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Novel whey-derived peptides and their role in angiotensin converting enzyme inhibition: a molecular docking and *in vitro* approach

A thesis submitted as a partial fulfilment for the degree of Doctor of Philosophy

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Declaration of authorship

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Sincerely,

Yara Chamata

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September 2022

Acknowledgments

There are many people who I am immensely grateful to for having supported me in various ways throughout this extremely challenging, yet equally enriching, journey of completing a PhD thesis in unordinary circumstances. First and foremost, I would like to dedicate this thesis to my family, and especially to my mother, the strongest and kindest woman I have ever known. I am forever in awe of your bravery and your dedication to provide me with an excellent education. Being raised by an independent and progressive mother made me the self-sufficient and resilient woman that I am today, and for that I am eternally grateful. I would also like to thank my sister, and my aunt Nada for the love and support to keep pushing forward throughout this research. I am extremely blessed to have such strong women in my life as role models. Sandra, you're my hero. Don't worry, you will always be the only "real" doctor in the family. To my grandfather, grandmother, and father, in loving memory.

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"Il faut imaginer Sisyphe heureux" – Albert Camus

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Chamata, Y., Watson, K. A., & Jauregi, P. (2020). Whey-derived peptides interactions with ACE by molecular docking as a potential predictive tool of natural ACE inhibitors. *International Journal of Molecular Sciences*, *21*(3), 864.

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General Abstract

Research in the production of anti-hypertensive activity demonstrates that several milk/whey derived peptides possess high *in vitro* angiotensin I-converting enzyme (ACE) inhibitory activity. However, in some cases, poor correlation between the *in vitro* ACE inhibitory activity and the *in vivo* anti-hypertensive activity has been observed. The main aim of this study is to investigate whether molecular docking can be used as an effective prediction tool to identify milk protein derived angiotensin I-converting enzyme (ACE) inhibitors, prior to testing the candidate peptides *in vivo*. Firstly, a molecular docking method was developed to elucidate the structure-activity relationship of peptide sequences present in whey/milk protein hydrolysates with previously reported high *in vitro* ACE inhibitory activities (Chapter Two). Main amino acid residues at the binding site of the human ACE formed strong hydrogen bonds with whey-derived peptide sequences IPP, LIVTQ, IIAE, LVYPFP, in common with anti-hypertensive drugs such as Sampatrilat, Captopril, Lisinopril and Elanapril. The molecular docking results indicate that these natural peptides may be potent ACE inhibitors. The developed molecular docking method was then employed to investigate the molecular interactions between specific amino acid residues at the binding site of human angiotensin II-converting enzyme (ACE2), and the whey-protein derived peptide sequences (Chapter Three). IPP, LIVTQ, IIAE, and LVYPFP all formed strong hydrogen bonds and salt bridge interactions with key residues in the active site of human ACE2, in common with known potent pharmaceutical ACE2 inhibitors. Therefore, IPP, IIAE, LIVTQ, and LVYPFP are suggested as potential candidates to be used in the treatment of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) via inhibition of the host cell receptor ACE2. Also, in contrast with well-known anti-hypertensive drugs, these peptides have a dual inhibitory action against both ACE and ACE2, and are natural and dietary derived, which may represent advantages in the treatment of coronavirus disease 2019 (COVID-19).

To further investigate whether molecular docking can be used as a reliable pre-screening tool to identify milk protein-derived bioactive peptides and predict their ACE inhibitory activities, whey protein isolate was subjected to a simulated gastric digestion procedure that mimics the chemical and physiological conditions of the gastro-intestinal tract to process whey proteins. Whey protein-derived hydrolysates were pre-purified by solid phase extraction, and the various hydrolysate fractions were then characterised in terms of their *in vitro* ACE inhibitory activities. The chemical characterisation of each fraction was carried out using mass spectrometry (MS) to identify the main peptides contributing to the ACE inhibitory activity measured in each fraction. Some novel

sequences were identified, such as AIPPK, TPEVDDE, PFPGPI, and VELLKHKP. To gain insight into which peptide sequences are most likely contributing to the ACE inhibitory activities measured in the various hydrolysate fractions, the whey protein-derived peptide sequences were then docked into the binding site of human ACE. The docking results, together with comparisons with ACE inhibitory drugs, provided strong evidence for the ACE inhibitory activities of whey protein-derived peptides AIPPK, PFPGPI, TPEVDDE and VELLKHKP. Finally, the results obtained indicate that experimental measurement of ACE inhibitory activities and docking results of AIPPK, PFPGPI, TPEVDDE and VELLKHKP are in agreement. This shows the potential effectiveness of molecular docking as a pre-screening tool to identify milk protein-derived bioactive peptides. Further research is needed to assess the *in vivo* anti-hypertensive effects of whey protein-derived bioactive peptides.

CHAPTER ONE Introduction

1.1 Introduction to this work

Angiotensin I-converting enzyme (ACE) inhibitors have been the main drugs used for effectively lowering blood pressure and preventing the development of cardiovascular diseases (CVDs), the leading cause of death globally (World Health Organisation, 2021). Recently, there has been an increasing scientific and commercial interest in functional foods. A dietary approach to stop hypertension (DASH) has been recognised as an effective strategy to reduce the incidence of CVDs, with evidence of other cardiometabolic advantages (Chiavaroli et al., 2019). Furthermore, there is growing scientific evidence of the strategic role of bioactive peptides (BAPs) in this approach. Milk proteins are the main source of ACE inhibitory peptides (Nongonierma et al., 2016), with Val-Pro-Pro and Ile-Pro-Pro first identified from commercial milk in 1995 (Nakamura et al., 1995). Since then, a significant number of studies on the anti-hypertensive activity of milk-derived proteins have been described in various reviews (FitzGerald et al., 2004; Hernandez-Ledesma et al., 2011; Korhonen & Pihlanto, 2006; Li et al., 2019). Despite the increasing interest in milk-derived BAPs as alternatives in hypertension management, the exact mode of their interaction with ACE is not fully understood. Also, there is a poor correlation between *in vitro* and *in vivo* effects when different individual peptides or protein hydrolysates are compared. Hence, the evidence of the ACE inhibitory activity of milk derived peptides in humans still needs to be strengthened (Hernández-Ledesma et al., 2011; Pripp, 2008; Wang et al., 2020).

ACE inhibitors are commonly discovered using classical approaches which include enzymatic hydrolysis of proteins and release of BAP fragments, followed by identification of BAP sequences by various isolation and purification methods (Li-Chan et al., 2015; Li et al., 2004). However, these investigation techniques are both costly and time-consuming for researchers and manufacturers, and there is uncertainty regarding the bioactivity of the protein hydrolysates, which must be confirmed. Furthermore, although some structure-activity relationships have been established for milk protein derived peptides, they are still quite generic and thus could not be solely used to predict the ACE inhibitory activity of peptide sequences (Pan et al., 2011). Molecular docking enables the investigation of specific and potentially crucial interactions between peptide sequences and specific binding sites of ACE, which could help to obtain a better prediction of bioactivity *in vivo*. This allows for novel sequences, which may be more relevant to human health, to be identified (Nongonierma &

Fitzgerald, 2017). Additionally, these techniques present many advantages such as less chemical/reagent consumption, faster rate of results acquisition, and resultant cost effectiveness.

Therefore, molecular docking can be a powerful tool to a pre-screen potentially BAPs prior to their testing *in vivo*, without the need to go through cumbersome purification processes. This may constitute an important strategy in the development of novel therapeutics and functional foods fortified with ACE inhibitory peptides as a sustainable solution to combat CVDs.

1.2 Aims of the project and specific objectives

In the first stage of this research, the aim is to gain insights into the structure-activity relationship of peptide sequences present in whey/milk protein hydrolysates with high ACE inhibitory activity. Using a molecular docking approach, the potential interactions between peptides produced from whey proteins (previously reported as high ACE inhibitors), and human ACE will be investigated. The molecular docking method will also be validated by docking well-established pharmaceutical ACE inhibitors, which are used in the treatment of hypertension, into the human ACE receptor (Chapter two).

Hypertension is regulated by the renin–angiotensin system (RAS), through mainly modulating two enzymes: ACE and angiotensin converting enzyme-2 (ACE2). Considering the high sequence similarity and sequence identity between ACE and ACE2, the significance of ACE2 as a SARS-CoV-2 receptor, and the reported reduced risk of mortality and disease associated with use of ACE inhibitors among COVID-19 patients; the second aim of this research is to obtain an improved understanding of the function of these natural peptides present in whey/milk protein hydrolysates as RAS inhibitors. To this end, the molecular interactions between these peptides, and human ACE2 will be explored by using the same molecular docking methodology developed in Chapter two (Chapter three).

The final aim of this research is to further explore whether this molecular docking approach can be employed as a pre-screening tool for anti-hypertensive peptide sequences. To this end, whey proteins will be subjected to the INFOGEST digestion protocol; a static digestion approach where digestion parameters are based on existing physiological data. The hydrolysate fractions produced under these conditions, and with high *in vitro* ACE inhibitory activity will be characterised, and the predominant peptide sequences in these hydrolysates will be identified by mass spectrophotometry techniques. The sequences with the highest *in vitro* ACE inhibitory activities will then be further investigated using the developed molecular docking method. Finally, the correlation between the experimental results and the molecular docking data will be assessed.

Therefore, the overall aim of this work is to explore whether molecular docking can be used as an effective prediction tool to identify milk protein derived ACE inhibitors and achieve a better estimation of their anti-hypertensive activity, prior to testing the candidate peptides *in vivo*.

1.3 Background

1.3.1. Bioactive peptides from food-derived proteins

Diet is being increasingly linked to the prevalence of diseases, such as CVD, hypertension, obesity, diabetes, and even cancer (Anand et al., 2015). In response to the increased awareness about the relationship between food and health, there has been an increasing demand and manufacture of functional foods. These are generally defined as a food that can provide a health benefit to one or more bodily functions, beyond that of basic nutrition (Action, E. C., 1999).

Many dietary proteins contain different peptide sequences encrypted within their primary structures, and which exert beneficial effects on human health. Food-derived BAPs refer to different amino acid sequence fragments, normally ranging in size from 2 to 50 amino acid residues and produced from food proteins. BAPs exhibit great benefits for decreasing the risk of chronic diseases, such as the blood pressure-lowering, lipid lowering, cholesterol-lowering, immune-boosting, anti-inflammatory, antimicrobial, antithrombotic, anticoagulative, and antiproliferative effects (Lopez-Exposito & Recio, 2008; López-Fandiño, 2006; Martínez-Augustin et al., 2014; Meisel & FitzGerald, 2003). Additionally, over the past decades, the chemically synthesised pharmaceuticals have been displaying adverse side effects. Therefore, the far-reaching significance of these BAPs to promote human health has been recognized by researchers, which has consequently aroused growing scientific and commercial interest in BAPs (Hartmann & Meisel, 2007; Martínez-Augustin et al., 2014).

1.1.2 Milk proteins as a source of bioactive peptides

Although BAPs have been found in other animal sources, such as fish and eggs, and in plant sources, such as rice, corn, and soybean; milk proteins are the most important source of BAPs (Korhonen & Pihlanto, 2006; Nongonierma et al., 2016). Bovine milk contains 30-35 g of protein per litre of which 76-80% are caseins, classified as α-, β- and k-caseins, and 20-24% whey proteins (Fox & McSweeney, 1998). Various types of whey are whey protein isolates, whey protein concentrates, and whey protein hydrolysates. Whey can also be classified based on pH, as the sweet whey (pH 6 & 7) and acidic whey (pH < 5) (Tallapragada & Rayavarapu, 2019). Found in sweet whey, bovine

glycomacropeptide (GMP) is released from the hydrolysis of κ-casein by the action of chymosin and is known to exert various biological activities (Brody, 2000; Eigel et al., 1984).

Whey also contains α-lactalbumin (α-La), β- lactoglobulin (β-Lg), and other minor proteins. Upon the degradation of milk proteins, peptide fragments with many biological effects that can be different from those of the parent protein, are released. Several BAPs in milk proteins have been identified (Meisel, 1998), and they serve an array of biological activities, including ACE inhibition. Table 1.1 lists some of the major biologically active milk peptides together with their possible biological functions. The multifunctional properties of biologically active milk peptides and their positive impact on human physiology and metabolism are therefore increasingly acknowledged (Kitts & Weiler, 2003).

Table 1.1 Bioactivities of some peptides derived from milk proteins (Adapted from Marcone et al., 2017).

