptide IGPGPFSR

The octapeptide sequence IGPGPFSR was commercially synthesised by the solid-phase method by standard Fmoc chemistry from Pepmic Solution, Suzhou, China. The purity of the peptide was about ≥ 95% which was further confirmed by high-performance liquid chromatography (HPLC).

Enzyme Kinetics of the ACE-Inhibitory Peptide IGPGPFSR

ACE activity was measured with different concentrations of substrate: (0.5, 1and 2 mM of HHL), while enzyme concentration (20 mU/ml) remained constant. The concentration of inhibitory peptide was 1 mg/ml in the inhibitory reaction. Kinetic parameters (K_m and V_{max}) and mode of inhibition were analyzed from the Lineweaver–Burk Plot (Ni et al. 2012).

Isothermal Titration Calorimetry (ITC)

Isothermal titration calorimetry (ITC) study was performed by using the Microcali TC200, GE Healthcare Bioscience Limited. Both the enzyme and the substrate were prepared in sodium borate buffer (pH-8.3) and the enzyme–substrate reaction was carried out at 37 °C. The control reaction cell was loaded with 350 µl of 0.357 µmol/l ACE and was titrated with HHL (1 mM/l) with 20 identical injections. 2 µl of injection volume and a time gap of 120 s between the injections hydrolysed the substrate completely. ITC cell with the same volume of ACE, 50 µl of peptide (0.09 mg/ml and 0.9 mg/ml), prepared with the same buffer was used to study the inhibition reaction. Then the mixture was titrated with 1 mM/L of HHL. Only buffer was injected into the reaction cell as a negative control. Heat change was calculated against a thermally stabilized reference cell and the binding mechanism of the inhibitory peptide was interpreted by studying the peaks (Ni et al. 2012).

Site-Specific Molecular Docking

Peptide Modelling

Molecular dynamics (MD) simulations are frequently used to study the structural behaviour of biomolecular systems (Hospital et al. 2015). MD simulations provide atom-level insights into the mechanistic properties and dynamics of biomolecules over time (Klepeis et al. 2009). Physicochemical properties and secondary structure of the peptide-IGPGPFSR were predicted using PEPDRAW and PEP2D. The peptide was modelled by de novo modelling using PEP-FOLD 3.5 via the RPBS web portal (Singh et al. 2019). A total of 200 models were generated in two steps, involving the addition of side chains with the OSCAR-star program and subsequent minimisation with Gromacs (Lamiable et al. 2016). Generated models were clustered based on the sOPEP score, and the model with the lowest sOPEP score in the largest cluster was selected for further analysis. The peptide was minimised by converging the heavy atoms to RMSD 0.3 Å, using the Protein Preparation Wizard, Schrödinger (Liang et al. 2011). The minimised structure was validated using the ProSA web server (Protein Preparation Wizard: Schrödinger Release 2015–1).

Target Modelling

Structure of the human testicular ACE complexed with captopril, an inhibitor, was retrieved from the Protein Data Bank (PDB) (PDB ID: 1UZF) (Wiederstein and Sippl 2007; Natesh et al. 2004). The missing amino acids in the structure were filled in by homology modelling using Modeller

version 9.24 (Natesh et al. 2004). The structure obtained by homology modelling was prepared using the Protein Preparation Wizard, Schrödinger (Berman et al. 2002). During preparation, the hydrogen bond network was optimised by reorientation of the hydroxyl and thiol groups, the amide moieties of asparagine and glutamine, and the imidazole ring of histidine. The protonation states of the ionisable amino acids, aspartic acid, glutamic acid, and histidine, were predicted using PROPKA at pH 7.0. The structure was finally minimised by converging the heavy atoms to RMSD 0.3 Å. The structure of the target was validated using QMean, ProSA, and Ramachandran plot analysis (Webb and Sali 2016; Benkert et al. 2008; Laskowski et al. 1993).

