**Predictive modelling of angiotensin converting enzyme inhibitory dipeptides**

Roseanne Norris[1], Fergal Casey[2], Richard J. FitzGerald[1]*, Denis Shields[2]* and Catherine Mooney[2]

[1] Department of Life Sciences and [1*]Food for Health Ireland,
University of Limerick, Ireland.
[2] Complex and Adaptive Systems Laboratory,
Conway Institute of Biomolecular and Biomedical Sciences, and
School of Medicine and Medical Science, University College Dublin, Ireland.

*Joint Corresponding Authors: R. J. FitzGerald; mailing address: Department of Life Sciences, University of Limerick, Limerick, Ireland; Tel.: +353 (0) 61 202598; Fax: +353 (0) 61 331490; E-mail: dick.fitzgerald@ul.ie
and Denis Shields; mailing address: School of Medicine & Medical Sciences, University College Dublin, Dublin, Ireland; Tel.: +353 (0) 1 7165344; E-mail: denis.shields@ucd.ie

# Abstract

The ability of docking to predict angiotensin converting enzyme (ACE) inhibitory dipeptide sequences was assessed using AutoDock Vina. All potential dipeptides and phospho-dipeptides were docked and scored. Peptide intestinal stability was assessed using a prediction amino acid clustering model. Selected dipeptides having AutoDock Vina scores ≤ -8.1 and predicted to be 'stable' intestinally were characterised using LIGPLOT and for ACE-inhibitory potency. Two newly identified ACE-inhibitory dipeptides Asp-Trp and Trp-Pro having Vina scores of -8.3 and -8.6 gave $IC_{50}$ values of 257.93 ± 4.23 and 217.27 ± 15.74 μM, respectively. LIGPLOT analysis indicated no zinc interaction for these dipeptides. Phospho-dipeptides were predicted to have a good affinity for ACE. However, the experimentally determined $IC_{50}$ results did not correlate since, for example, Trp-pThr and Pro-pTyr having Vina scores of -8.5 and –8.1, respectively, displayed $IC_{50}$ values > 500 μM. While docking allowed identification of new ACE inhibitory dipeptides, it may not be a fully reliable predictive tool in all cases.



Key Words: ACE inhibition, dipeptides, predictive modelling, AutoDock Vina, intestinal stability.

# 1.0 Introduction

Angiotensin converting enzyme (ACE, EC 3.4.15.1) is a key target for the treatment of hypertension due to its involvement in a number of blood pressure-related systems, i.e., the renin-angiotensin system (RAS) and the kinin nitric oxide system (KNOS). In the RAS, ACE cleaves angiotensin I into the potent vasoconstrictor angiotensin II, and in the KNOS, ACE inactivates the hypotensive peptide, bradykinin (Eriksson, Danilczyk, & Penninger, 2002). Therefore, excessive action of ACE leads to increased vasoconstriction and hypertension. The discovery of the first ACE-inhibitory peptides in the venom of the snake *Bothropa jararace* in the 1970*s* led to the development of synthetic ACE inhibitors such as Captopril, Lisinopril and Enalapril (Meyer, Essenburg, Smith, & Kaplan, 1982). However, the use of these pharmacological ACE inhibitors is associated with a range of side-effects including angiodema, cough, skin rashes, reduced renal function and fetal abnormalities (Libby, Bonow, Mann & Zipes, 2008).

There has been an ongoing search for natural food-derived ACE inhibitory peptides. These peptides have been identified in a variety of food proteins and become active when released from their parent proteins through enzymatic hydrolysis in food processing and/or digestion (Murray and FitzGerald, 2007). Although these peptides are less potent than synthetic ACE inhibitors ($IC_{50}$ values in μM range), they have potential as active components in the diet by integration into functional food products. At present, the search for ACE inhibitory peptides has focused primarily on the production and characterisation of peptides isolated from microbial fermentation or enzymatic digests of proteins with limited studies involving the relationship between structure and activity of peptide inhibitors (FitzGerald & Meisel, 2000). However,