Protein precursor	Peptide fragment	Bioactivities observed	References
β-casein	VPP	ACE inhibition*	Maeno et al., 1996; Yamamoto et al., 1994
	IPP		
	LTLTDVEYPQRDMPIQ	ACE inhibition	FitzGerald, & Meisel, 2000
	YPFPGPI	ACE inhibition, opioid	
	YPFPGPIPNSL		
	LTLTDVE YPQRDMPIQ	ACE inhibition	Haileselassie et al., 1999
	PGPIP		
	YPFPGPIPNSL	Opioid	Meisel, 1986
	YPFPGI	Opioid, ACE Inhibition, Immunomodulatory	Cieślińska et al., 2012
	YPFPG	Opioid, ACE Inhibition	Maruyama et al., 1985
	AVPYPQR	ACE Inhibition	Maruyama et al., 1985
	YQQPVLGPVR	ACE Inhibition, Immunomodulatory	Maruyama et al., 1982
	KVLPVPQ	ACE inhibition	Maeno et al., 1996
	PGPIPN	Immunomodulatory	Migliore-Samour et al., 1989
	LLY		
	YPVEP	Immunomodulatory	Kitazawa et al., 2007
	EPVLGPVRGPFP	ACE inhibition	Hayes et al., 2007
	RELEELNVPGEIVESLSSSEESITR	Calcium binding	Sato et al., 1991
αs_1-casein	RYLGYLE	Opioid	Loukas et al., 1983
	RYLGYL		
	YLGYLE		
	FFVAPFPEVFGK	ACE inhibition	Smacchi & Gobbetti, 2000
	FFVAP		
	FPEWFGK	ACE inhibition	Maruyama et al., 1982
	TTMPLW	ACE inhibition, Immunomodulatory	Karaki et al., 1990
	LGTQYTDAPSFSDIPNPIGSENSEK	ACE inhibition	Minervini et al., 2003
αs_2-casein	QKALNEINQF	Antimicrobial, ACE inhibition	Srinivas et al., 2010
	TKKTKLTEEEKNRL	ACE inhibition	
k-Casein	MAIPPKKNQDK	Antithrombotic	Chabance et al., 1995; Jolles et al., 1986
	NQDK		

	MAIPPL		
	SRYPSY	Anti-Opioid	Yoshikawa et al., 1986
	YIPIQYVLSR	Anti-Opioid	Chiba et al., 1989
	MAIPPKKNQDK	Antithrombotic	Jolles et al., 1986
	YPSY	Opioid agonist	Patten et al., 2011
α-lactalbumin	YGLF	Opioid agonist, ACE inhibition	Nurminen et al., 2000
	VAGTWYHIRL	ACE inhibition	Pihlanto et al., 2000; Mullally et al., 1997
	GLF	Antithrombotic	Chabance et al., 1998; Fiat et al., 1993
	RGDGLF		
	MHIRL	Antioxidant	Hernandez-Ledesma et al. (2005)
	YVEEL		
β-lactoglobulin	TLLF	Non-opioid, ACE-inhibition	Mullally et al., 1997
	ALPMHIR	ACE inhibition	Fitzgerald & Meisel, 1999
	HIRL	Hypocholesterolemic activity	Mullally et al., 1996; Yamauchi et al., 2003
	LAMA	Anti-hypertensive (ACE & endothelin-1 inhibition)	Pihlanto & Korhonen, 2000; Pihlanto-Leppälä et al., 2000; Mullally et al., 1997
	VKF		
Bovine Serum Albumin	YGFQDA	Opioid	Tani et al., 1994
	ALKAWSVAR	ACE inhibition	Fitzgerald et al., 1999
Lactoferrin	FKCRRWQWRMKKLGAPSICURRAF/A	Antimicrobial	Meisel & Bockelmann, 1999

*Angiontensin-converting enzyme (ACE) inhibition.

1.4 Angiotensin I-converting enzyme inhibitory peptides

Hypertension, often referred to as high blood pressure represents a main risk factor for developing CVDs (Aguiar et al., 2021; Hippauf et al., 2015). According to the World Health Organization (WHO), these diseases have become the leading cause of death globally. In 2019, 17.9 million people were estimated to die from CVDs, accounting for 32% of all deaths worldwide (WHO, 2021). Hypertension is a common chronic disease in which the arteries are exposed to a persistently raised pressure as the blood flows within them. Traditionally, it has been defined by diastolic blood pressure ≥ 90 mmHg and/or systolic blood pressure ≥ 140 mmHg, at rest (Krakoff et al., 2014). Hypertension is associated with many serious debilitating conditions, such as heart failure, stroke and renal failure,

and myocardial infraction (Wei et al. 2019). Unhealthy living habits, unhealthy diet, severe psychological and emotional distress, and other factors linked to physical functions such as obesity and aging could be defined as risk factors for hypertension prevalence (Udenigwe & Mohan, 2014).

Nutrition is considered to play an increasingly significant role in the prevention of CVDs (Boschin et al., 2014; Iwaniak et al., 2014). Over the last 25 years, the rising incidence of CVDs has turned into a public health priority, and most specifically the prevention of CVDs through lifestyle and diet interventions (Casas et al., 2018). The DASH diet is characterised by a high intake of fruits and vegetables, low fat dairy, whole grain products, and a low intake of saturated fat, red meat, and processed meats. A large body of evidence confirms that following the DASH dietary plan is associated with decrease in blood pressure (Saneei et al., 2014). Taking into consideration this finding, and the fact that hypertension is a major global challenge, many studies have been performed to determine the anti-hypertensive properties of foods.

1.4.1 ACE inhibitory peptides derived from milk proteins

Most natural ACE inhibitory peptides usually contain 2-20 amino acid residues (Nongonierma & Fitzgerald, 2015). Hydrolysates of caseins (αs1-casein, β-casein and κ-casein), whey proteins (αLa, βLg and bovine serum albumin), fractions-enriched individual milk proteins, and whole milk proteins have been shown to be a good precursor of ACE inhibitory peptides (FitzGeraldet al.,2004). Table 1.2 shows some examples of the most potent ACE inhibitory peptides derived from bovine caseins and whey proteins that have been reported in the literature.

Table 1.2 ACE inhibitory peptides derived from bovine caseins and whey proteins (Adapted from Fitzgerald et al., 2004 and Ortiz-Chao, 2008).

Protein source	Peptide sequence[a]	Treatment or origin	IC_{50} (μM)[b]	Reference
α_{s1}CN	RYLGY	Pepsin	0.7	del Mar Contreras et al., 2009
	VAP	Synthetic	2.0	Maruyama et al., 1987b
	FFVAP	Trypsin	6.0	Maruyama et al., 1985
	AYFYPEL	Pepsin	6.6	del Mar Contreras et al., 2009
	FVAP	Synthetic	10.0	Maruyama et al., 1987b
	RY	Fermentation	10.5	Hernández-Ledesma et al., 2005b
	RPKHPIKHQ	Synthetic	13.4	Saito et al., 2000

	TTMPLW	Trypsin	16.0	Maruyama et al., 1987a
	FFVAPFPEVFGK	Trypsin	18.0	Tauzin et al., 2002
	PLW	Synthetic	18.0	Maruyama et al., 1987a
	YKVPQL	Proteinase of *Lactobacillus helveticus* CP790	22.0	Maeno et al., 1996
	MKP	Subtilisin, bacillolysin, and trypsin	0.3	Yamada et al., 2013
α_{s2}CN	FALPQY	Trypsin	4.3	Tauzin et al., 2002
	FALPQYLK	Trypsin	4.3	Tauzin et al., 2002
	RY	Synthetic	10.5	Hernández-Ledesma et al., 2005a
	YQK	Pepsin and trypsin	11.1	Xue et al., 2018
	FPQYLQY	Trypsin	14.0	Tauzin et al., 2002
	TVY	Trypsin	15.0	Tauzin et al., 2002
	YQKFPQY	Pepsin	20.1	del Mar Contreras et al., 2009
	VRYL	Synthetic	24.1	Gómez-Ruiz et al., 2004a
	NMAINPSK	Trypsin	60.0	Tauzin et al., 2002
	IPY	Synthetic	206.0	Gómez-Ruiz et al., 2004a
	ALNEINQFY	Trypsin	219.0	Tauzin et al., 2002
	ALNEINQFYQK	Trypsin	264.0	Tauzin et al., 2002
β-CN	LHLPLP	Synthetic	2.9	Kohmura et al., 1989
	LVYPFPGPIPNSLPQNIP	Thermolysin	4.0	Otte et al., 2007
	IPP	Fermentation	5.0	Nakamura et al., 1995
	YPFPGPIPNSLPQNIPP	Thermolysin	5.0	Otte et al., 2007
	KVLPVP	Synthetic	5.0	Maeno et al., 1996
	VPP	Fermentation	9.0	Nakamura et al., 1995
	YPFPGPIPN	Synthetic	14.8	Saito et al., 2000
	NLHLPLPLL	Synthetic	15.0	Robert et al., 2004
	AVPYPQR	Trypsin	15.0	Maruyama et al., 1985
	IHPFAQTQSLVYP	Synthetic	19.0	Kohmura et al., 1990
	LVYPFP	*Bifidobacterium bifidum* MF 20/5	132	Gonzalez-Gonzalez et al., 2013
κ-CN	IPP	Fermentation	5.0	Nakamura et al., 1995
	DERF	AS1.398 neutral protease	21	Jiang et al., 2010
	RYPSYG	AS1.398 neutral protease	54	Jiang et al., 2010
	YP	Fermentation	720.0	Yamamoto et al., 1999
α-La	YGGVSLPEW	Thermolysin	1.0	Otte et al., 2007
	YGLF	Synthetic	1.3	Nurminen et al., 2000
	GVSLPEW	Thermolysin	2.0	Otte et al., 2007
	VSLPEW	Thermolysin	3.0	Otte et al., 2007
	LKGYGGVSLPEW	Thermolysin	5.0	Otte et al., 2007
	WLAHK	Trypsin	77.0	Pihlanto-Leppälä et al., 2000
	VGINYWLAHK	Trypsin	327.0	Pihlanto-Leppälä et al., 2000
	LF	Synthetic	349.1	Mullally et al., 1996
Bovine serum	ALKAWSVAR	Synthetic	3.0	Chiba & Yoshikawa, 1991

albumin	FP	Proteinase K	315.0	Abubakar et al., 1998
β-Lg	SAPLRVY	Protease N Amano	8	Ortiz-Chao et al., 2009
	LQKW	Thermolysin	34.7	Hernández-Ledesma et al., 2007b
	ALPMHIR	Trypsin/Synthetic	42.6	Mullally et al., 1997
	LLF	Thermolysin	82.4	Hernández-Ledesma et al., 2007b
	YL	Synthetic	122.1	Mullally et al., 1996
	IIAE	Protease N "Amano"	128	Welderufael et al., 2012
	IPA	Proteinase K	141.0	Abubakar et al., 1998
	LIVTQ	Neutrase	170.0	Schlothauer et al., 2005
	LDIKQ	Neutrase	170.0	Schlothauer et al., 2005

[a] One letter amino acid code.

[b] Concentration of peptide mediating 50% of ACE activity.

As can be seen from table 1.2., milk is a rich source of ACE inhibitory peptides including the isoleucine-proline-proline (IPP) and valine-proline-proline (VPP), and the polypeptide phenylalanine-phenylalanine-valine-alanine-proline-phenylalanine-proline-glutamate-valine-phenylalanine-glycine-lysine (FFVAPFPEVFGK). The effect of these peptides on the main cardiovascular parameters, such as blood pressure, have been evaluated in several clinical studies (Cicero et al., 2013; Guo et al., 2020). Particularly, IPP and VPP have been reported to exhibit an anti-hypertensive effect, more evident in Asian patients, signifying a potential genetic-dependent effect, as already reported for some synthetic anti-hypertensive drugs (Cicero et al., 2011a). Other data demonstrate that these tripeptides were also able to exert a positive effect on pulse wave velocity (a measurement of arterial stiffness) in mildly hypertensive patients, without any adverse effects being raised (Cicero et al., 2011b, 2016).

1.4.2 Angiotensin I-converting enzyme (ACE)

ACE (dipeptidyl carboxypeptidase, EC 3.4.15.1) is a zinc metallopeptidase, found in male genital, vascular endothelial, neuro-epithelial, and absorptive epithelial cells (Acharya et al., 2003; Li et al.,2004; Wei et al., 1992). This enzyme displays both endopeptidase and exopeptidase activities, and acts on a wide range of substrates (Sturrocket al., 2004). ACE consists of two isoforms transcribed by the same gene: a larger one referred to as somatic ACE (sACE) that is expressed in most tissues and has 1277 amino acids, and a smaller one referred to as testis ACE (tACE) that is found in adult testis and has 701 amino acids (Nateshet al., 2003; Tzakos et al., 2003). Somatic ACE has two homologous catalytic domains (N- and C- domains), and each domain has distinct functional

and physiochemical properties, as well as a separate functional active site (Corradi et al., 2006). The two catalytic domains of ACE differ in terms of both inhibitor and substrate specificity, as well as chloride activation (Georgiadis et al., 2003; Natesh et al., 2004). Although both domains are effective in angiotensin I (AT1) cleavage, studies suggest that the C-domain is the dominant angiotensin-converting site (Natesh et al., 2003; Tzakos & Gerothanassis, 2005).

ACE is a key enzyme for regulating blood pressure in the renin-angiotensin system (RAS). Renin cleaves the N-terminal segment of angiotensinogen from the biologically inert AT1 (Figure 1.1). ACE then hydrolyses AT1 by cleaving the carboxyl terminal His-Leu dipeptide from the inactive AT1 into the active angiotensin II (AT2), a potent vasoconstrictor responsible for the development of hypertension (Chen et al., 2009; Donkor et al., 2007; Natesh et al., 2003; Tzakos et al., 2003). ACE also indirectly influences the kallikrein–kinin system, by promoting the inactivation and degradation of the catalytic function of bradykinin, a vasodilator involved in blood pressure control (Korhonen & Philanto, 2007; Natesh et al., 2003; Tzakos et al., 2003). Apart from its vasodilatory properties, bradykinin also has cardioprotective properties which stimulate endothelial production of nitric oxide. By repressing AT2 production and restraining bradykinin degradation, ACE inhibitory peptides control the increase of blood pressure (Korhonen & Philanto, 2007). Consequently, milk and whey protein derived ACE inhibitory peptides could exhibit *in vitro* ACE inhibiting activity or *in vivo* anti-hypertensive effect.