Protein-Peptide Docking

The peptide was docked to the binding site of the anti-hypersensitive drug, captopril, in ACE using the SP-peptide docking algorithm of Glide, Schrödinger. The grid was prepared around the binding site of captopril in human ACE, and the coordinates of the center of the grid were 42.64, 34.95, and 44.24 respectively. Dimensions of the grid were 30 Å × 30 Å × 30 Å along the x-, y-, and z-axes. The peptide was docked to ACE using the SP-peptide docking mode of Glide, Schrödinger (Friesner et al. 2004). A total of 100 poses were set for post-docking minimisation, and the top-scoring pose was selected as the optimum solution. The docking protocol was validated by re-docking captopril to the binding site, using the same grid and docking parameters.
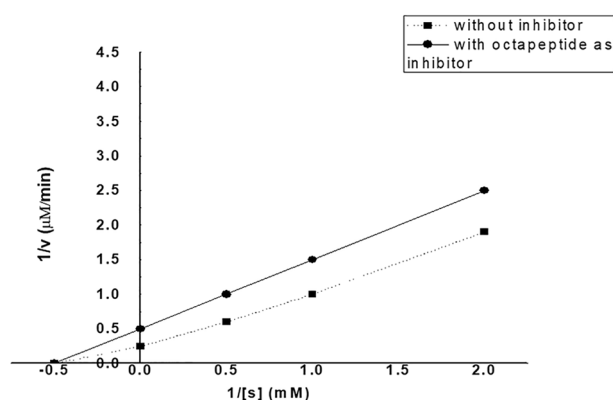
Fig. 1 Lineweaver–Burk plot of ACE activity with inhibitory peptide IGPGPFSR. Control (■) without the inhibitor, 1 mg/ml of octapeptide (●) as inhibitor. 1/V and 1/[S] depicts the reciprocal of velocity and substrate concentration, respectively

Molecular Dynamics (MD) Simulation

The ACE-peptide complex was simulated for 250 ns using Desmond, for determining complex stability and analysing the protein-peptide interactions (Bowers 2006). The system was prepared by solvating in an orthorhombic shell of TIP4P solvent, and the boundary was set to 10 Å. The system was first neutralised, followed by the addition of Na^+ and Cl^- ions until the concentration of the solution reached 0.15 M and then simulated for 250 ns using the NPT ensemble, at a temperature of 310 K and a pressure of about 1.01325 bar. The trajectory was analysed with Desmond. RMSD values of the receptor and peptide, RMSF values of the receptor, and receptor-peptide interactions were determined.

Table 1 Kinetic parameters of the octapeptide IGPGPFSR identified from the ultrafiltrate (<3 kDa) of *Lamellidens marginalis* Alcalase2.4L 120 mins protein hydrolysate

	K_m(mM)	V_{max} (μM/min)	R^2
ACE+HHL (no inhibitor)	2.68	3.48	0.98
ACE+HHL+octapeptide (IGPGPFSR)	2	2	0.99

All values are presented as mean±standard deviation for triplicate experiments

Statistical Analysis

All experiments were carried out in triplicate and data are presented as mean±standard deviation (SD). Data comparison and analysis were done using Origin 8.1 (Origin Lab Corporation, Northampton, MA, USA). A *T*-Test ($p < 0.05$) was employed to determine the significant difference between the means.

Results and Discussion

Enzyme Kinetic Study of the ACE-Inhibitory Peptide IGPGPFSR

Inhibitory kinetics elucidates the type of enzyme inhibition i.e., whether a particular inhibitor inhibits a reaction in a competitive, non-competitive, or un-competitive way. Figure 1 represents the enzyme–substrate reaction in the presence and absence of the peptide IGPGPFSR as an inhibitor. The Lineweaver–Burk plot reveals that the inhibition is of a non-competitive type as the lines intersect at the 1/[s] axis. The graph further indicates that the inhibitory peptide binds with the ACE molecule whether the substrate HHL is bound or not. But binding of IGPGPFSR with ACE prevents the release of the end product hippuric acid (HA). The V_{max} of the inhibited reaction is lower than the enzyme–substrate reaction without the inhibitor (Table 1). Moreover, the K_m
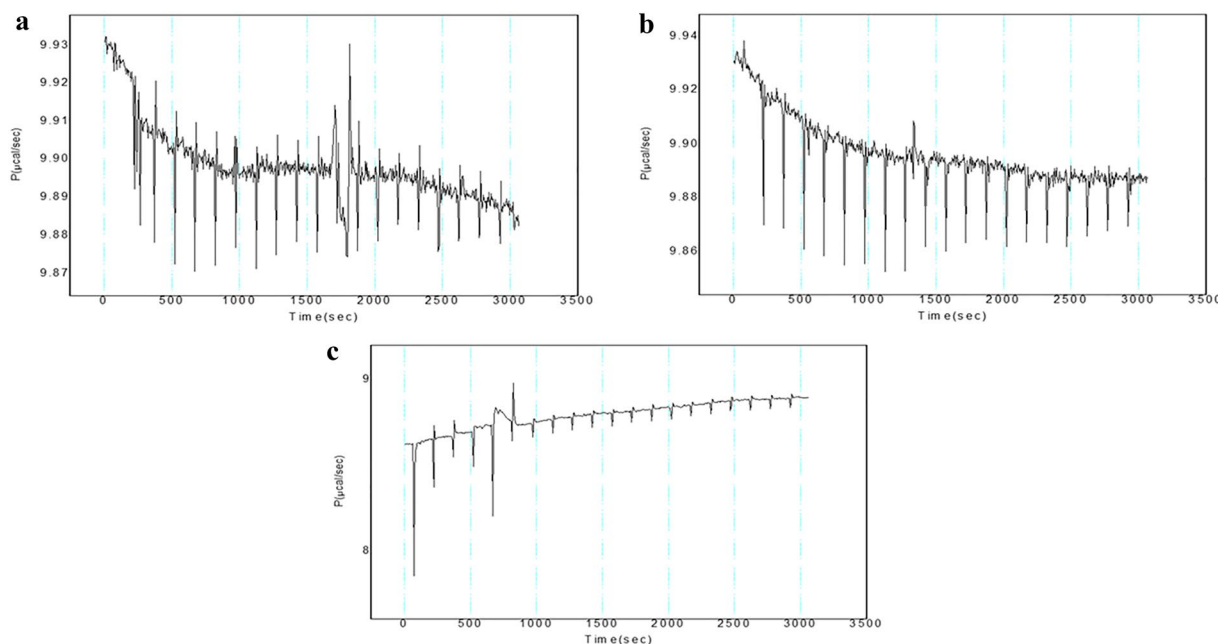
Fig. 2 Isothermal titration calorimetric analysis. **a** Binding of ACE to HHL at a concentration of 1 mM without any inhibitor at pH-8.3. **b** Interaction of HHL, ACE and the inhibitory peptide IGPGPFSR at a concentration of 0.09 mg/ml. **c** with inhibitory peptide at a concentration of 0.9 mg/ml