some structure-activity features of ACE inhibitory peptides have been identified. Potent ACE-inhibitory peptides are generally short sequences (2-12 amino acids) and contain aromatic or hydrophobic residues at their C-terminus, with many containing Pro, Trp and Lys residues (Murray, & FitzGerald, 2007; López-Fandiño, Otte, & van Camp, 2006; FitzGerald & Meisel, 2000; Pripp, Isaksson, & Stepaniak, 2004). ACE also seems to prefer positively charged functional groups at the N-terminus. Bulky and hydrophobic side chains seem to be features favoured by ACE inhibitory dipeptides (Wu, Aluko, & Nakai, 2006a). ACE, being a metallopeptidase, contains a Zinc binding motif, HEXXH (X = any amino acid residue), which is located on both the C- and N-domains of somatic ACE (Ehlers & Riordan, 1991). The active sites of the two-domain somatic ACE are located within the cleft of the two domains. The $Zn^{2+}$ molecule binds to the two histidines of the motif, and is known to directly interact with inhibitors at the active site. The cleft is partially covered by an N-terminal 'lid' thus allowing for only small peptides to reach the active sites (Natesh, Schwager, Sturrock & Acharya, 2003). Everything that is known about the structure-activity relationship of these peptides has been derived from qualitative analysis with chemically synthesized peptides or with analogues that have similar structures to known inhibitors, work which is both time-consuming and costly.

Quantitative computational methods such as quantitative structure-activity relationship modelling (QSAR), artificial neural networks (ANN), and virtual substrate docking are tools increasingly been applied in medicinal and pharmaceutical drug discovery. They allow for a statistical prediction of the potential of a small molecule (ligand) to bind to a macromolecule (receptor), while allowing for a better understanding of the molecular mechanisms involved (Pripp, Isakasson, Stepaniak, Sorhaug, & Ardo**,** 2005). There is significant scope for predictive approaches to be

applied as an alternative method for identifying bioactive sequences in food proteins. (Pripp, Isakasson, Stepaniak, Sorhaug, & Ardo**,** 2005). There has been a small number of QSAR and ANN studies carried out to date that have focused on ACE-inhibitory peptides from food (Wu Alka, & Nakai 2006a; Wu Alka, & Nakai 2006b; Majumder & Wu, 2010; Meisel, Walsh, Murray & FitzGerald, 2006). Docking studies have potential in the area of ACE-inhibitory peptides as it means many peptides can be virtually screened for their theoretical inhibitory activity (Pripp, 2007). All possible docking or binding conformations can be assessed for their binding affinity to a receptor, and then 'scored' on their potential complementarities. Although experimental verification is still required, if a correlation between theoretical and actual ACE-inhibitory potential is found, target peptides of interest may be identified without the need for a time-consuming conventional peptide discovery strategy (Majumder & Wu, 2010). Furthermore, quantitatively relating peptide sequence to inhibitory activity allows for physicochemical interpretation and for further molecularly-designed optimisation experiments (Pripp, 2007).

AutoDock Vina is a molecular docking program which can be used for virtual screening. In the case of ACE, this programme allows the docking of peptide sequences with the active site, e.g., against PDB structure 1UZF (complex of captopril and human testicular angiotensin converting enzyme, Natesh, Schwager, Evans, Sturrock, & Acharya, 2004). Additional *in silico* approaches to characterise ligand-protein interactions are also available. The LIGPLOT programme allows the generation of schematic diagrams of protein-ligand interactions (Wallace, Laskowski, & Thornton, 1995) and has the potential to provide useful information on key interactive events during binding. The intestinal stability and thus the bioavailability of peptides is central to their ability to mediate a physiological response (Vermerissen,

van Camp, & Verstraete, 2004). The intestinal stability of peptides may be assessed using an amino acid clustering model (Foltz, van Buren, Klaffke, & Duchateau, 2009). The model measures how stable a peptide may be under simulated small intestinal digestion conditions.

The objective of this study was firstly to assess the potential of using AutoDock Vina and LIGPLOT analysis along with small intestinal stability modelling for the *in silico* identification of potent ACE-inhibitory dipeptides. The second objective was to quantify the *in vitro* ACE-inhibitory potency of the identified dipeptides in order to validate the potential of the predictive computational methods in the discovery of potent ACE-inhibitory peptides.