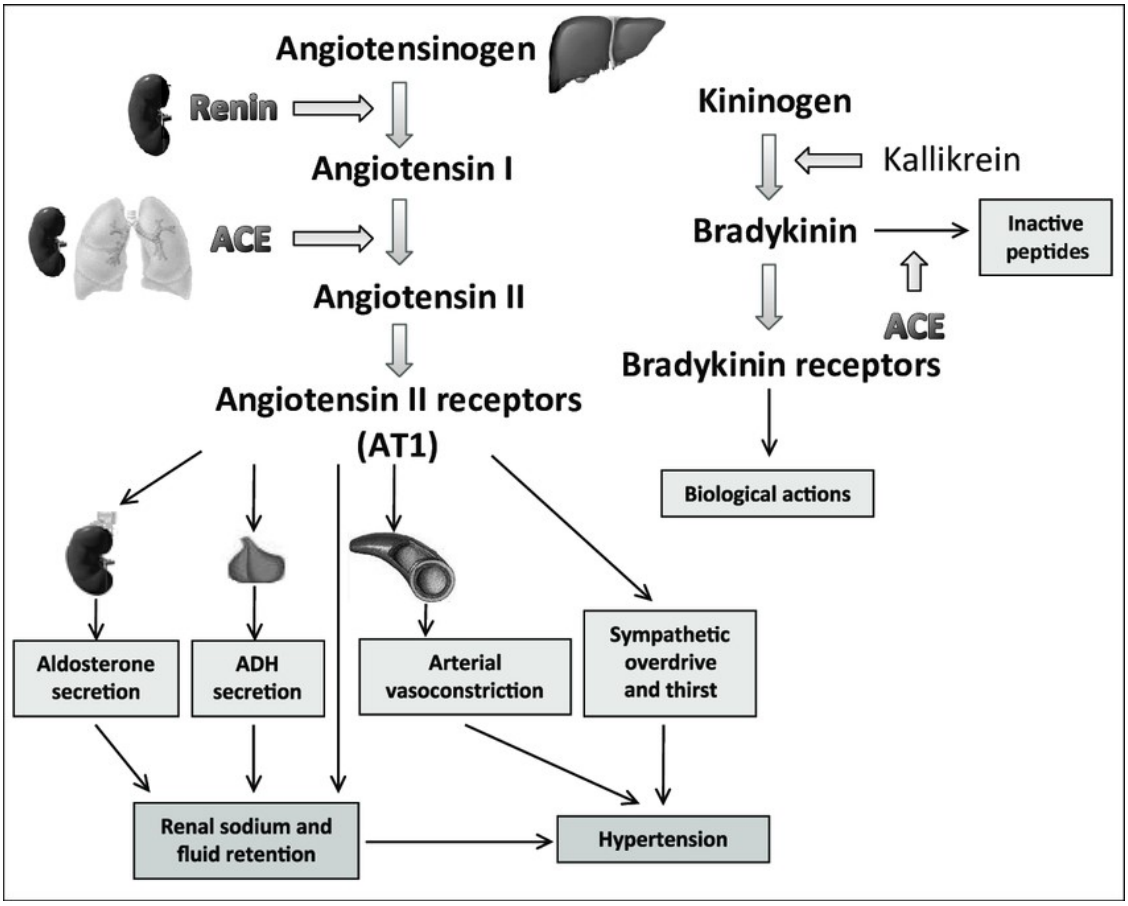

Figure 1.1 The role of ACE in the RAS and kallikrein-kinin systems. Taken from Santos et al., (2012).

1.4.3 Structural implications of bioactive peptides on ACE inhibition

The three-dimensional design and mechanism of action of ACE has been highlighted in numerous X-ray crystallographic studies (Cozier et al., 2018; Cozier et al., 2021; Sturrock et al., 2019). However, the structure-activity relationship of ACE inhibitors has to date not been fully characterised (Daskaya et al., 2017; Mirzapour-Kouhdasht et al., 2022; Pripp et al., 2005; Wu et al., 2006). To obtain a better understanding of the structure of the most potent ACE inhibitors and correlate such findings to a model structure, research has been carried out with various ACE peptide inhibitors and substrates (Guy et al., 2003; Quirós et al., 2009; Wu et al., 2006).

Structure-activity correlations among different substrates and inhibitors show that binding of a substrate to ACE is strongly influenced by the C-terminal tripeptide sequence; these three amino acid residues have been demonstrated to play a major role in competitive binding to the active site of the enzyme (Cheung et al., 1980; Cushman & Cheung, 1971; Meisel et al., 2006; Petrillo & Ondetti, 1982). R1-R2-R3-OH is the general structure of ACE substrates, whereby; ACE cleaves R1 from R2, where R1 is the protected (non-cleaved) amino acid or peptide, R2 is any amino acid that does not

contain proline, and R3 is an amino acid with a free carboxyl terminal, except for glutamic acid (Erdös, 1975). These observations were later confirmed by Cheung et al (1980), who also found that ACE binds weakly to substrates with dicarboxylic amino acids at the C-terminal, together with peptides with a Pro residue in the penultimate position. Cheung et al., (1980) also investigated the importance of the C-terminal sequences of ACE inhibitory peptides, and the binding of two peptides hippuryl-phenylalanine-arginine (Hip-Phe-Arg) and hippuryl-histidyl-leucine (Hip-His-Leu) that have similar C-terminal dipeptides with that of bradykinin and AT1, the two biologically active substrates of ACE. Findings of this work show the two peptides' similar binding affinity trends with AT1 and bradykinin, thus indicating substrate specificity of ACE (Cheung et al., 1980).

It has been established that the activity of BAPs depends on their inherent amino acid composition and sequence (Dallas et al., 2015; Meisel et al., 2003). Shorter peptides, up to 12 amino acids with hydrophobic and positively charged amino acids at the carboxyl end, are more likely to interact with ACE. In terms of favourable structure–function relationships for high ACE inhibitory activity, dipeptides including bulky and hydrophobic amino acids are more potent, whereas tripeptides having aromatic amino acids at the C-terminus end, positively charged amino acids in the middle and hydrophobic amino acids at the N-terminus end, are the most potent (Welderufael et al., 2012). Kobayashi et al. (2008) investigated the effects of aromatic amino acids in the third position of the tripeptides on ACE inhibitory activity. They found that the difference in the ACE inhibitory activity between the BAPs (IKW, LKW, IKY and LKF) resulted from the aromatic amino acids W, Y and F. The highest inhibitory activity was presented by LKW, with the largest amino acid at the C-terminus. Accordingly, ACE inhibitory activity is affected by the size of the amino acid, as well as its hydrophobicity (Kobayashi et al., 2008). In the same study, Kobayashi et al. (2008) examined the effects of the charged amino acid in the second position, and they reported that to obtain a high inhibitory activity, it is essential to have a positively charged residue next to an aromatic residue. They also highlighted that the tripeptide sequence consisting of either I or L and positively charged amino acids and aromatic amino acids are likely to have a high ACE inhibitory activity. The charged amino acid takes part in binding to ACE, while the bulky aromatic amino acid prevents access between substrates and the active site of ACE (Kobayashi et al., 2008). Some studies have indicated that tripeptides show higher ACE inhibitory activity, and the C terminal end of the tripeptides substantially affects binding to ACE. Hydrophobic amino acid residues or proline residues at the carboxyl end are important for ACE inhibition, and inhibitors containing these residues are resistant to digestion (Pan et al., 2011).

Pripp et al., (2004) developed a quantitative structure-activity relationship (QSAR) using ACE inhibitory peptides derived from milk proteins and formed by up to six amino acids. In this work,

they demonstrated that increased side hydrophobicity and absence of positive charge at the C-terminal enhanced the peptides' ACE inhibitory activities, whereas increased side chain size of the amino acid next to the C-terminal end reduced it. More recently, Sagardia et al., (2013) developed a new QSAR model for ACE inhibitory oligopeptides. They showed that proline residues, aromatic amino acids (W, F), and branched-chain aliphatic amino acids (L, I, V) at the C-terminus contribute to the increase in the peptides' ACE inhibitory potential (Aluko 2015; Hernández-Ledesma et al., 2011; Kapel et al. 2006; Pan et al., 2011). Further, some studies reported that amino acid residues at the N-terminus also had a significant effect on ACE inhibitory activity (García-Tejedor et al. 2015; Ishiguro et al. 2012; Li et al. 2016). In fact, the ACE inhibitory activity of peptides was found to be positively correlated with the presence of aliphatic amino acids (V, L, G, I, A) at the N-Terminal.

1.4.4 Milk-derived ACE inhibitory peptides as an alternative to synthetic drugs in hypertension treatment

Captopril (1-[(2S)-3-mercapto-2-methylpropionyl]-L-proline, Fig. 1.2) was the first orally active and non-peptide ACE inhibitor potent enough to be clinically useful (Petrillo & Ondetti, 1982). This was developed at Squib and was a blockbuster drug that inspired the production of generations of similar anti-hypertensive drugs such as Enalapril, Alecepril and Lisinopril (Acharya et al., 2003; Smith & Vane, 2003).

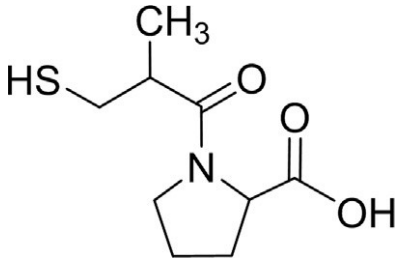

Figure 1.2 Chemical structure of Captopril, a potent ACE inhibitor.

The most potent ACE inhibitors, such as Lisinopril, Captopril, and Enalaparil, exhibit their anti-hypertensive activity through inhibition of both C- and N- domains of sACE and tACE (Tzakos et al., 2003; Natesh et al., 2004). These drugs interact with ACE through hydrophobic interactions and hydrogen bonds at the ACE subsites S1, S2, S1'and S2'. They also make a direct interaction with the catalytic Zn^{2+} ion (Andujar-Sanchez et al.,2004; Natesh et al., 2003; Natesh et al., 2004; Tzakos & Gerothanassis, 2005). Captopril has a thiol group that can coordinate with zinc (II) ions (FitzGerald et al., 2004), whereas Lisinopril and Enalaprilat possess different ligands that interact with zinc (II) ions;

Enalaprilat has a zinc (II)-coordinating carboxyl group and Lisinopril a carboxylate group that binds to active site zinc (II) (Tzakos & Gerothanassis, 2005).

Sampatrilat ((S, S, S)-N-{1-[2-carboxy-3-(N-mesyllysylamino) propyl]-1-cyclopentylcarbonyl} tyrosine) (Figure 1.3) is a potent dual inhibitor of ACE and neutral endopeptidase. Based on this dual inhibitory action, this drug could potentially provide a greater benefit than traditional ACE inhibitors in the treatment of hypertension (Venn et al., 1998; Wallis et al., 1998).

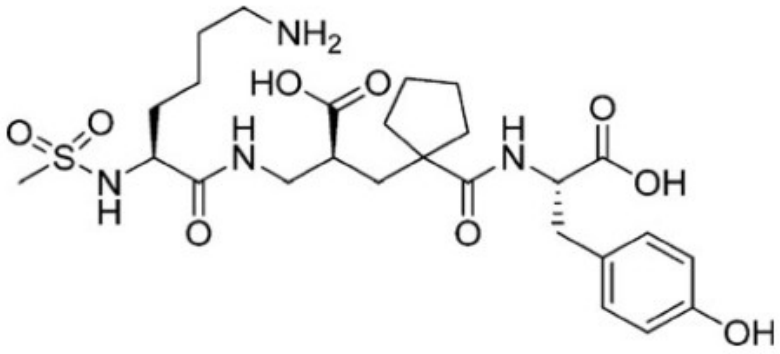

Figure 1.3 Chemical structure of Sampatrilat, a potent dual inhibitor of ACE.

Captopril is significantly more potent than any of the more potent milk-derived ACE inhibitory peptides reported in the literature thus far. An IC_{50} of 0.006 µM is reported for Captopril, using hippuryl-histidyl-leucine as substrate (Mullally et al., 1996). For instance, the most potent milk-derived peptides IPP and VPP have IC_{50} values of 5.0 and 9.0 µM, respectively (Ehlers et al., 2011; Nakamura et al., 1995), which indicates these are between 800-1500 times less potent than the synthetic drug Captopril.

1.4.4 Measurement of in vitro ACE inhibitory activity

The establishment of a simple, reliable, and sensitive *in vitro* ACE inhibition assay is desirable to facilitate identification and isolation of ACE inhibitory peptides. Different methods for the measurement of ACE activity have been reported in the literature, including high-performance liquid chromatography (HPLC), spectrophotometric and fluorometric methods (Chen et al., 2013). Various substrates are used to measure ACE activity. The most common are the synthetic peptides hippuryl-L-histidyl-L-leucine (HHL) and furanacryloyl-L-phenylalanylglycyl-glycine (FAPGG) (Carmel and Yaron, 1978, Sentandreu and Toldra, 2006). In most studies, the assay used to measure ACE inhibitory activity is based on the method developed by Cushman and Cheung (1971). This method relies on

the hydrolysis of the peptide HHL by ACE to produce hippuric acid (HA) which is then extracted with ethyl acetate, and spectrophotometric assays are then used to determine its concentration. In other studies, where modifications of this method have been reported, the ethyl extraction method was substituted with specific binding of His-Leu with 2,4,6-trinitrobenzene sulphonate (TNBS) (Matsui et al., 1992) or a specific reaction of HA with benzene sulfonyl chloride (Li et al., 2005). However, several drawbacks of this method have been reported. An organic solvent is used to extract the product from the reaction, which allows for an additional source of error. Also, an incubation time of 30 minutes is necessary to yield a significant amount of product, which further prolongs the experiment. Additionally, both HHL and HA compounds show significant absorption at 228 nm, which could result in an overestimation of the amount of HA, subsequently leading to an overestimate of ACE inhibitory activity (Chen et al., 2013).