value in both cases reflects that the inhibitor does not compete with the substrate for binding with ACE which again confirms the non-competitive nature of inhibition.

Isothermal Titration Calorimetry

ITC experiments are specifically used for mechanistic assessment of inhibition based on the thermal parameters of enzyme–substrate reaction (Dey et al. 2021). Figure 2 shows a significant difference between the titration curves in the absence and presence of the ACE inhibitory peptide. Substrate HHL, titrated with the enzyme into the ITC cell results in the hydrolysis of HHL into HA by ACE. Figure 2a depicts the complete binding of HHL to ACE resulting in a higher titration peak with an enthalpy change ($\Delta H = -5.66*10^{-6}$ kJ/mol). But the reaction proceeds towards equilibrium, as the catalytic sites get saturated with the increased amount of product i.e., HA. The thermogram indicates that the net heat rate decreases with the gradual decrease of the peak in size. At the end of the titration, full saturation has been achieved and background heat of the dilution peak is observed.

Addition of the inhibitory peptide IGPGPFSR (0.09 mg/ml) into the ITC leads to a slow but gradual decrease in the conversion of HHL to HA with K_i of 0.03 ± 16. Figure 2b shows that during the experiment, substrate HHL continuously binds to ACE while the inhibitory peptide IGPGPFSR binds to ACE at a different site than HHL. This study further suggests that when both HHL and inhibitor peptide IGPGPFSR bind to ACE, an enzyme–substrate-inhibitor (ESI) complex is produced which results in little or no product formation. On the other hand, an increase in inhibitor concentration to 0.9 mg/ml (Fig. 2c), results in a decrease in the rate of substrate binding ($Ki = 69.7 \pm 96$) and ultimately leads to no product formation.

This study indicates that although a little amount of product (HA) is formed at a low concentration of inhibitor in the reaction mixture, HA production is almost nil at the higher concentration of the inhibitory peptide. ITC study thus validates the inhibitory kinetics that binding of the antihypertensive peptide (IGPGPFSR) to ACE produces an inhibitory effect by limiting product formation in a non-competitive way. Therefore, a docking simulation study was performed to explore the underlying mechanism of ACE inhibition by IGPGPFSR.

Molecular Docking of IGPGPFSR onto ACE

Site-specific molecular docking was carried out to confirm the non-competitive nature of inhibition between ACE and the peptide IGPGPFSR.