# 2.0 Materials and Methods

## 2.1 Materials

Captopril, enalapril maleate, lisinopril, rabbit lung acetone powder, and sodium tetraborate decahydrate (borax) were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). *o*-Aminobenzoylglycl-*ρ*-nitro-*L*-phenylalanyl-*L*-proline (Abz-Gly-Phe-($NO_2$)-Pro) and o-aminobenzoylglycine (Abz-Gly-OH) were from Bachem Feinchemikalien (Bubendorf, Switzerland). Synthetic peptides Pro-Tyr, Trp-Thr, Tyr-Thr, Pro-pTyr., pTyr-pSer, Trp-Pro, Trp-pThr, Tyr-Ser, pTyr-pThr and Asp-Trp (where p represents a phosphorylated residue) were obtained from GenScript Corporation (Piscataway, NJ, USA).

## 2.2 Computational analysis

AutoDock Vina was used to dock all 529 possible dipeptides (from the twenty standard amino acids and three phosphorylated amino acids, i.e., Tyr, Ser and Thr) and six synthetic drug inhibitors (captopril, enalaprilat, lisinopril, RXPA380, kAF and kAW) against chain A of the PDB structure 1UZF (Natesh, Schwager, Evans, Sturrock, & Acharya, 2004). The structures of the six drug inhibitors were taken from their respective PDB files (see Table 1) and the initial poses of the PDB-formatted structures of dipeptides were generated using the Open Babel Package, version 2.1.1 (Guha *et al*., 2006). AutoDockTools (ADT) was used to prepare the ligands and the 1UZF receptor and to determine the 'search space'. Dipeptides and inhibitors were then docked with the PDB structure 1UZF giving a Vina score i.e., the predicted affinity of the molecule to bind to the PDB structure, calculated in kcal/mol. A more negative score indicates that a molecule (ligand) is more likely to dock with the structure (receptor) and achieve more favourable interactions. The Vina scoring system was tuned using PDBbind and is described in Trott & Olsen (2010).

LIGPLOTs were generated for the six drug inhibitors (using their PDB files as input) and the highest ranked dipeptides, i.e., having Vina scores $\leq$ -8.1 (using the Vina docking poses) in complex with ACE according to the protocol described by Wallace, Laskowski, & Thornton (1995).

Dipeptides were assessed for their intestinal stability using an amino acid clustering model adopted from Foltz, van Buren, Klaffke, & Duchateau (2009). This model rates dipeptides as 'stable,' 'neutral' or 'unstable' with regard to small intestinal stability using correlations between the N- and C-terminal amino acids of dipeptides and the stability of the dipeptide in the intestine.

Histograms and scatter plots were generated using the R package (R Development Core Team, 2004).

## 2.3 ACE activity assay

ACE inhibitory activity was determined using a fluorometric microtitre assay as described by Sentandreu and Toldrá (2006) with some modifications. ACE was extracted from rabbit lung acetone powder as described by Murray, Walsh & FitzGerald (2004). The reaction was carried out as follows: 50 µl of enzyme extract was added to 100 µl of assay buffer (100 mM sodium borate buffer, 300 mM NaCl, pH 8.3) and preincubated for 5 min at 37 °C. The reaction was initiated with the addition of the synthetic substrate Abz-Gly-Phe-($NO_2$)-Pro (0.45 mM) dissolved in assay buffer. The assay was run continuously for 30 min and fluorescence from the release of product (Abz-Gly) was quantified at time zero and time 30 min using a BioTek Synergy HT plate reader (©BioTek Instruments Inc., VT, USA). Excitation and emission wavelengths were 360 and 400 nm, respectively. One unit of ACE activity (U/ml) was defined as the amount of enzyme hydrolysing 1 µmol of Abz-Gly-Phe-($NO_2$)-Pro per min at 37 °C.

For inhibition studies, an ACE activity of 10 mU/ml was used for each determination by diluting the enzyme solution appropriately with assay buffer. Dipeptides were analysed at final concentrations of 500, 250, 100, 50, 10, 1, 0.5, 0.1, 0.05, and 0.01 µM. For the synthetic ACE inhibitors, determinations were carried out at concentrations of 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, 0.0001 µM. The reaction was carried out as above except 50 µl of inhibitor and 50 µl of buffer was added in place of 100 µl of buffer. The % inhibition of ACE for each reaction was calculated using the formula below. Experiments were carried out as independent duplicates assayed in triplicate. $IC_{50}$ values (i.e., the concentration of inhibitor that inhibits 50 % of ACE activity) were calculated automatically using GraphPad® Prism

4.0 sigmoidal dose response plots of inhibitor concentration (μM) versus % inhibition. The values were expressed as the mean $IC_{50}$ ± standard deviation.

$$Inhibition\,(\%) = \left(\frac{WithoutInhibitor - WithInhibitor}{WithoutInhibitor}\right) x\,100$$

Where

- Without Inhibitor = fluorescence units/min without inhibitor
- With Inhibitor = fluorescence units/min with inhibitor.