Homquist et al (1979) described another colorimetric method utilising the peptide FAPGG as a substrate of ACE. This assay was based on the blue shift of the absorption spectrum between 328 and 352 nm that occurs upon hydrolysis of FAPGG to FAP and GG. Various modifications such as fixed time conditions (Murray et al., 2004), employment of 96-wells microtiter plates (Otte et al., 2007) or HPLC determination (Anzenbacherová et al., 2001) were also applied. Some researchers have compared the performance of the ACE inhibition assays using FAPGG and HHL as substrates (Shalaby et al., 2006). Both methods showed a similar performance, but FAPGG seemed to be more stable than HHL in solution. Additionally, the assay with FAPGG as substrate consumed less chemicals per sample. In another study, ACE inhibition was measured using synthetic substrates FAPGG and HHL, and natural substrate angiotensin I. According to a SWOT (strengths, weaknesses, opportunities, and threats) analysis of all substrates used and all tests performed, it was found that the ACE inhibition assay utilising FAPGG as a substrate was the easiest and quickest (Henda et al., 2013). Indeed, FAPGG has a faster hydrolysis process compared to that of HHL, as reported by Bunning et al. (1983). Also, considering kinetic parameters, FAPGG has a Km = $3x10^{-4}$ M and a kcat = 19000 min^{-1}, which compares favourably with Km = $2.4x10^{-3}$ M and kcat = 15600 min^{-1} for HHL. Further, considering the time needed to prepare the plate, perform data collection, and treatment, an IC_{50} can be obtained for two or more inhibitors within 30 minutes. FAPGG is also reasonably cheap, and a spectrophotometer is easy to use. This method developed by Homquist et al., 1979 is hence useful for screening fractions obtained from hydrolysates or for quickly evaluating ACE inhibitory potential and comparing biological activities from one batch to another (Henda et al., 2013).

1.4.5 In vivo ACE inhibitory activity

The *in vivo* ACE inhibitory activity of peptides is generally investigated in spontaneously hypertensive rats (SHRs) by a periodic blood pressure measurement following intravenous or intraperitoneal injection and oral gavage (Jao et al., 2012). The hypotensive effect of milk-derived peptides has been demonstrated in hypertensive rats (Geerlings et al., 2006; Jakala et al., 2009; Liu et al., 2011; Muguerza et al., 2006; Sánchez et al., 2011; Seppo et al., 2003). The clinical efficacy of anti-hypertensive BAPs substantively depends on two factors: the absorption of peptides into the blood stream, and their resistance to degradation by gastrointestinal enzymes. In terms of blood pressure reduction, the clinical results will be different based on the amino acid sequence of the BAP, and these two factors (Aluko et al., 2015). The evidence obtained in human clinical studies was largely compatible with the results from SHRs (Martin & Deussen, 2019). Indeed, the significant blood-pressure lowering effects of milk-derived peptides in human subjects have been reported in several clinical studies (Aihara et al, 2005; Beltrán-Barrientos et al., 2018; Boelsma & Kloek, 2010; Cadée et al., 2007; Mizuno et al., 2005; Pins & Keenan, 2006). Furthermore, many clinical trials were carried out using the well-known ACE inhibitory tripeptides IPP and VPP, and their efficacy to moderately reduce systolic blood pressure has been highlighted in both human and animal studies (Cicero et al., 2011; Cicero et al., 2013; Pripp, 2008; Xu et al., 2008).

1.4.6 Discrepancies between in vitro and in vivo ACE inhibitory activities

As evident from the preceding sections, many milk-derived peptides with *in vitro* ACE inhibitory activity have been characterised in recent years. The anti-hypertensive effects of these peptides have also been documented in SHRs and in human trials. However, in some cases, poor correlation between the *in vitro* ACE inhibitory activity of milk-derived peptides and the *in vivo* anti-hypertensive activity has been observed. This discrepancy can be explained by bioaccessibility and bioavailability issues, stability, and reactivity of the peptides within the food, gut, and by the microbiota (Sánchez-Rivera et al., 2012; Wu et al., 2017). Bioavailability is an essential factor in gaining health benefits from a food component (Rein et al., 2013). To display their bioavailability, ACE inhibitory peptides must remain active during gastrointestinal digestion and absorption before reaching the bloodstream and then the cardiovascular system (Jao et al., 2012). The inconsistency in the peptides' *in vivo* and *in vitro* effects can thus also be partly due to digestion which renders less

BAP sequences (Sánchez-Rivera et al., 2014). The bioavailability of a peptide, and consequently its ACE inhibitory activity may also be affected by amino acid composition and the chain length of the peptides during gastrointestinal digestion. Proline- and hydroxyproline-containing peptides and tripeptides, having proline-proline at their C-termini, were reported to be resistant to degradation by digestive enzymes (Vermeirssen et al., 2004), whereas oligopeptides are more difficult to keep intact during absorption, as these are more easily hydrolysed by different proteases and peptidases (Ding et al., 2016).

Furthermore, according to several studies, food-derived peptides can exert their blood pressure-lowering effects not only through ACE inhibition, but also via other emerging anti-hypertensive mechanisms of ACE inhibitory peptides, such as upregulation of angiotensin-converting enzyme 2 (ACE2), endothelin-converting enzyme (ECE) inhibition, improvement of endothelial function, and reduced vascular oxidation and inflammation. Evidence has shown that BAPs can also interact with AT2 receptors, arginine-nitric oxide pathway, RAS-related renin and Ca^{2+} channels, which may allow them to mediate their anti-hypertensive effects, together with ACE inhibition (Fernández-Musoles et al., 2010; Majumder & Wu, 2014; Udenigwe & Mohan, 2014).

1.4.7 In silico approaches for the identification of ACE inhibitory peptides

ACE inhibitors are commonly discovered using classical approaches which involve: (i) identifying a suitable protein source; (ii) enzymatic hydrolysis of protein and release of BAP fragments; (iii) isolation and purification of peptides by chromatographic systems or membrane separation; and (iv) validating the activities and structures of the identified peptides (Li-Chan et al., 2015; Li et al., 2004). However, these investigational techniques are both costly and time-consuming for researchers and manufacturers, and there is uncertainty regarding the bioactivity of the protein hydrolysates, which must be confirmed.

Bioinformatics provides software tools and methods aimed at understanding, analysing, and interpreting large quantities of biological data by combining biology, computer science and statistical techniques. This field has been extensively used in biological sciences for *in silico* analysis to gain a better understanding of the biological basis of disease (Tu et al, 2018). Furthermore, since the association between functional food ingredients and disease/health has attracted increasing attention, the application of bioinformatics in the study of food compounds is being widely examined (Valdés et al., 2017). In some cases, some multifunctional peptides have a unique primary structure that can resist proteolysis, and/or can induce more than one physiological activity due to

their overlapping sequences (Agyei et al., 2016). Bioinformatics can help determine how the structure of BAPs relates to their activity, thus it can minimise the number of experiments that must be performed to generate BAPs from food. Recently, due to the challenges encountered by more classical experimental approaches, bioinformatics has been frequently used to identify BAPs in proteins (Ji et al., 2018). Also known as *in silico* analysis, bioinformatics includes computational methods that consist of databases, software, and online tools, applied to interpret, curate, and manage information related to biological systems (Li-Chan et al., 2015).

In silico molecular docking methodologies are one of the most applied and important virtual screening methods in novel drug design strategies (Di Muzio et al., 2017; López-Camacho et al., 2015; Yuriev et al., 2015). These methods can provide an economical and comparatively fast alternative to standard experimental methodologies for compound screening (Sousa et al., 2010; Pereira et al., 2016). The aim of molecular docking is to predict the affinities and binding modes of small ligands within the binding site of a particular receptor protein. Molecular docking normally involves four main procedures: selection and preparation of a protein structure, preparation of ligand, docking, and analysis of the results, as shown in Figure 1.4. Molecular docking methodologies have two important goals: (i) finding correct binding poses and, (ii) accurately predicting the binding affinities for each of those poses. More accurate predictions of these binding affinities and binding poses can significantly reduce experimental efforts, and consequently provide suggestions of candidates for bioactive compounds (Shin et al., 2013).

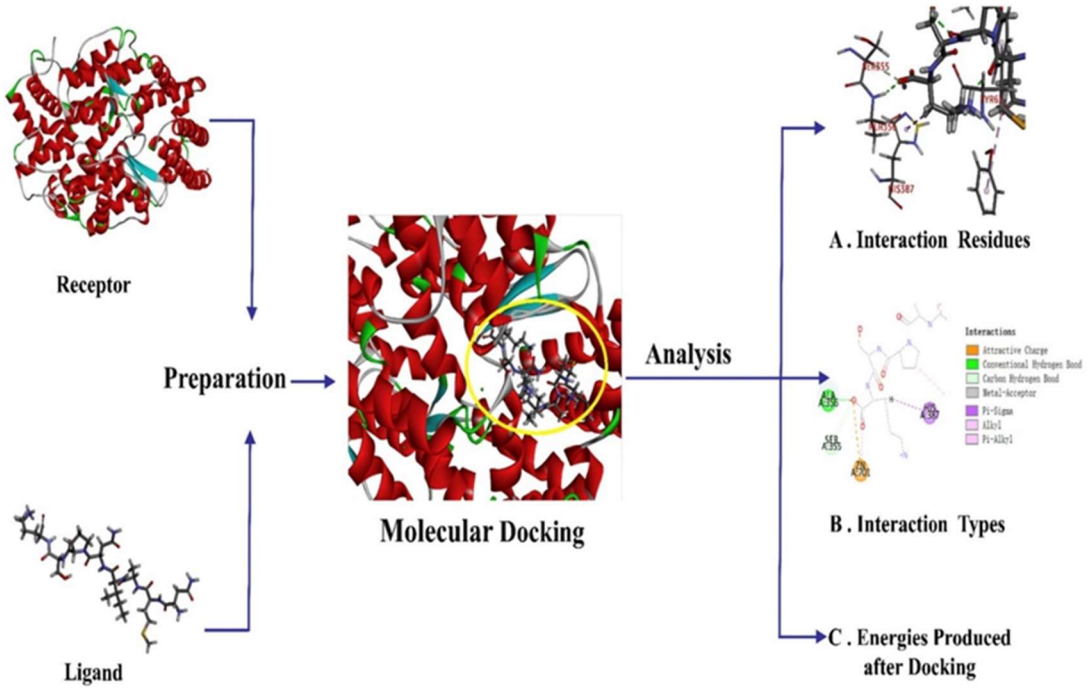

Figure 1.4 General procedure for molecular docking. Taken from Tu et al., (2018).

Several docking algorithms used in multiple studies to predict potent ACE inhibitory peptides encrypted in food proteins (García-Mora et al., 2017; Guo et al., 2017; Sangsawad et al., 2018; Shi et al., 2017; Wang et al., 2017; Wu et al., 2016; Yu et al., 2018), and more specifically milk proteins, are coming to light (Ashok & Aparna, 2017; Lin et al., 2017). Using molecular docking, specific and potentially crucial interactions between receptor and ligand could be realised, and novel sequences, which are more relevant to human health from a stability and bioavailability perspective identified. Molecular docking enables the investigation of the interactions of peptide sequences with specific binding sites of ACE, which could help to obtain a better prediction of bioactivity *in vivo*. This can be a powerful tool that can be used to pre-screen potentially BAPs prior to their testing *in vivo* (Nongonierma & Fitzgerald, 2017).

1.5 Bioactive peptides produced from *in vitro* digestion

Traditionally, BAPs derived from milk proteins can be produced using the following methods:

(1) Enzymatic hydrolysis by different enzymes, such as trypsin, pepsin, and papain (FitzGerald et al., 2004; Gobbetti et al., 2004; Gobbetti et al., 2007; Korhonen & Pihlanto, 2006; Meisel and FitzGerald, 2003; Yamamoto et al., 2003)
(2) Hydrolysis by proteolytic microorganisms, such as lactobacillus (FitzGerald and Murray, 2006; Gobbetti et al., 2007; Matar et al., 2003)
(3) A combination of methods 1 and 2 (Chen et al., 2007; Tsai et al., 2008).

In this section, examples of methods (1) and (2) will be provided, with a focus on production of BAPs from whey proteins. As casein-derived peptides were the first source of BAPs to be reported and identified in milk proteins, production of BAPs from caseins is more advanced. Caseins are considered an important source of a broad variety of potent BAPs, namely peptides with anti-hypertensive activity. As such, multiple strategies and processes have been developed for generation of these peptides, with some of them leading to commercialised products.
Furthermore, an increasing number of recent studies have demonstrated the *in vitro* bioactivities of whey-derived peptides in relation to a wide range of biological functions. However, a limited number of processes to generate such peptides have been developed. Therefore, a review mainly focused on the generation of BAPs derived from whey proteins will follow.

1.5.1. Enzymatic hydrolysis by different enzymes

Enzymatic hydrolysis is the most common way to generate BAPs. Those from milk and whey proteins have been produced using a wide range of proteases from various sources. Porcine or bovine derived digestive enzymes are the most employed proteins for hydrolysis of whey and subsequent generation of BAPs, such as pepsin, trypsin, chymotrypsin, and kallikrein (Korhonen and Pihlanto 2006; Korhonen 2009). Found in the gastrointestinal tract, these enzymes break down dietary proteins into their peptide and amino acid components:

- Pepsin (E.C. 3.4.23.1) is the principal acid protease in the stomach. It is an aspartic endopeptidase that displays a broad specificity. Synthesised in the gastric mucosa, then secreted into the stomach, pepsin is released as a zymogen called pepsinogen which is stable in alkaline and neutral conditions but is converted into active pepsin when it reaches the acidic pH of the stomach (Tang, 1998).