Peptide Modeling

Approximately, 200 models are generated and clustered based on sOPEP score using the online software PEP-FOLD 3.5. Results of clustering reveal that cluster-2 is the largest one comprising 30 models. The model with the lowest
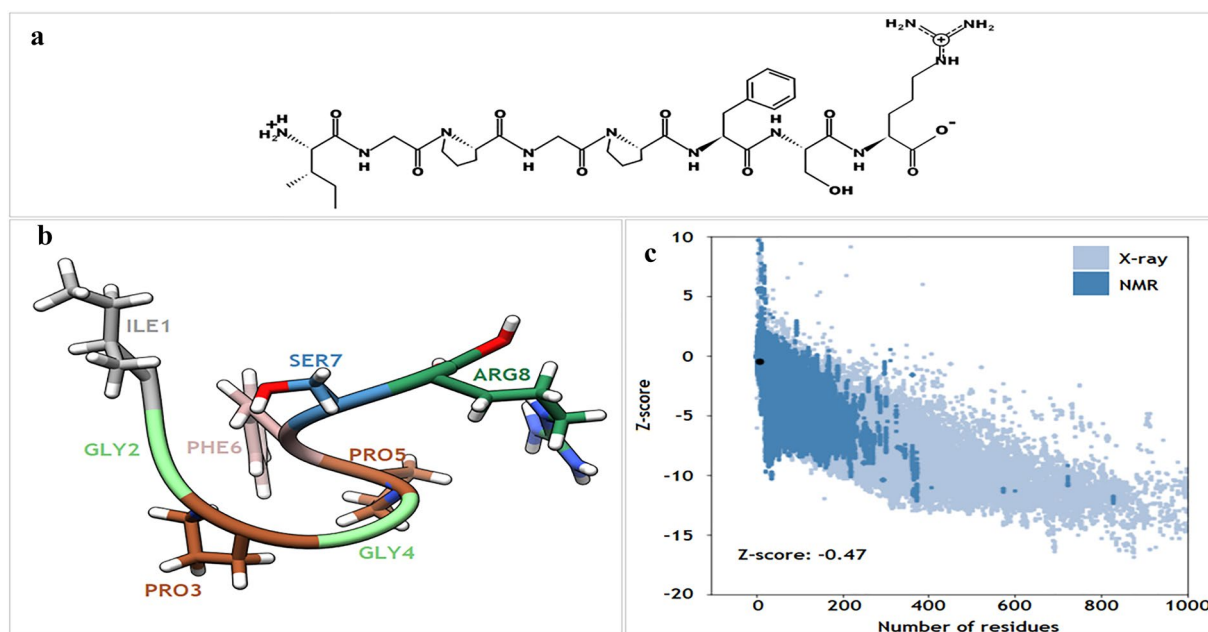

Fig. 3 **a** 2D structure of octapeptide IGPGPFSR. **b** 3-dimentional structure of the peptide in ribbon representation shows different amino acids in separate colours. **c** Validation of the quality of the peptide model with ProS reveals that the model quality (Z-score: −0.47) is comparable to that of PDB structures determined using NMR
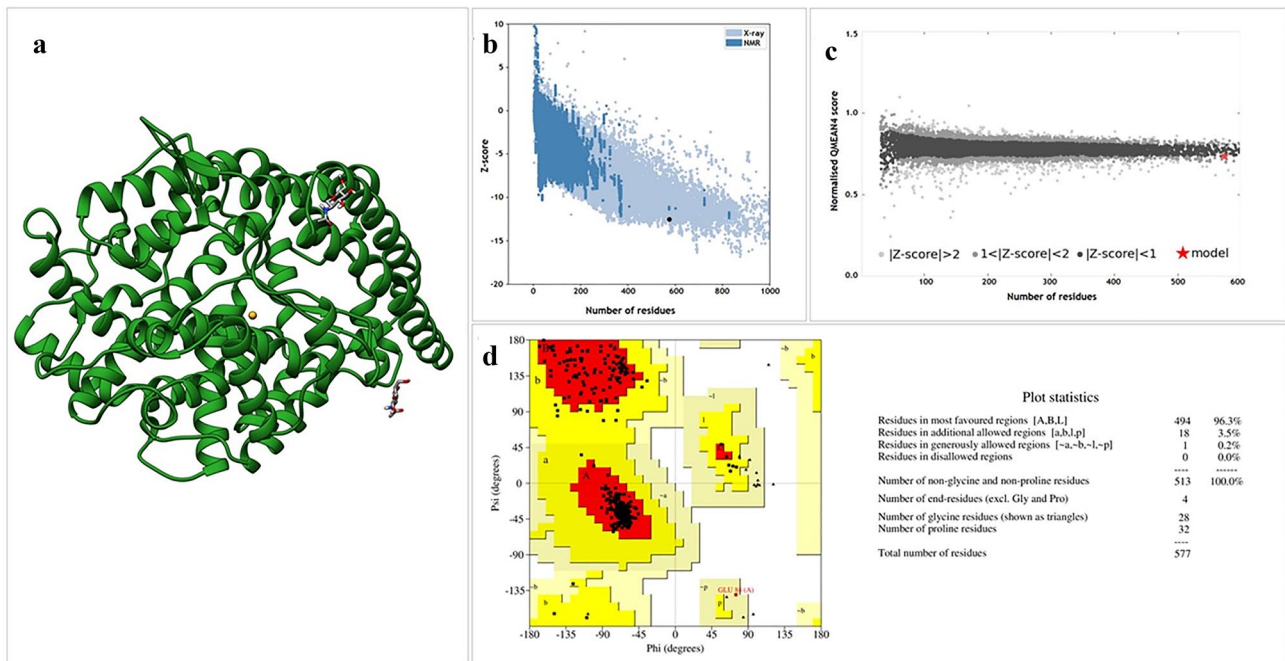
Fig. 4 a Homology model of the ACE receptor constructed by homology modelling, in ribbon representation. The zinc atom is represented by yellow sphere, and the ligands in CPK colour represent two molecules of NAG. **b** Validation of the model of ACE by ProSA reveals that the quality of the ACE model is comparable to protein structures determined by X-ray crystallography. **c** Qmean value reveals good model quality. **d** Validation of the homology model of ACE by Ramachandran plot analysis
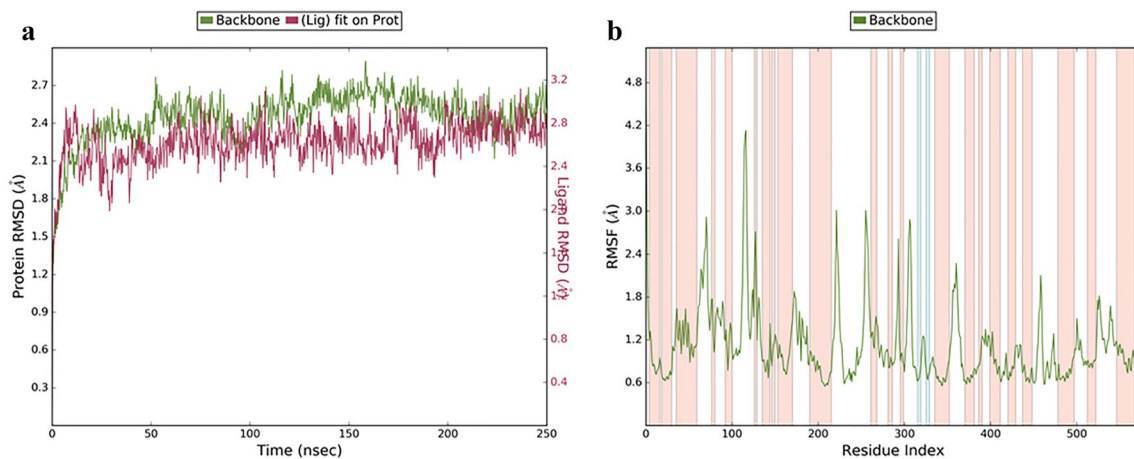