# 3.0 Results and Discussion

## 3.1 Predictive identification of potent ACE inhibitory dipeptides

A total of 535 structures (529 dipeptides and six drug inhibitors) were given a Vina score for each individual pose using AutoDock Vina. Frequency distribution graphs of the highest Vina score for (a) all dipeptides, (b) non-phosphorylated dipeptides and (c) phosphorylated dipeptides were composed (Figure 1). For all dipeptides, the distribution was negatively-skewed, with the greatest distribution of dipeptides scoring between -6 and -7. A similar distribution pattern was seen with the non-phosphorylated dipeptides. For phosphorylated dipeptides, the distribution followed a more normal distribution, with a considerable number of phosphorylated peptides attaining highly negative Vina scores. These results would suggest that phosphorylated dipeptides may have better interactions with the ACE active site compared to non-phosphorylated peptides. The scatter plot of Vina scores with ACE-

inhibitory log $IC_{50}$ values for all dipeptides was composed using $IC_{50}$ values taken from the literature (Wu, Aluko, & Nakai, 2006a; Murray & FitzGerald, 2007; Iwankia, Dziuba, & Niklewicz, 2005). This analysis demonstrated that Vina scores correlate positively with both $IC_{50}$ ( r=0.52) and with log $IC_{50}$ ( r=0.69), Figure 2 illustrates the relationship using the log transformation, as it is easier to visualise. While values were taken from the above references in this instance, it must be noted that variation to the reported $IC_{50}$ values for a given dipeptide have been found to occur with different assay conditions. This includes the units of ACE activity used in the assay (Murray, Walsh, & FitzGerald, 2004), the source of ACE activity and the substrate (Vermeirssen, van Camp, & Verstraete, 2002). Amino acid clustering model analysis indicated that some of the dipeptides ranked in the top 10 %, on the basis of having Vina score values $\leq$ - 8.1, were potentially intestinally stable. Of the 49 dipeptides with a Vina score $\leq$ -8.1, 21 dipeptides (43 %) were phosphorylated. Analysis with the amino acid clustering model found that 8 out of the 21 phosphorylated dipeptides (38 %) to be small intestinally stable or neutral (Table 2). The stability of bioactive peptide sequences in the intestine is generally essential as their therapeutic effects may be dependent on the ability to reach target organs in an intact and active form. Intestinally stable di- and tripeptides can be transported across the brush border membrane by the PepT1 transmembrane peptide transporter (Vermerissen, van Camp, & Verstraete, 2004). It was decided to focus on the docking of dipeptides only in this study as increasing the size of the docked peptide may lead to less reliable docking results.

## 3.2 ACE inhibitory activity

A selection of dipeptides which were ranked in the top 10 % by AutoDock Vina and which were also identified as 'stable' from the intestinal stability model were synthesised and were characterised for ACE inhibitory potency *in vitro*. Three synthetic ACE inhibitors, i.e., captopril, lisinopril and enalapril along with the non-phosphorylated equivalents of selected phosphorylated dipeptides were also assessed for ACE-inhibitory potency (Table 3). The synthetic drug inhibitors gave $IC_{50}$ values in agreement with literature values. All the phosphorylated peptides tested resulted in $IC_{50}$ values >500 uM. Although these peptides were predicted by AutoDock Vina as being good inhibitors of ACE, the experimentally determined high $IC_{50}$ values indicates that phosphorylation does not enhance ACE inhibitory potency. Surprisingly captopril, a potent synthetic ACE inhibitor with an $IC_{50}$ value in the low nM region (Murray, Walsh, & FitzGerald, 2004) was not ranked in the top 10% on AutoDock Vina analysis. These results suggest that Vina scores may not be a reliable predictive tool for assessment of ACE inhibitory potency. The practical use of docking is to provide help in prioritising peptides for subsequent testing, which is made feasible when its rankings of compounds or peptides is combined with other factors, such as predictions of intestinal stability. There is a substantial potential for error and the enrichment of true positive peptides in the top ranking hits may be relatively low. While our analysis suggested that phospho-dipeptides were favoured by docking, none of the tested phospho-dipeptides had a very strong affinity for ACE (Table 3).