- Trypsin (EC 3.4.21.4) is a serine endopeptidase and one of several enzymes found in the digestive systems of all animals, including mammals, fish, and invertebrates. It is stable and exhibits maximum activity in a slightly alkaline environment (pH of around 8) with moderate concentrations of $CaCl_2$ (20 mM). Trypsin is produced as a pre-proenzyme by the pancreas, and then transported to the Golgi complex where it is stored within secretory granules as the pro-enzyme trypsinogen. Once activated, trypsin plays a crucial role in protein digestion by activating the pro-enzymes of all digestive enzymes, such as chymotrypsin (Halfon & Craik, 1998). Trypsin cleaves peptide bonds at the C-terminal side of lysine and arginine residues, i.e., amino acids with basic side chains.

- Chymotrypsin (EC 3.4.21.1) is a serine endopeptidase that is identified as the second major protease component of pancreatic juice after trypsin. It is synthesised in the acinar cells of the pancreas as a zymogen, chymotrypsinogen. Chymotrypsin acts upon peptide bonds at the C-terminal side of aromatic amino acid (tryptophan, tyrosine, phenylalanine, methionine, and leucine) residues. It preferentially cleaves peptide bonds where the carboxyl side of the amide bond (the P1 position) is a tryptophan, tyrosine, or phenylalanine, as these amino acids have an aromatic ring in their side chain that fits into the hydrophobic pocket of the enzyme (Gráf et al., 1998).

1.5.2 Microbial fermentation

A vast variety of microbial proteinases have been employed to generate BAPs from whey. Sources include *Bacillus* spp., *Aspergillus* spp., *Penicillium citrinum*, *Cryphonectria parasitica*, *Rhizomucor miehei*, and *Rhizopus niveus* (Law, 2002). Although the use of microbial enzymes has been more limited, these enzymes can have certain advantages over enzymes extracted from animal sources; (i) Transportation costs: Microbial proteinases can be produced anywhere in the world, (ii) It is easy to predict the enzyme yield from the fermentation parameters and, (iii) It is easier to avoid challenges related to animal borne diseases and enzyme availability (Law, 2002). Examples of microbial proteinases include Thermolysin, Proteinase K, and Subtilisin:

- Thermolysin (E.C. 3.4.24.27) is produced by the Gram-positive thermophilic bacterium *Bacillus thermoproteolyticus*. It is a thermostable neutral metallopeptidase that cleaves peptide bonds at the N-terminus of hydrophobic residues such as phenylalanine, leucine, valine, and isoleucine. In the P1 position, a hydrophobic amino acid residue is preferred, whereas in P2 and P2', phenylalanine or alanine is preferred to glycine, with the order of preference being Leu>Ala>Phe>Gly (Beynon & Beaumont, 1998; Bond, 1996).

- Proteinase K (E.C. 3.4.21.64) is synthesized by *Tritirachium album* fermentation. It is a member of the subtilisin family of proteinases and an alkaline endolytic enzyme that hydrolyses peptide bonds at the C-terminus of aliphatic, aromatic, or hydrophobic amino acid residues in position P1, with a preference for aromatic and hydrophobic residues (Bond, 1996; Saenger, 1998).

- Subtilisin (E.C. 3.4.21.62) is a serine endoprotease that was first isolated from *Bacillus subtilis*. The term subtilisin also includes enzymes secreted by various *Bacillus* species. Commercially available as alcalase, Subtilisin Carlsberg is an alkaline protease secreted by *B. licheniformis*. Alcalase has been widely used to generate BAPs derived from whey proteins due to their broad specificity for peptide bonds. These microbial enzymes are stable over a range of pH 6-10 and have two subsites with strong preferences. S1 subsite has an affinity

for large non β-branched hydrophobic side chains, whereas S4 subsite has a strong affinity for hydrophobic side chains (Ballinger & Wells, 1998).

The release of BAPs from milk proteins through microbial proteolysis is well documented. Numerous researchers have reported that *Lactobacillus helveticus* strains are particularly capable of generating ACE inhibitory peptides, most notably IPP and VPP, with their anti-hypertensive effects being demonstrated in human studies and rat models (Aihara et al., 2005; Mizushima et al., 2004; Seppo et al., 2003). Cheese starter and yogurt bacteria, as well as probiotic bacteria, have also been shown to release BAPs from milk during fermentation (Donkor et al., 2007). In particular, the fermentation of milk with a commercial starter culture mixture of lactic acid bacteria (LAB) strains, succeeded by hydrolysis with a microbial protease, was found to significantly increase the milk hydrolysates' ACE inhibitory activities. Additionally, the anti-hypertensive effects of the hydrolysates containing ACE inhibitory peptides GVW and GTW were established in an animal model study including SHRs (Chen et al., 2007). Various novel ACE inhibitory peptides derived from milk, following fermentation with *Enterococcus faecalis* strains isolated from raw milk, were also identified by Quiros et al, (2007). In fact, several dairy LAB cultures, and probiotic strains such as *Bifidobacterium lactis*, *Lactobacillus acidophilus*, and *Lactobacillus casei* were reported to release ACE inhibitory peptides during growth (Donkor et al., 2007).

Some researchers have isolated and identified proteolytic strains from dairy products to produce BAPs derived from milk whey proteins. The yeast *Kluyveromyces marxianus* screened from a traditional Tibet dairy product was shown to generate highly active proteases, leading to the release of ACE inhibitory peptides from whey proteins (Li et al., 2015). The release of ACE inhibitory BAPs during the fermentation process of whey by its native microbiota was recently documented by Mazorra-Manzano et al., (2020). Whey hydrolysates with potential anti-hypertensive activity, fermented by the probiotic *Lactobacillus acidophilus* strain were also identified by Skrzypczak et al., (2019).

In the food industry, various enzyme combinations of proteinases and other gastrointestinal enzymes, such as alcalase, pancreatin, and thermolysin, together with enzymes from fungal and bacterial sources, have been used to generate BAPs from different proteins (Korhonen & Pihlanto, 2003). In a study by Otte et al. (2007), five proteases (thermolysin, trypsin, proteinase K, pepsin, and *Bacillus licheniformis* protease) were used to hydrolyse milk proteins *in vitro*. The highest ACE inhibition was shown in the samples hydrolysed with thermolysin, with whey protein-derived BAPs displaying an IC_{50} of 90-400 mg/mL and α-La hydrolysates. A high ACE inhibitory activity with an IC_{50} value of 0.041 mg/mL of whey proteins produced using *in vitro* gastrointestinal digestion was also

reported by Vermeirssen et al. (2005). In addition, following digestion with pancreatin and pepsin, BAPs with high antioxidant and ACE inhibitory activities were identified in hydrolysates of infant formula and human milk (Hernandez-Ledesma et al., 2007). More recently, whey proteins hydrolysed by alcalase at 55 °C and pH 9 for 8 hours were found to have an antioxidant activity of 1.18 ± 0.015 µmol Trolox/mg protein (Athira et al., 2015).

Furthermore, whey protein-derived BAPs have been produced using commercial enzyme mixtures. Novel commercial proteolytic preparations are regularly being introduced onto the market. Compared to the more purified enzymatic preparations, the advantages of using such commercial mixtures can be that a wider range of BAPs can be generated at reduced cost (Smyth & FitzGerald, 1998; Mullally et al., 1994; Mullally et al., 1995). However, most of these enzymes are not well characterised, which could hinder their potential for being applied to the generation and identification of BAPs (Fráckowiak & Kennedy, 2009).

1.5.3 In vitro simulation of gastrointestinal food digestion

Due to the growing demand for BAPs being the driving force for the development of functional foods, the identification and characterisation of food-derived BAPs has become an emerging research topic (Tadesse & Emire, 2020). However, the ability to translate novel research findings into practical applications to produce BAPs is not developing at the same pace. Among the main factors behind this delay is the peptides' low bioavailability following oral administration, which often results in a lack of correlation between the *in vitro* bioactivities and *in vivo* functions (Santos et al., 2019; Wang et al., 2021). To reach the target organs where they can exert their health-promoting impact, BAPs must resist the low pH in the stomach as well as the action of the digestive enzymes during the transport of peptides through the gastrointestinal tract (bioaccessible) and across the intestinal epithelial barrier to become bioavailable (FitzGerald et al., 2004; Roufik et al., 2006; Quirós et al., 2009).

Furthermore, due to milk proteins displaying completely different properties, milk is considered to be unique in its digestion kinetics. The coagulation of the casein fraction of milk proteins, induced by pepsin under acidic conditions, results in a prolonged gastric digestion. In contrast, soluble whey proteins have been found to reach the intestinal compartment much more rapidly (Boirie et al., 1997). Consequently, to evaluate the bioaccessibility and bioavailability, and thereby the *in vivo* effects of the whey-derived peptides, the identification of BAPs directly in milk is insufficient. It is

also critical to follow the complex digestive processes within the human digestive tract in more detail (Brodkorb et al., 2019; Sánchez-Rivera et al., 2014; Santos et al., 2019; Zhang et al., 2017).

1.5.3.1 *In vitro* digestion systems and methods

Various types of *in vitro* digestion methods are commonly applied for food. Based on the modelling approach, these can be broken down into static and dynamic methods. The aim of these models is to simulate the physiological conditions of the upper gastrointestinal tract (oral, gastric, and small intestinal phases). The suitability of many dynamic models for simulating the digestion of food and pharmaceutical products has been demonstrated in various studies (Kong et al., 2010; Ménard et al., 2014; Minekus et al., 2015; Molly et al., 1993; Wickham et al., 2009). However, these methods are complex, expensive to set up and maintain, and consequently may not be accessible to many food researchers.

In contrast, due to their simplicity, static models have been commonly used for many decades. These methods use a constant pH for each digestive phase, and a constant ratio of food to electrolytes and enzymes (Boisen et al., 1997; Kaukonen et al., 2004; Maldonado- Valderrama et al., 2010). Also, the utility of static *in vitro* digestion models in predicting outcomes of *in vitro* digestions has been demonstrated in various studies (Bohn et al., 2018; Sanchón et al., 2018). These standardised static models are employed to simulate the gastrointestinal behaviour of pharmaceutical products (United States Pharmacopeia methods). To assess the *in vitro* bioaccessibility of soil contaminants (Oomen et al., 2004), such as mycotoxins and heavy metals in food (Versantvoort et al., 2005), other static models were also developed and standardised by the Bioaccessibility Research Group of Europe (BARGE) (Wragg et al., 2009).

1.5.3.2 INFOGEST static in vitro simulation of gastrointestinal food digestion

Although they were based on available physiological data described by breakthrough papers (Dressman et al., 1990; Wentworth et al., 1982), the purpose, experimental conditions, and endpoints of the static methods developed by the United States Pharmacopeia, and BARGE group were shown to be unsuitable for the digestion of food. This is due to the variability and complexity of food structures, together with the prevalence of very different research questions in food technology and science (Hurr et al., 2011). The urgent need for harmonisation of digestion conditions was therefore identified. To this end, a standardised protocol based on an international

consensus was established by the international INFOGEST network of multidisciplinary experts from more than 35 countries (Dupont et al., 2011; http://www.cost-infogest.eu). Generally referred to as the INFOGEST method, this approach consists of a set of digestive parameters for a static *in vitro* simulation of adult digestion, reliable for food. Based on existing *in vivo* physiological data, parameters such as pH, time of digestion, enzymes, bile, and dilution were justified and discussed in great detail (Minekus et al., 2014).

The digestion procedure is summarised in Figure 1.5. Briefly, the method consists of exposing the food to three digestive phases: oral, gastric, and intestinal (Minekus et al., 2014).

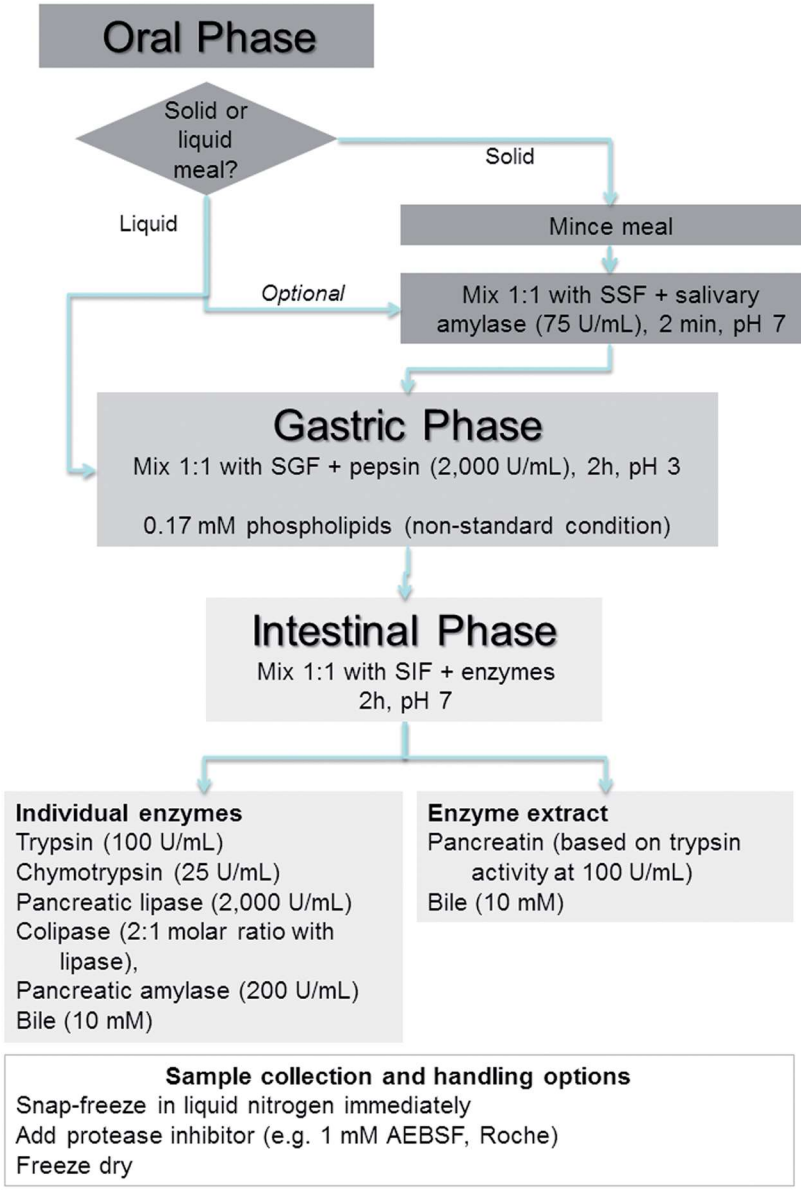

Figure 1.5. Flow diagram of the INFOGEST digestion method (Minekus et al., 2014). The simulated digestive conditions include SSF (Simulated Salivary Fluid), SGF (Simulated Gastric Fluid) and SIF (Simulated Intestinal Fluid). At each corresponding digestion phase, enzyme activities are expressed in units per mL of final digestion mixture.