Fig. 5 a RMSD values of the protein backbone (green) and peptide ligand (red) over the 250 ns trajectory. The RMSD values become steady after the initial 50 ns, and remains steady thereafter, indicating that the system had reached equilibrium. **b** RMSF values of the protein backbone (in Å) over the 250 ns trajectory. The alpha helices and beta strand regions are highlighted in red and blue, respectively. These regions being more rigid than coiled regions, showing lower values of RMSF than that of the unstructured regions

sOPEP energy (− 2.75), has been selected and minimized with the Protein Preparation Wizard: Schrödinger (Fig. 3a, b). Figure 3c, obtained from ProSA emphasizes that the quality of the peptide is comparable with that of the structures of similar size in the PDB that have been determined using NMR.

Validation results of the structure of human ACE with ProSA show that the quality of the modelled structure (Fig. 4a) is comparable to that of proteins of similar size in the PDB, determined by X-ray crystallography (Fig. 4b). Results of QMean validation reveal that the normalised QMEAN score of the ACE model is comparable to those of

Fig. 6 Schematic diagram of the peptide-ACE interactions that stabilised the complex throughout the trajectory. The occupancy of the peptide-protein interactions over the 250 ns trajectory are indicated in %. The hydrogen bonds with the main chains and side chains are indicated by solid and dashed purple lines, respectively. The occupancy of all the interactions is > 60%, indicating that the interactions are highly stable and important for stabilising the peptide-ACE complex
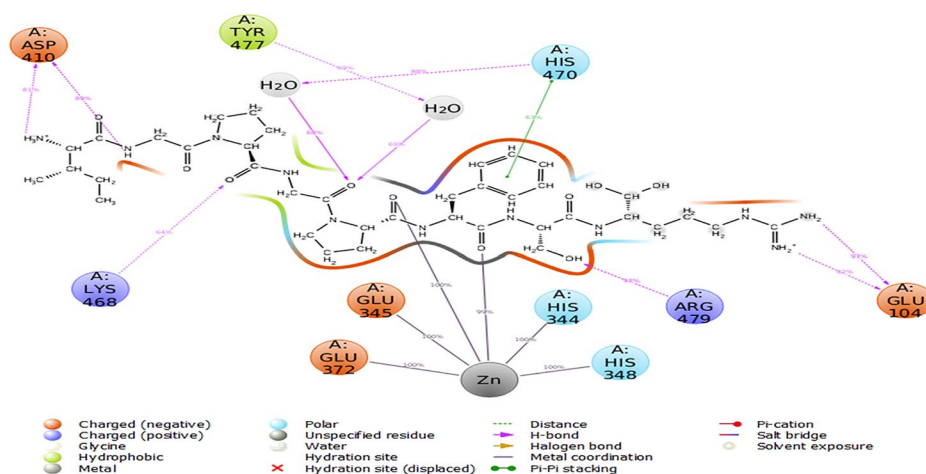
proteins of similar size in the PDB (Fig. 4c). Ramachandran plot analysis depicts that 96.3% of residues are in the most favoured regions of the plot, and none of the residues are in the disallowed regions (Fig. 4d). For a model of good quality, over 90% of residues should fall within the most favoured regions. These results thus indicate that the model of the ACE protein is of good quality.