It has been shown that direct inhibitor-zinc interaction occurs during inhibition at the ACE active site (Bicknel, Holmquist, Lee, Martin, & Riordan, 1987). The LIGPLOTs for the six drug inhibitors, two non-phospho-dipeptides, Asp-Trp and Trp-Pro, and two phospho-dipeptides, Pro-pTyr and Trp-pThr, are shown in Supplemental Figure 3). This Figure shows that both Asp-Trp and Trp-Pro having Vina scores of

-8.3 and -8.6, respectively, did not interact with zinc on LIGPLOT analysis in the present study (Figure 3 g and h). Therefore, it is possible that neither of these peptides interact with zinc *in vivo*. In contrast, LIGPLOTs for the PDB structures listed in Table 1 show the drugs interacting with zinc in all six cases. When the drugs were then re-docked the Zinc interactions were only maintained for captopril, lisinopril and kAW (Figure 3 b, c and f), but not for enalaprilat, RXPA380 or kAF (Figure 3 a, d and e). Figure 3 i and j show two possible views on how the phosphor groups in the phospho-dipeptides could interact with the ACE pocket. For Pro-pTyr (Figure 3 i) the dipeptide can be seen interacting with zinc with no interaction between the phosphor group and the binding site, whereas for Trp-pThr (Figure 3 j) there is no interaction with zinc but the phosphor group interacts with Ala 354 and His 353. Enalapril can also be seen to interact with Ala 354, while all the drugs interact with His 353 except lisinopril. The LIGPLOTs for pTyr-pThr and pTyr-pSer show that similar to Pro-pTyr the pTyr phosphor group does not interat with ACE, however in both cases the other phosphor groups, pThr and pSer, interact with zinc, His 513 and Asn 277 and zinc, Tyr 523, His 353 and His 513 respectively. This may explain the favourable Vina scoring of the phospho-dipeptides, but these interactions may not be achieved *in vivo*.

Of all the dipeptides tested, Asp-Trp and Trp-Pro were found to have the highest ACE inhibitory potency with $IC_{50}$ values of 257.93 ± 4.23 and 217.27 ± 15.74 μM, respectively (Table 3). To our knowledge, no previous studies appear to report on the ACE-inhibitory activity for these peptides. Dipeptides with similar $IC_{50}$ values have been reported in the literature including Gly-Tyr, Gly-Phe, Met-Tyr and Arg-Pro which have ACE $IC_{50}$ values of 257.04, 275.42, 194.98, and 181.97 μM, respectively (Murray & FitzGerald, 2007; Wu, Aluko, & Nakai, 2006a). Although these may not

be regarded as considerably potent peptides in terms of ACE-inhibitory activity *in vitro*, dipeptides with similar $IC_{50}$ values are reported to have a significant antihypertensive effect. A proline containing dipeptide, Tyr-Pro, purified from a *Lactobacillus helveticus* CPN4 fermented yoghurt product was found to have a significant effect on systolic blood pressure in spontaneously hypertensive rats after oral administration. However, this peptide was reported to have a relatively high *in vitro* $IC_{50}$ value (720 uM, Yamamato, Maeno, & Takano, 1999). This finding highlights the importance of considering a combination of factors when assessing a peptides' potential bioactive ability such as bioavailability, stomach and intestinal stability. Therefore, assumptions on hypotensive capability cannot be made on *in vitro* $IC_{50}$ values alone.

Food proteins containing Trp-Pro include wheat gliadin, barley hordein, and chicken myosin while Asp-Trp can be found in bovine lactoferrin, rice oryzain, and barley tubulin (Iwaniak, Dziuba, & Niklewicz, 2005). Therefore, the targeted release of these dipeptides may lead to the generation of protein hydrolysates having enhanced functional food potential. However, *in vivo* studies are firstly required to confirm the hypotensive activity of these dipeptides.