The oral phase is where solid foods are mechanically broken down in the mouth, through the process of mastication (chewing). The texture of solid food samples in the oral cavity is significantly transformed by salivation and mastication, leading to the formation of a bolus, prior to swallowing (Peyron et al. 2004). The oral phase involves dilution of the solid food masses to achieve a final ratio of food to simulated salivary fluid (SSF) electrolyte stock solution of 50:50 (w/v). The amount of SSF must be high enough to reach a paste-like consistency, and thereby simulate the lubrication and wetting of solids by salivation. Liquid foods don't need to be chewed and so it is optional for liquid food samples to be exposed to the oral phase (Minekus et al., 2014). Besides chewing, the other essential factor for solids is the addition of saliva containing the enzyme α-amylase (EC 3.2.1.1). Human salivary α-amylase (75 U mL^{-1}) is thus added to the digestion mixture, followed by $CaCl_2$ (0.75mM). The recommended contact time of food with salivary α-amylase is limited to 2 min at 37 °C and pH 7 (Pedersen et al., 2002). Following oral processing, solid food is emptied from the oral cavity (Okada et al., 2007). Transit times of food in the oesophagus is a short process lasting a few seconds (Nguyen et al., 1997), with no reported effect on food digestion (Gardner et al., 2002).

The gastric phase is where the liquid food or oral bolus, is diluted 50:50 (v/v) with simulated gastric fluid (SGF) and the gastric enzyme porcine pepsin (EC 3.4.23.1, 2,000 U mL^{-1}), and incubated under agitation for 2 hours at pH 3.0 and 37 °C. The exposure time in the gastric phase is recommended for a period of 2 hours, because this duration represents the half emptying of a reasonably nutritious and semi-solid meal (Dressman et al. 1990). Despite commonly held beliefs about a very low pH (1-2) in the stomach, the pH in the gastric compartment is very dynamic and dependent on the buffering capacity of the food (Carrière et al. 1991; Kalantzi et al. 2006). Following entry of food into the stomach, pH usually increases to 5 and above, before the pH is lowered to values required for optimal enzyme activity after secretion of hydrochloric acid (Kalantzi et al., 2006). Gastric lipase is mainly active between pH 4 and 6 (Carriere et al., 1991), whereas acidic conditions with pH ranging from 2 to 4 are required for optimal pepsin enzyme activity (Kalantzi et al., 2006). To match the 2-hour recommendation for the duration of the gastric phase, Minekus et al., (2014) suggested the use of a static pH value of 3, representing a mean value for a common meal over that period. Also, some mixing either by stirring or shaking of the food sample is recommended to mimic the physical environment of the gastric compartment.

Once the simulated gastric phase of digestion is completed, the food is transferred to a simulation of the digestion that takes place in the small intestine. The intestinal phase is where the gastric chyme is diluted 50:50 (v/v) with simulated intestinal fluid (SIF), bile salts and pancreatic enzyme (either

pancreatin from porcine pancreas or individual enzymes) and incubated at pH 7 for a further 2 hours. Based on the trypsin activity, pancreatin is added to achieve 100 U mL^{-1} in the final mixture but if the food contains high amounts of fat, pancreatin is added to obtain a concentration of 2,000 U mL^{-1} lipase activity in the final mixture. Also, individual enzymes are alternatively added to achieve the following activities in the final digestion mixture: bovine chymotrypsin (EC 3.4.21.1) (25 U mL^{-1}), porcine trypsin (EC 3.4.21.4) (100 U mL^{-1}), porcine pancreatic α-amylase (EC 3.2.1.1) (200 U mL^{-1}), and porcine pancreatic lipase (EC 3.1.1.3) (2,000 U mL^{-1}). Since they play an important role in the digestion of both protein and lipid, bile salts and $CaCl_2$ are added to reach a concentration of 10 mM and 0.3 mM in the final digestion mixture, respectively. Minekus et al., (2014) suggested the period of simulated intestinal digestion to be 2 hours, based on normal transit times in the human gut. To mimic the pH during passage through the small intestine, an average value of pH 7 is also recommended (Carriere et al., 1991; Kopf-Bolanz et al., 2012; Versantvoort et al., 2012).

1.5.3.3 Correlation between *in vivo* and *in vitro* data on milk digestion

Using skim milk powder (SMP) as a standardised food matrix, the success of the INFOGEST method was experimentally tested by Egger et al., (2016). The results showed that the harmonised digestion protocol significantly enhanced the comparability of experimental results to *in vivo* data. Whether the *in vitro* method indeed was comparable to the *in vivo* digestion, was still, however, an open question. To answer this second and key research question, an *in vivo* trial was performed where pigs were fed the same SMP that was formerly digested with the *in vitro* method. Egger et al., (2017) found that, considering the digestion of proteins, the gastric and intestinal phases indeed reflected the *in vivo* digestion in the pigs. The performance of the harmonised digestion protocol also has been validated on milk proteins using human digests (Sanchón et al., 2018; Miralles et al., 2021).

Since its publication in 2014, the INFOGEST method has been cited more than 650 times in Web of Science, and thus has received a Highly Cited Paper status for agricultural sciences. This *in vitro* digestion method also has been extensively used with a variety of foods for various purposes (Brodkorb et al., 2019). BAPs from several milk proteins have been successfully produced using digestive enzymes and combinations of various proteinases (del Mar Contreras et al., 2009), including potent whey proteins derived ACE inhibitory peptides (Hernandez-Ledesma et al., 2002; Picariello et al., 2010; Picariello et al., 2013). *In vitro* digestion methods could thus be employed to identify ACE inhibitory, or other BAPs, from milk proteins (Larder et al., 2021; Tagliazucchi et al., 2017).

1.6 Angiotensin II-converting enzyme inhibitory peptides and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

Originally, RAS was defined as an endocrine system that targets both central and peripheral receptors. However, substantial evidence shows a tissue-based RAS that influences local cellular actions (Chappell, 2016), and exerts several autocrine/paracrine effects associated with tissue homeostasis and physiology (Bader, 2010). Indeed, RAS was demonstrated to play crucial roles in cellular growth, proliferation, differentiation, migration, and apoptosis, together with inflammation and extracellular matrix (ECM) remodelling (Ribeiro-Oliveira et al., 2008).

The RAS pathway is composed of a cascade of proteases that generate several bioactive molecules (Chappell, 2016). Besides the classical components of the RAS (ACE, renin, Ang II, AT1 and AT2 receptors) that were reviewed in Section 1.4.2, receptors such as ACE2, and novel peptides such as angiotensin 1–7 (Ang (1–7)) also seem to play a key role in the regulation of the RAS pathway (Santos, 2014).

1.6.1 The classical and counter-regulatory renin–angiotensin system (RAS) pathways

The glycoproteolytic enzyme renin is produced by specialised secretory cells called juxtaglomerular (JG) cells, which are found within the afferent arterioles of the kidney and are activated in response to reduced blood pressure (Nehme et al., 2019; Ren et al., 2019) (Figure 1.6). The liver-derived angiotensinogen is the main substrate for renin. Once it has been released into the blood, renin cleaves angiotensinogen to form the physiologically inactive peptide angiotensin I (Ang I). Ang I is then further hydrolysed into the potent vasoconstrictor Ang II by ACE and exerts its important physiological effects via two distinct receptors, AT1 and AT2. The vast majority of the deleterious classical Ang II effects such as cardiovascular inflammation and vasoconstriction are mediated by AT1 receptors (Nguyen Dinh Cat & Touyz, 2011). The precise physiological role of the AT2 receptor remains unclear, and studies reporting its functions remain under debate. However, it seems that the AT2 receptor is associated with vasodilatory effects, and there is evidence to suggest that AT2 receptors could counteract the AT1 receptor-mediated vasoconstrictor effects of Ang II (Padia & Carey, 2013; Lemarié & Schiffrin, 2010).

Discovered as a homologue of ACE, ACE2 was first identified in 2000 (Tipnis et al., 2000). ACE2 is an 805 amino-acid Type-I membrane-anchored glycoprotein that functions as a monocarboxypeptidase and zinc metalloenzyme (Tikellis & Thomas, 2012). ACE2 is primarily located in vascular endothelial

cells, and the renal tubular epithelium (Donoghue et al., 2000). Ang II acts as the major substrate for ACE2 (Donoghue et al., 2000; Rice et al., 2004). Ang (1–7) and angiotensin 1–9 (Ang (1–9)) are vasodepressor peptides that oppose the vasoconstrictor actions of Ang II. ACE2 cleaves the amino acid leucine at the C-terminal of Ang I to generate Ang (1-9) (Donoghue et al., 2000; Ocaranza et al. 2006). Ang (1-9) is consequently hydrolysed by neutral endopeptidase 24.11 (NEP) and ACE, to produce the biologically active peptide Ang (1-7) (Rice et al. 2004). Originally defined for its ability to generate Ang (1–9) from Ang I, ACE2 has also been shown to remove the amino acid phenylalanine at the C-terminal of vasoconstrictor Ang II, thus directly generating Ang (1–7) (Vickers et al., 2002). Furthermore, the generation of Ang (1-7) directly from Ang II seems to be physiologically and biochemically more pertinent. ACE2 is hence considered a negative regulator of the RAS because it increases vasodilator Ang-(1-7) and Ang-(1-9) levels and reduces vasoconstrictor Ang II levels (Santos et al, 2003). First described in 1986, Mas is a G protein-coupled receptor that was, at first, thought to be a receptor of Ang II (Jackson et al., 1988). It was not until 2003 that the specific binding of Ang (1–7) to the Mas receptor was finally demonstrated. The identification of Mas as a receptor of Ang (1-7) was another fundamental step to establish the significance of Ang (1-7) (Santos et al. 2003). The non-classical ACE2/Ang (1-7)/Mas axis is now accepted to counteract the majority of the detrimental effects of the classical ACE/Ang II/AT1 receptor axis (Ferreira & Santos 2002, Figure 1.6). Therefore, ACE2 is considered an important counter regulatory enzyme in the RAS pathway that plays a significant role in modulating blood pressure (Patel et al., 2016).

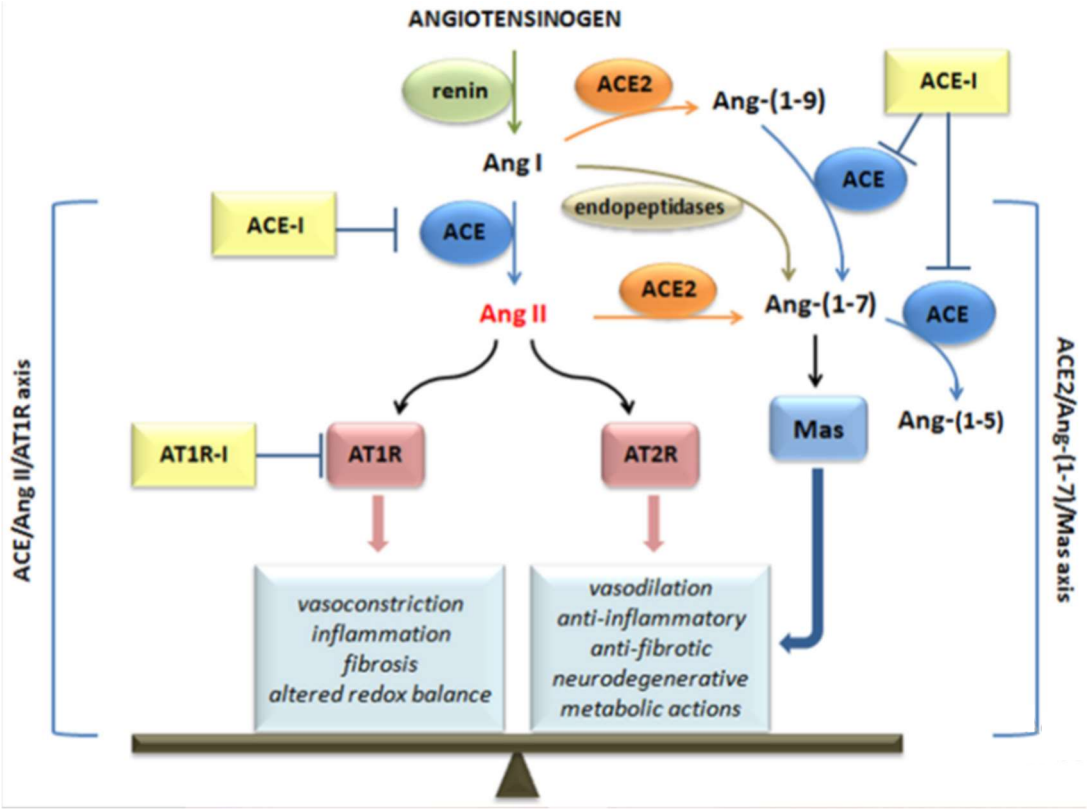

Figure 1.6 Overview of the renin-angiotensin system (RAS) cascade (D'ardes et al., 2020).