Peptide Docking and MD Simulation

The docking protocol was validated by re-docking captopril to the binding site using the same grid and docking parameters used for docking the peptide. The RMSD between the docked pose and the experimental structure of captopril is 0.333 Å; which indicates that the docking protocol is capable of predicting the native binding pose of ligands. The docking score of the peptide IGPGPFSR is -5.28 on the binding with ACE. The peptide receptor complex was subjected to MD simulation for 250 ns. Figure 5a shows, throughout the trajectory (≤ 2.0 Å), the RMSD values of the receptor backbone and the peptide becomes steady after 250 ns, and remain steady thereafter, throughout the trajectory, suggesting that the system has reached equilibrium. Analysis of RMSF values of the protein backbone expresses further that higher RMSF refers to the regions containing loops while lower RMSF values signify α-helices and β strands (Fig. 5b). These secondary structures are rigid and undergo less alteration. However, as loops and coils are flexible and prone to variations, thus evidence of higher values of RMSF.

The active site of ACE comprises of HEXXH…E motif which includes His 383, Glu384, His 387, and Glu 411 and a zinc atom. ACE constitutes 2 sub-sites, S1′ and S2′ which are mainly occupied by competitive inhibitors. As per the crystallographic structure (PDB code 1UZF), the inhibitor occupies the S1′ and S2′ sub-sites of the active site and restricts the binding of HHL (Ciulli and Abell 2005). But in this study, IGPGPFSR does not occupy the S1′ and

S2′ sub-sites, rather ACE-IGPGPFSR complex is stabilized by 8 hydrogen bonds, out of which 2 are water-mediated hydrogen bonds (Fig. 6). The frequency of the hydrogen bonds is above 80% when amino acids: Asp410, Lys468, His470, Arg479, and Glu104 of ACE interacts with Ile1, Pro3, Gly4, Ser7 and Arg8 of the peptides respectively. These interactions are crucial for stabilizing the peptide-ACE complex. Phe6 undergoes one pi-pi stacking interaction with His470 with 100% frequency and plays an important role in stabilizing the complex. Only Pro5 and Phe6 interact with the zinc (Zn^{+2}) ion at a frequency of 100% and 99% respectively which changes the position of the ion from the active site and results in the conformational alteration of the enzyme. As a result, HHL may bind to the active site but be unable to form a product (Hippuric acid) (Hospital et al. 2015). Li et al. 2020 demonstrated the structure–activity relationship study of ACE with the anti-SARS octapeptide AVLQSGFR. Presence of phenylalanine (F) and arginine (R) at the C-terminal increases the ACE inhibitory activity. Moreover, the presence of a positive charge on the guanidine group on arginine residue induces the inhibitory activity of the octapeptide. Molecular docking thus confirms that the low molecular weight bioactive peptide IGPGPFSR inhibits ACE activity in a non-competitive manner.

Conclusion

Taken together the present observations made in this study suggest that the low molecular weight, novel antihypertensive peptide IGPGPFSR isolated from the freshwater edible bivalve *Lamellidens marginalis* muscle protein hydrolysate is a potent inhibitor of ACE. Enzyme kinetics in combination with ITC supports that this octapeptide inhibits the release of hippuric acid in a non-competitive manner. Molecular docking reveals that the enzyme-inhibitor complex is stabilized by eight (8) hydrogen bonds. Interaction

of Pro5 and Phe6 with zinc (Zn^{+2}) present in the active site alters the conformation of the enzyme which results in little or no conversion of HHL to HA which further confirms that IGPGPFSR is a non-competitive inhibitor of ACE. The results indicate that this novel ACE inhibitor can be used as an auxiliary for nutraceutical therapy against hypertension. Moreover, the underlying mechanism of ACE inhibition by this bioactive peptide IGPGPFSR will help in widening our knowledge of drug designing against enzyme targets. Further in vivo study will be needed to explore the bioavailability of this novel antihypertensive peptide.

Authors Contributions Conceptualisation: TKD, PD, JC, Methodology: MD, AH, RC, TKD, Formal analysis and Investigation: MD, AH, RC, AG, TKD, SR, Writing- Original Draft preparation: MD, JC, Writing- Review and editing: MD, PD, JC, Resources: PD, JC, Supervision: PD, JC.