# 4.0 Conclusions

Scoring of dipeptides by docking and determination of intestinal stability predicted a number of phosphorylated and non-phosphorylated dipeptides to have ACE inhibitory activity. The $IC_{50}$ values determined *in vitro* indicated that the phospho-dipeptides were not potent inhibitors of ACE. However, the study did uncover two relatively potent non-phosphorylated dipeptides Asp-Trp and Trp-Pro with $IC_{50}$ values of 257.

93 ± 4.23 and 217.27 ± 15.74 μM, respectively. These sequences can be found in many food protein sources.

## Acknowledgements

Financial support for this work was provided by the Irish Research Council for Science, Engineering and Technology (IRCSET) in the form of a studentship to author Norris and Science Foundation Ireland (SFI, grant number 08/IN.1/B1864).

## References

Acharya, K.R., Sturrock, E.D., Riordan, J.F. & Ehlers, M.R.W. (2003). ACE revisited: A new target for structure-based drug design. *Nature Reviews Drug Discovery*, 2, 891-902

Anthony, C.S., Corradi, H.R., Schwager, S.L.U., Redelinghuys, P., Georgiadis, D., Dive, V., Acharya, K.R. (2010). The N-domain of human angiotensin I-converting enzyme: the role of N-glycosylation and the crystal structure in complex with an N-domain specific phosphinic inhibitor RXP407. *Journal of Biological Chemistry*, 283, 35685-35693.

Bicknell, R., Holmquist, B., Lee, F.S., Martin, M.T. & Riordan, J.F. (1987). Electronic spectroscopy of cobalt angiotensin converting enzyme and its inhibitor complexes. *Biochemistry*, 26, 7291-7297.

Corradi, H.R., Schwager, S.L.U., Nchinda, A.T., Sturrock, E.D. & Acharya, K.R. (2006). Crystal structure of the N-domain of human somatic angiotensin I-converting enzyme provides a structural basis for domain-specific inhibitor design. *Journal of Molecular Biology,* 47, 5942-5950.

Ehlers, M.M. (2006). Safety issues associated with the use of angiotensin-converting enzyme inhibitors. *Expert Opinion on Drug Safety,* 5, 739–740.

Ehlers, M.R.W. & Riordan, J.F. (1991). Angiotensin-converting enzyme: Zinc- and inhibitor-binding stoichiometries of the somatic and testis lsozyme. *Biochemistry*, 30, 7118-7126.

Eriksson, U., Danilczyk, U. & Penninger, J.M. (2002). Just the beginning: novel functions for angiotensin converting enzymes. *Current Biology*, 12, R745-752.

FitzGerald, R.J. Meisel, H. (2000). Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. *British Journal of Nutrition*, 84, S33-S37.

Foltz, M., van Buren, L., Klaffke, W. & Duchateau, G.S. (2009). Modeling of the relationship between dipeptide structure and dipeptide stability, permeability, and ACE inhibitory activity. *Journal of Food Science*, 74, H243-H251.

Guha, R., Howard, M.T., Hutchison, G.R., Murray-Rust, P., Rzepa, H., Steinbeck, C., Wegner, J. & Willighagen, E.L. (2006). The Blue Obelisk – interoperability in chemical informatics. *Journal of Chemical Information and Modeling*, 46, 991-998.

Iwaniak, A., Dziuba, J., & Niklewicz M. (2005). The BIOPEP database-a tool for the in silico method of classification of food proteins as the source of peptides with antihypertensive activity. Acta Alimentaria, 34(4):417-425.

Libby, P., Bonow, R.O., Mann, D.L. & Zipes, D.P. (2008). *Braunwald's Heart Disease: A textbook of cardiovascular Medicine*. (8th ed.). Philadelphia: Saunders.

López-Fandino, R., Otte, J. & van Camp, J. (2006). Physiological, chemical and technological aspects of milk-protein peptides with antihypertensive and ACE-inhibitory activity. *International Dairy Journal*, 16, 1277-1293.

Majumder, K. & Wu, J. (2010). A new approach for the identification of noval antihypertensive peptides from egg proteins by QSAR and bioinformatics. *Food Research International*, 43, 1371-1378.