Discovery of this novel ACE2/Ang (1-7)/Mas axis, which has been newly added to the RAS system, has significantly transformed our understanding of the regulatory mechanisms of this pathway. It is now commonly acknowledged that the RAS is a dual system with two opposing axes: the beneficial/counter-regulatory axis consisting of ACE2/Ang-(1–7)/Mas, and the largely harmful axis consisting of ACE/Ang II/AT1. Studies have corroborated the participation of the ACE2/Ang (1-7)/Mas axis in the regulation of blood pressure, and most importantly, in the pathogenesis of CVDs. Historically, in clinical interventions targeting the RAS and its pathogenic actions, ACE and Ang II have been the focus. However, the importance of ACE2 in maintaining the balance of the RAS has been demonstrated in many recent studies, with some indicating that ACE2 may even be more significant than ACE in regulating local levels of Ang (1-7) and Ang II in the cardiovascular system. Indeed, acquired or genetic deficiency of ACE2 was associated with reduced levels of Ang (1-7), and increased levels of Ang II (Tikellis et al., 2012). Furthermore, some data have correlated endothelial ACE2 overexpression with reduced blood pressure, indicating that ACE2 functions as a negative regulator of the RAS in spontaneously hypertensive stroke-prone rats (SHRSP) (Rentzsch et al., 2008). In other animal models, the overexpression of ACE2 cardiomyocyte was also associated with reduced effects of hypertension (Der Sarkissian et al., 2008), and other beneficial effects, such as

ameliorated myocardial performance, and survival in animal models of heart failure (Crackower et al., 2002; Ferreira et al., 2001; Nakamura et al., 2008; Yamamoto et al., 2006). Thus, these novel RAS components open new avenues for the development of novel cardiovascular therapeutic strategies targeting the ACE2/Ang (1-7)/Mas axis (Ferreira et al. 2012).

1.6.2. ACE and ACE2

Despite sharing 42% sequence identity and 61% sequence similarity with the catalytic domain of ACE (Donoghue et al., 2000), ACE2 differs from ACE in terms of substrate specificity. While ACE is a dipeptidyl carboxypeptidase, with two zinc-binding motifs (HEXXH, where X is any amino acid) at both its N- and C-terminal catalytic domains; ACE2 functions exclusively as a monocarboxypeptidase, presenting only one zinc-binding motif at its N-terminus (Donogue et al., 2000; Guy et al., 2003; Tipnis et al., 2000). Further distinguishing its specificity from that of ACE, ACE2 appears to be insensitive to conventional ACE inhibitors, such as Captopril, Lisinopril, and Enalapril (Rice et al., 2004).

In addition, ACE and ACE2 are structurally well conserved, as demonstrated in an analysis of key active site residues between these two proteins. Also, there is a high similarity between the catalytic domains of ACE and ACE2. As a result, the catalytic mechanism of ACE2 is closely similar to that of ACE. However, distinctive structural differences are found between the active site pockets of ACE and ACE2, due to differences in substrate specificity in the ligand-binding pockets, mainly in the binding at the S2' subsite. The cavity in ACE is larger than that of ACE2, which provides an additional space for amino acid binding in the specificity pocket. Also, the S1 subsite of ACE2 is smaller than that of ACE (Guy et al., 2003).

1.6.3. ACE2: The cell entry receptor for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

Following the severe acute respiratory syndrome coronavirus (SARS-CoV) outbreak in 2003, ACE2 was shown to be the receptor for viral entry into bronchial epithelial cells (Li et al., 2003). Findings have also revealed that SARS-CoV-2 hijacks ACE2 to enter the host cells in humans, using its SARS-COV-2 spike protein (S protein) (Scobey et al., 2013; Qiu et al., 2020; Zhou et al., 2020). The first electron micrograph of SARS-CoV-2 describes how it uses the highly glycosylated S protein to enter host cells (Wrapp et al.,2020). The SARS-CoV S protein is functionally divided into two subunits,

denoted S1 and S2 which are responsible for binding to the host cell receptor, and fusion of the viral and cellular membranes, respectively. S1 and S2 are not covalently bound in the pre-fusion conformation (Cui et al., 2019; Rothan & Byrareddy, 2020; Tortorici & Veesler, 2019). Triggered by the S1 subunit and a host–cell receptor binding (RBD) destabilising the pre-fusion trimer, S protein undergoes a drastic structural rearrangement to fuse the viral membrane with the host cell membrane. This results in S2 subunit transitioning to a highly stable post-fusion conformation, together with S1 subunit shedding (de Wilde et al., 2017). Furthermore, the S2 subunit was found to share 99% identity with that of SARS-CoV (Chan et al, 2020), and so it is not surprising that ACE2 is the human cell receptor of SARS-CoV-2.

ACE2 is a type I integral membrane glycoprotein, comprised of the N-terminal peptidase domain (PD) providing a direct binding site for S protein, and the C-terminal collectrin-like domain (CLD) (Yan et al., 2020). SARS-CoV-2 was shown to bind to ACE2 with much higher affinity (10- to 20-fold higher) than the binding of the SARS-CoV S protein (Wrapp et al, 2020), which explains why SARS-CoV-2 is highly contagious. A high expression of ACE2 is mainly present in the tongue and mouth, which facilitates SARS-CoV-2 entry into the cells. ACE2 can also be found on alveolar epithelial cells type I and type II in the lower lungs (Zhao, 2020). After infection, the S protein present on the viral envelope binds to ACE2 on the alveolar surface. This binding activates the clathrin-mediated endocytosis of the ACE2- SARS-CoV-2 complex, including fusion at the cell membrane. Once SARS-CoV-2 enters the target cell, it exploits the alveolar cells' endogenous transcriptional machinery to replicate itself, leading to viral spread through the lung (Perico et al., 2020).

1.6.4 Milk-derived peptides as promising therapeutic candidates

Finding effective and safe compounds that can obstruct entry of SARS-CoV-2 into host cells via ACE2 is a pressing matter for the scientific community. With the aim of being able to commercialise such inhibitory compounds, molecules from natural sources are prioritised due to their low toxicity and lack of associated side effects. In this regard, an active area of current research is the effects of milk-derived BAPs, as health-promoting functional components (Mohanty et al., 2016). Peptide sequences from whey proteins have been found to express various bioactivities, including immunomodulatory actions. In fact, among the different studies characterising immunologically active peptides (Kyo et al., 2001; Sultan et al., 2014), milk proteins represent the vast majority of peptides exhibiting immunomodulatory functions (Cross et al., 2000; Gill et al., 2000; Pihlanto-leppala, 2000).

Recently, in a computer-based *in silico* analysis, β-Lg derived peptides exerting an antiviral activity against SARS-CoV-2 were identified and assessed as promising candidates in the treatment of COVID-19 (Çakır et al., 2021). Similarly, the potential of milk-derived peptides as ACE2 inhibitors was investigated in another recent molecular docking-based screening. The binding mechanisms of milk derived GWELPLL and IQKVAGTW with the best docking potentials for ACE2 were analysed by employing a Molecular Dynamics (MD) simulation approach. Both peptides GWELPLL and IQKVAGTW displayed promising stabilising properties for human cell receptor ACE2 (Pradeep et al., 2021).

Furthermore, controversy has arisen in the scientific community regarding the hypothetical relationship between treatment with ACE inhibitors and COVID-19 disease. However, numerous studies reported a significant association between use of ACE inhibitors, and reduced risk of disease (Mehta et al., 2020; Zhang et al., 2020; Hippisley-Cox et al., 2020). Although there is lack of evidence to demonstrate the effect of ACE inhibitors' use on ACE2 expression and thereby SARS-CoV-2 infectivity, the bulk of experimental evidence indicates that ACE inhibitors may reduce the action of Ang II, and consequently attenuate Ang II-driven acute lung injury (South et al., 2020; Supe et al., 2016; Zambelli et al., 2015). ACE inhibitors thus could offer promise as potential novel therapeutics to treat COVID-19 disease (Kuba et al., 2005; Gu et al., 2016; Imai et al., 2005; Ye et al., 2020).

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CHAPTER TWO Whey-Derived Peptides Interactions with ACE by Molecular Docking as a Potential Predictive Tool of Natural Ace Inhibitors

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Contribution towards PhD thesis: My responsibilities included conducting the literature search, the molecular docking studies, and the formal data analysis and visualisation. Then, I wrote the initial draft of the manuscript for publication, and lastly, I finalised the published manuscript presented below after including the comments and feedback received from co-authors Paula Jauregui and Kim Watson, as well as journal reviewers.

2.1. Introduction

The number of people with unhealthy living habits who have developed cardiovascular disease (CVD) has increased in recent years. The WHO reported that an estimated 17.9 million people lose their lives as a result of cardiovascular disease every year (WHO, 2015). CVDs have become the leading cause of death globally (Celermajer et al., 2012). High blood pressure (hypertension) is one of the most important well-defined risk factors for CVD (Cannon et al, 2008), therefore, cardiovascular diseases can be prevented with blood-pressure lowering treatment. Hypertension is regulated by the renin-angiotensin system (RAS), through modulating the angiotensin-converting enzyme ACE, bradykinin, and other factors (Aluko et al., 2009; Chen et al., 2009; Donkor et al., 2007).

ACE (dipeptidyl carboxypeptidase, EC 3.4.15.1) is a zinc metallopeptidase, found in male genital, vascular endothelial, neuro-epithelial, and absorptive epithelial cells (Acharya et al, 2003; Li et al., 2004; Wei et al., 1991), and displays both endopeptidase and exopeptidase activities, acting on a wide range of substrates (Sturrock et al., 2004). ACE is a key enzyme for regulating blood pressure in the renin-angiotensin system. Renin cleaves the N-terminal segment of angiotensinogen from the biologically inert AT-1. ACE then hydrolyzes AT-1 by cleaving the carboxyl terminal His-Leu dipeptide from the inactive AT-1 to the active angiotensin II (AT-2), a potent vasoconstrictor responsible for the development of hypertension [Chen et al, 2009; Donkor et al, 2007; Natesh et al., 2003; Tzakos et al.,

2003). ACE also indirectly influences the kallikrein–kinin system, by promoting the inactivation and degradation of the catalytic function of bradykinin, a vasodilator involved in blood pressure control (Natesh et al., 2003; Tzakos et al., 2003). By repressing AT-2 production and restraining bradykinin degradation, ACE inhibitory peptides control the increase of blood pressure (Tzakos et al., 2003).

Consequently, ACE inhibiting natural products have been vigorously investigated during the last decades, due to their potential in lowering blood pressure during hypertension. Among various types of bioactive peptides, ACE-inhibitory peptides from food sources have been most extensively studied for their potential use as natural alternatives to drugs for reducing blood pressure through binding and inhibiting ACE, and thus preventing and managing hypertension (Fitzgerald et al., 2004; Miguel et al., 2005). Food-derived peptides are believed to represent a healthier and more natural alternative source for chronic treatment of hypertension. Moreover, and although the inhibitory capacity of food-derived peptides is lower than that of chemically designed anti-hypertensive drugs, such as Captopril, Sampatrilat, Lisinopril, and Enalapril, it is thought that food-derived peptides are safer than pharmaceutical drugs due to their lack of some drug-associated adverse side effects such as angioedema, skin rashes, and dry cough (Beltrami et al., 2006; Donkor et al, 2007). However, considering the lack of consensus in their physiological anti-hypertensive effects in different human populations, the role of food peptides in regulating blood pressure is still a subject of ongoing debate (Cicero et al., 2011; Fekete et al., 2013; Geleijnse et al., 2010).

Although different animal and plant proteins have been used in the development of functional foods providing anti-hypertensive activity, milk is the main source of anti-hypertensive ACE inhibitory peptides reported to date (Martínez-Maqueda et al., 2010). Milk is made up of 3.5% proteins of which 80% are caseins, classified as α-, β- and k-caseins, and 20% whey proteins. Whey contains α-lactalbumin, β- lactoglobulin and other minor proteins. Upon the degradation of milk proteins, peptide fragments with many biological effects that can be different from those of the parent protein, are released. Several bioactive peptides in milk proteins have been identified (Meisel, 1998), and they serve an array of biological activities, including angiotensin-converting enzyme (ACE) inhibition, antimicrobial, antioxidative functions, dipeptidyl peptidase IV (DPP-IV) inhibition, opioid agonist and antagonist activities, immunomodulation, and mineral binding (Kitts et al., 2003). Several milk/whey derived peptides possess high *in vitro* ACE inhibitory activity; particularly, hydrolysates of whey proteins, caseinates, fractions-enriched in individual milk proteins, and whole milk proteins have been reported to be a good source of ACE-inhibitory peptides (Fitzgerald et al., 2004). Ile-Pro-Pro (IPP) has been identified as the most potent ACE inhibitor from milk protein, and it is derived from casein

(Nakamura et al., 1995). The anti-hypertensive activity of this tripeptide has been demonstrated in several animal studies and human trials (Ehlers et al., 2011). However, in some cases, poor correlation between the *in vitro* ACE inhibitory activity of milk-derived peptides and the *in vivo* anti-hypertensive activity has been observed. This can be partly due to digestion which renders less active peptide sequences and/or due to their low bioavailability (Sánchez-Rivera et al., 2014). Also, anti-hypertensive activity may be exerted by mechanisms other than ACE inhibition (Majumder & Wu, 2014; Udenigwe & Mohan, 2014), e.g., specific ACE inhibitors were demonstrated to increase the risk of microscopic colitis in a recent study, suggesting that milk-derived peptides may exert their anti-hypertensive activity through the microbiome (Masclee et al., 2015).