Declarations

Conflict of interest There is no conflict of interest to declare.

Ethical Approval Neither animal nor human studies were conducted by any of the authors.

References

Benkert P, Tosatto SCE, Schomburg D (2008) QMEAN: a comprehensive scoring function for model quality assessment. Proteins 71:261–277. https://doi.org/10.1002/prot.21715

Berman HM, Battistuz T, Bhat TN, Bluhm WF, Bourne PE, Burkhardt K et al (2002) The protein data bank. Acta Crystallogr Sect D Biol Crystallogr 58:899–907. https://doi.org/10.1107/S0907444902003451

Bowers KJ (2006) Scalable algorithms for molecular dynamics simulations on commodity clusters. in Proceedings of the 2006 ACM/IEEE Conference on Supercomputing, SC'06 84 (ACM Press, 2006) https://doi.org/10.1145/1188455.1188544.

Ciulli A, Abell C (2005) Biophysical tools to monitor enzyme-ligand interactions of enzymes involved in vitamin biosynthesis. Biochem Soc Trans 33:767. https://doi.org/10.1042/bst0330767

Corvol P, Williams TA (1998) Handbook of Proteolytic Enzymes. Academic, London

Curtis JM, Dennis D, Waddell DS, Macgillivray T, Ewart HS (2002) Determination of angiotensin-converting enzyme inhibitory peptide Leu-Lys-Pro-Asn-Met (LKPNM) in bonito muscle hydrolysates by LC–MS/MS. J Agric Food Chem 50:3919–3925. https://doi.org/10.1021/jf011684c

Dey TK, Chatterjee R, Mandal RS, Roychowdhury A, Paul D, Roy S, Pateiro M, Das AK, Lorenzo JM, Dhar P (2021) ACE inhibitory peptides from *Bellamya bengalensis* protein hydrolysates: in vitro and in silico molecular assessment. Processes 9:1–13. https://doi.org/10.3390/pr9081316

Dikmen CD, Yucetepe A, Guler FK, Daskaya H, Ozcelik B (2017) Angiotensin-1-converting enzyme (ACE)-inhibitory peptides from plants. Nutrients 9:316. https://doi.org/10.3390/nu9040316

Ferreira SH, Bartelt DC, Greene LJ (1970) Isolation of bradykinin-potentiating peptides from *Bothrops jararaca* venom. Biochemistry 9:2583–2593. https://doi.org/10.1021/bi00815a005

Friesner RA, Banks JL, Murphy RB, Halgren TA, Klicic JJ, Mainz DT et al (2004) Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. J Med Chem 47:1739–1749. https://doi.org/10.1021/jm0306430

Haldar A, Das M, Dey TK, Dhar P, Chakrabarti J (2022) Isolation of an antihypertensive bioactive peptide from the freshwater mussel *Lamellidens marginalis*. Int J Food Nutr Sci 11(1):1–8

He HL, Liu D, Ma CB (2013) Review on the angiotensin-I-converting enzyme (ACE) inhibitor peptides from marine proteins. Appl Biochem Biotechnol 169:738–749. https://doi.org/10.1007/s12010-012-0024-y

Hospital A, Goñi JR, Orozco M, Gelpí JL (2015) Molecular dynamics simulations: advances and applications. Adv Appl Bioinform Chem 8:37–47

Jacob HJ (1999) Physiological genetics: application to hypertension research. Clin Exp Pharm Phys 26:530–535. https://doi.org/10.1046/j.1440-1681.1999.03078.x

Klepeis JL, Larsen KL, Dror RO, Shaw DE (2009) Long-timescale molecular dynamics simulations of protein structure and function. Curr Opin Struct Biol 19:120–127. https://doi.org/10.1016/j.sbi.2009.03.004

Lamiable A, Thevenet P, Rey J, Vavrusa M, Derreumaux P, Tuffery P (2016) PEP-FOLD3: faster de novo structure prediction for linear peptides in solution and in complex. Nucleic Acids Res 44:449–454. https://doi.org/10.1093/nar/gkw329

Laskowski RA, MacArthur MW, Moss SJ, Thornton M (1993) PROCHECK: a program to check the stereochemical quality of protein structures. J Appl Crystallogr 26:283–291. https://doi.org/10.1107/S0021889892009944

Ledesma BH, Recio I, Ramos M, Amigo L (2002) Preparation of ovine and caprine b-lactoglobulin hydrolysates with ACE-inhibitory activity. Identification of active peptides from caprine b-lactoglobulin hydrolysed with thermolysin. Int Dairy J 12:805–812. https://doi.org/10.1016/S0958-6946(02)00080-8