Meisel, H., Walsh, D.J., Murray, M. & FitzGerald, R.J. (2006). ACE Inhibitory peptides. In Y. Mine, & F. Shahidi (Eds.) *Nutraceutical Proteins and Peptides in Health and Disease* (pp.269-316). Florida: CRC Press.

Meyer, R.F.., Essenburg, A.D., Smith, R.D. & Kaplan, H.R. (1982). Angiotensin converting enzyme inhibitors: modifications of a tripeptide analogue. *Journal of Medicinal Chemistry*, 25, 996-999.

Murray, B,A, Walsh, D.J. & FitzGerald, R.J. (2004). Modification of the furanacryloyl-L-phenylalanylglycylglycine assay for determination of angiotensin-I-converting enzyme inhibitory activity. *Journal of Biochemical and Biophysical Methods*, 59, 127-37.

Murray, B.A. & FitzGerald, R.J. (2007). Angiotensin converting enzyme inhibitory peptides derived from food proteins: Biochemistry, bioactivity and production. *Current Pharmaceutical Design*, 13, 773-91.

Natesh, R., Schwager, S.L.U., Evans, H.R., Sturrock, E.D. & Acharya, K.R. (2003). Crystal structure of the human angiotensin-converting enzyme-lisinopril complex. *Nature*, 421, 551-554.

Natesh, R., Schwager, S.L.U., Evans, H.R., Sturrock, E.D. & Acharya, K.R. (2004). Structural details on the binding of antihypertensive drugs Captopril and Enalaprilat to human testicular angiotensin I-converting enzyme. *Biochemistry*, 43, 8718-8724.

Pripp, A.H., Isaksson, T. & Stepaniak, L. (2004). Quantitative structure-activity relationship modelling of ACE inhibitory peptides derived from milk proteins. *European Food Research and Technology*, 219, 579-583

Pripp, A.H., Isakasson, T., Stepaniak, L., Sorhaug, T. & Ardo, Y. (2005). Quantitative structure activity relationship modelling of peptides and proteins as a tool in food science. *Trends in Food Science and Technology*, 16, 484-494.

Pripp, A.H. (2007). Docking and virtual screening of ACE inhibitory dipeptides. *The European Food Research & Technology*, 225, 589-592.

R Development Core Team (2004) R: A language and environment for statistical computing [computer program]. Vienna, Austria: R Foundation for Statistical Computing. http://www.R-project.org.

Sentandreu, M.A. & Toldrá, F. (2006). A fluorescence-based protocol for quantifying angiotensin-converting enzyme activity. *Nature Protocols*, 2423-2427.

Trott, O. & Olsen, A.J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimisation and multithreading. *Journal of Computational Chemistry*, 31, 455-461.

Vermeirssen, V., van Camp, J. & Verstraete, W. (2002). Optimisation and validation of an angiotensin-converting enzyme inhibition assay for the screening of bioactive peptides. *Journal of Biochemical and Biophysical Methods*, 4, 75-87.

Vermeirssen, V., van Camp, J. & Verstraete, W. (2004). Bioavailability of angiotensin I converting enzyme inhibitory peptides. *British Journal of Nutrition*, 92, 357-366.

Wallace, A.C., Laskowski, R.D. & Thornton, J.M. (1995). LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Engineering Design and Selection*, 8, 127-134.

Watermeyer, J.M., Kröger, W.L., O'Neill, H.G., Sewell, B.T. & Sturrock, E.D. (2008). Probing the basis of domain-dependent inhibition using novel ketone inhibitors of angiotensin-converting enzyme. *Biochemistry,* 47, 5942-5950.

Wu, J., Aluko, R.E. & Nakai, S. (2006a). Structural requirements of angiotensin 1-converting enzyme inhibitory peptides: quantitative structure-activity relationship study of di- and tripeptides. *Journal of Agricultural & Food Chemistry*, 54, 732-738.

Wu, J., Aluko, R.E. & Nakai, S. (2006b). Structural requirements of angiotensin 1-converting enzyme inhibitory peptides: quantitative structure-activity relationship modelling of peptides containing 4-10 amino acid residues. *QSAR and Combinatorial Science*, 25, 873-880.

Yamamoto, N., Maeno, M. & Takano, T. (1999). Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by *Lactobacillus helveticus* CPN4. *Journal of Dairy Science*, 82,1388-1393.