Li GH, Le GW, Shi YH, Shrestha S (2004) Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. Nutr Res 24:469–486. https://doi.org/10.1016/j.nutres.2003.10.014

Li GH, Qu MR, Wan JZ, You JM (2007) Antihypertensive effect of rice protein hydrolysate with in vitro angiotensin I-converting enzyme inhibitory activity in spontaneously hypertensive rats. Asia Pac J Clin Nutr 16:75. https://doi.org/10.6133/APJCN.2007.16.S1.52

Li H, Ren J, Zhang Z, Chang N, Qin C (2020) Greener liquid phase synthesis and the ACE inhibitory structure-activity relationship of an anti-SARS octapeptide. Org Biomol Chem 18:8433–8442. https://doi.org/10.1039/D0OB01948H

Liang S, ZhengStandley DDM (2011) Fast and accurate prediction of protein side-chain conformations. Bioinformatics 27:2913–2914. https://doi.org/10.1093/bioinformatics/btr482

Murray BA, FitzGerald RJ (2007) Angiotensin converting enzyme inhibitory peptides derived from food proteins: biochemistry bioactivity and production. Curr Pharm Des 13:773–791. https://doi.org/10.2174/138161207780363068

Nakashima Y, Arihara K, Sasaki A, Ishikawa MH, Itoh SM (2002) Antihypertensive activities of peptides derived from porcine skeletal muscle myosin in spontaneously hypertensive rats. J Food Sci 67:434–437. https://doi.org/10.1111/j.1365-2621.2002.tb11424.x

Natesh R, Schwager SLU, Sturrock ED, Acharya KR (2003) Crystal structure of human angiotensin-converting enzyme –lisinopril

complex. Nature 421:551–554. https://doi.org/10.1038/nature01370

Natesh R, Schwager SLU, Evans HR, Sturrock ED, Acharya KR (2004) Structural details on the binding of antihypertensive drugs captopril and enalaprilat to human testicular angiotensin I-converting enzyme. Biochemistry 43:8718–8724. https://doi.org/10.1021/bi049480n

Ni H, Li L, Liu G, Hu SQ (2012) Inhibition mechanism and model of an angiotensin 1 converting enzyme (ACE)-inhibitory hexapeptide from yeast (Saccharomyces cerevisiae). PLoS ONE 7(5):1–7. https://doi.org/10.1371/journal.pone.0037077

Nongonierma AB, FitzGerald RJ (2015) The scientific evidence for the role of milk protein-derived bioactive peptides in humans: a Review. J Funct Foods 17:640–656

Prabhakar AK, Roy SP (2009) Ethno-medicinal uses of some shell fishes by people of Kosi River basin of North-Bihar India. Stud Ethno-Med 3(1):1–4

Quiro´s A, Da´valos A, Lasuncio´n MA, Ramos M, Recio I (2008) Bioavailability of the antihypertensive peptide LHLPLP: transepithelial flux of HLPLP. Int Dairy J 18:279–286

Saiga A, Tanabe S, Nishimura T (2003) Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. J Agric Food Chem 51(12):3661

Singh H, Singh S, Raghava GPS (2019) Peptide secondary structure prediction using evolutionary information. bioRxiv. https://doi.org/10.1101/558791

Tan J, Tian F, Lv Y, Liu W, Zhong L, Liu Y (2013) Yang L (2013) Integration of QSAR modelling and QM/MM analysis to investigate functional food peptides with antihypertensive activity. Mol Simul. https://doi.org/10.1080/08927022788247

Vermeirssen V, Camp JV, Verstraete W (2004) Bioavailability of angiotensin I converting enzyme inhibitory peptides. Br J Nutr 92:357–366. https://doi.org/10.1079/bjn20041189

Watermeyer JM, Kroger WL, Neill HGO, Sewell BT, Sturrock ED (2008) Probing the basis of domain-dependent inhibition using novel ketone inhibitors of angiotensin-converting enzyme. Biochemistry 47:5942–5950. https://doi.org/10.1021/bi8002605

Webb B, Sali A (2016) Comparative protein structure modeling using MODELLER. Curr Protoc Bioinform. https://doi.org/10.1002/cpbi.3

Wei L, Clauser E, Alhenc-Gelas F, Corvol P (1992) The two homologous domains of human angiotensin I-converting enzyme interact differently with competitive inhibitors. J Biol Chem 267:13398–13405. https://doi.org/10.1016/s0021-9258%2818%2942224-7

Wiederstein M, Sippl MJ (2007) ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Res 35:407–410. https://doi.org/10.1093/nar/gkm290

Yusuf S, Reddy S, Ounpuu S, Anand S (2001) Global burden of cardiovascular diseases. Part I: general considerations, the epidemiologic transition, risk factors, and impact of urbanization. Circulation 104:2746–2753. https://doi.org/10.1161/hc4601.099487