The activity of these peptides depends on their inherent amino acid composition and sequence (Dallas et al., 2015; Meisel & Fitzgerald, 2003). Shorter peptides up to 12 amino acids with hydrophobic and positively charged amino acids at the carboxyl end are more likely to interact with ACE (Welderufael et al., 2012). In terms of favourable structure–function relationship for high ACE-inhibitory activity, dipeptides including bulky and hydrophobic amino acids are more potent whereas tripeptides having aromatic amino acids at the C-terminus end, positively charged amino acids in the middle and hydrophobic amino acids at the N-terminus end, are most potent (Welderufael et al., 2012). Kobayashi et al. (2008) investigated the effects of aromatic amino acids in the third position of the tripeptides on ACE-inhibitory activity. They found that the difference in the ACE-inhibitory activity between the bioactive peptides (IKW, LKW, IKY, and LKF) resulted from the aromatic amino acids W, Y, and F. The highest inhibitory activity was presented by LKW, with the largest amino acid in the C-terminal. Accordingly, ACE-inhibitory activity is affected by the size of the amino acid, as well as its hydrophobicity (ACE (Welderufael et al., 2012). In the same study, Kobayashi et al. (2008) examined the effects of the charged amino acid in the second position, and they reported that in order to obtain a high inhibitory activity, it is essential to have a positively charged residue next to an aromatic residue. They also highlighted that the tripeptide sequence consisting of either I or L + positively charged amino acids + aromatic amino acids is likely to have high ACE-inhibitory activity. The charged amino acid takes part in binding by ACE while the bulky aromatic amino acid prevents the access between substrates and the active site of ACE. Some studies have indicated that tripeptides show higher ACE-inhibitory activity, and the C terminus end of the tripeptides substantially affects binding to ACE. Hydrophobic amino acid residues or Proline residues at the carboxyl end are important for ACE inhibition and inhibitors containing these residues are resistant to digestion (Pan et al., 2011).

ACE inhibitors are commonly discovered using classic investigation techniques which include hydrolysis of proteins with different proteolytic enzymes, isolation and purification of peptides using chromatographic systems, and synthesis of corresponding peptides for the confirmation of activity and structure (Li et al., 2004). Although some structure-activity relationships have been established for food protein derived peptides, they are still quite generic and thus could not be solely used to predict the ACE inhibitory activity of peptide sequences (Udenigwe, 2014). In order to avoid some challenges of the classical approach, such as having to apply cumbersome purification processes to isolate active peptides, the computer-based approach is considered a useful and effective method to identify novel peptides (Tu et al., 2017). A number of docking algorithms are being used in multiple studies to predict potent ACE inhibitory peptides encrypted in food proteins (García-Mora et al., 2017; Guo et al., 2017; Sangsawad et al., 2018; Shi et al., 2017; Wang et al., 2017; Wu et al., 2016; Yu et al., 2018), and more specifically in milk proteins (Ashok et al., 2017; Lin et al., 2017), whereby attempts to understand the interactions between receptor and ligand are being attempted (Nongonierma & FitzGerald, 2017).

Molecular docking enables the investigation of the specific interactions between certain peptide sequences and specific binding site residues in ACE, which could help to provide a better prediction of bioactivity *in vivo* through a molecular understanding of the structure-function relationship. Such an approach can be a powerful tool that can be used in pre-screening potentially bioactive peptides, prior to their testing *in vivo*.

Herein we investigate the potential interactions between whey protein derived peptides with high ACE inhibitory activity and human ACE, utilising a molecular docking approach. This study follows our previous work (Welderufael et al., 2011), where the peptides were produced by enzymatic hydrolysis of whey and were further fractionated for their chemical and activity characterisation. Moreover, the interactions between these peptides and ACE are compared with those of the ACE inhibitory drugs Sampatrilat, Captopril, Lisinopril, and Enalapril.

2.2. Results

2.2.1. Molecular Homology Between Human ACE and Rabbit ACE

According to the EMBOSS NEEDLE results (Figure 2.1), there is 93.4% of similarity between human ACE and rabbit ACE. As shown in figure 1, the structural comparison of these two enzymes indicates that

there is a close homology between the human ACE and the rabbit ACE, and that the active sites between human ACE and rabbit ACE are very similar. The rabbit ACE is generally used for the *in vitro* testing of ACE inhibition; hence it can be assumed that similar results will be obtained with human ACE. There have not been any previous studies that reported the homology between the human ACE and the rabbit ACE. In a study by Soubrier et al. (1988), amino-terminal sequence analysis was conducted between amino-terminal amino acid sequences of human ACE and other mammalians (rabbit, calf, pig, and mouse), and a high degree of similarity was found between human ACE and these mammalians.

```
ACE_HUMAN          1 MGAASGRRGPGLLLPLP-----LLLLLPPQPALALDPGLQPGNFSADEAG     45
                     ||||.|||||.||.|.|     ||||.||..||.|||||.||:|:|||||
ACE_RABIT          1 MGAAPGRRGPRLLRPPPPLLLLLLLLRPPPAALTLDPGLLPGDFAADEAG     50

ACE_HUMAN         46 AQLFAQSYNSSAEQVLFQSVAASWAHDTNITAENARRQEEAALLSQEFAE     95
                     |:|||.||||||||||:|.||||||||||||||||||.|||||||||
ACE_RABIT         51 ARLFASSYNSSAEQVLFRSTAASWAHDTNITAENARRQEEEALLSQEFAE    100

ACE_HUMAN         96 AWGQKAKELYEPIWQNFTDPQLRRIIGAVRTLGSANLPLAKRQQYNALLS    145
                     |||:|||||||:|:||||||||:||||||||||||.||||||||||||:|||
ACE_RABIT        101 AWGKKAKELYDPVWQNFTDPELRRIIGAVRTLGPANLPLAKRQQYNSLLS    150

ACE_HUMAN        146 NMSRIYSTAKVCLPNKTATCWSLDPDLTNILASSRSYAMLLFAWEGWHNA    195
                     |||:||||.|||.|||||:||||||||.||||||||||||||||||||||
ACE_RABIT        151 NMSQIYSTGKVCFPNKTASCWSLDPDLNNILASSRSYAMLLFAWEGWHNA    200

ACE_HUMAN        196 AGIPLKPLYEDFTALSNEAYKQDGFTDTGAYWRSWYNSPTFEDDLEHLYQ    245
                     .||||||||:|||||||||:||||:||||||||||:||||||:|||.:|.
ACE_RABIT        201 VGIPLKPLYQEFTALSNEAYRQDGFSDTGAYWRSWYDSPTFEEDLERIYH    250

ACE_HUMAN        246 QLEPLYLNLHAFVRRALHRRYGDRYINLRGPIPAHLLGDMWAQSWENIYD    295
                     ||||||||||:|||.||||||||||||||||||||:||||||:|||
ACE_RABIT        251 QLEPLYLNLHAYVRRVLHRRYGDRYINLRGPIPAHLLGNMWAQSWESIYD    300

ACE_HUMAN        296 MVVPFPDKPNLDVTSTMLQQGWNATHMFRVAEEFFTSLELSPMPPEFWEG    345
                     ||||||||||||||||:|:||||||||||||||||||.|.|||||||..
ACE_RABIT        301 MVVPFPDKPNLDVTSTMVQKGWNATHMFRVAEEFFTSLGLLPMPPEFWAE    350

ACE_HUMAN        346 SMLEKPADGREVVCHASAWDFYNRKDFRIKQCTRVTMDQLSTVHHEMGHI    395
                     ||||||.|||||||||||||||||||||||||:|||||||||||||||:
ACE_RABIT        351 SMLEKPEDGREVVCHASAWDFYNRKDFRIKQCTQVTMDQLSTVHHEMGHV    400
```

(I) residues are identical

(.) conserved change

(:) part of the residue is similar but not that conserved

Figure 2.1. EMBOSS NEEDLE multiple sequence alignment results. Colour coding is as follows: Yellow indicates identical residues at the active site, green indicates similar residues at the active site, and red indicates that a part of the residue is similar.

2.2.2. Molecular Docking

Molecular docking was conducted to elucidate the potential molecular interactions between the whey-protein derived peptide sequences and specific amino acids at the binding site of human ACE. The peptide sequences were docked into the binding site of the human ACE, using the X ray crystallographic structure of the human ACE receptor (PDB code 6F9V). The extracted co-crystallized ligand, Sampatrilat, (Cozier et al., 2018) was first re-docked into the prepared protein to be used for docking in order to validate the docking procedure. The RMSD between the docked conformation, as generated by the program PyMol, and the native co-crystallized ligand conformation was 0.1 Å, which was well within the 2 Å grid spacing used in the docking procedure, demonstrating that the docking method to be used was valid and reliable. Additionally, the interactions between the docked ligand and the prepared target receptor mimicked those observed in the crystal structure of the same protein.

Hydrogen bonds are a significant factor that contribute to the specificity and stability of protein-ligand interactions. Figures 2–5 and Tables 1–4 show the hydrogen bond interactions associated with each ligand and the surrounding ACE residues. IPP formed 3 hydrogen bonds with the ACE residues: one with Asp 354 and two with Gln 355 (Figure 2.2, Table 2.1). IIAE formed three hydrogen bonds with residues Thr 144, Gln 259, and Thr 358 (Figure 2.3, Table 2.2). LIVTQ formed three hydrogen bonds: one with Ala 332, one with Gln 355, and one with Thr 358 (Figure 2.4, Table 2.3). As for the ligand LVYPFP, five hydrogen bonds were formed with residues Asp 255, Ser 260, His 331, Arg 350, and Thr 358 (Figure 2.5, Table 2.4). It is interesting to note that several peptides had same H bonds in common: Thr 358 formed H bonds with three of the peptides, IIAE, LIVTQ, LVYPFP; Gln 355 with IPP and LIVTQ. Additionally, all except one of the amino acids in the active site of the ACE were polar (charged and non-charged) and some of these charged amino acids were also involved in salt bridge (electrostatic) interactions (Tables 2.1-2.4). IPP formed one salt-bridge interaction with residue Arg 350 (Figure 2.2, Table 2.1), whereas ligands IIAE and LIVTQ formed only one salt bridge interaction with residues Asp 140, and Asp 255, respectively (Figures 2.3 and 2.4, Tables 2.2 and 2.3). As for LVYPFP, two salt-bridge interactions were formed with residues Glu 262, and His 331 (Figure 2.5, Table 2.4). Both, LVYPFP and LIVTQ interacted with Asp 255 via H-bonding and a salt bridge, respectively; and, both IPP and LVYPFP interacted with Arg 350 via a salt bridge and H-bonding, respectively.

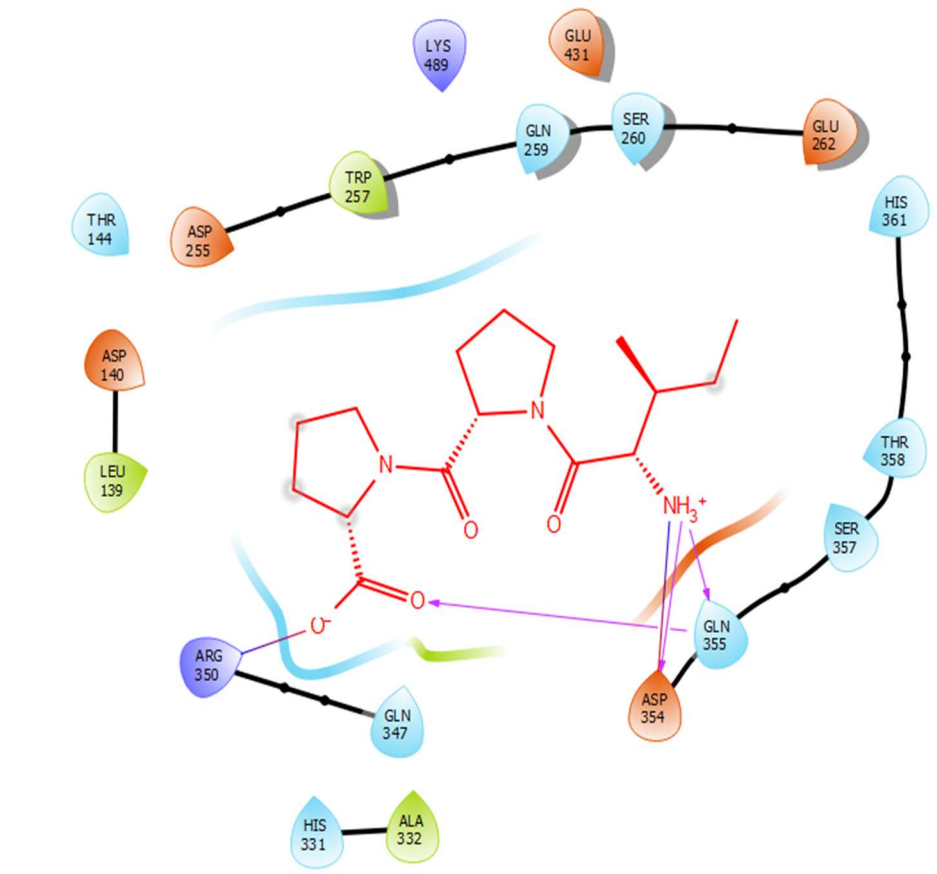

Figure 2.2. Docking results of the peptide IPP in the active site of human angiotensin I-converting enzyme (ACE). IPP is represented in red, interactions of human ACE residues with the peptide are indicated by arrows of different colours, with purple representing hydrogen bond interactions and blue arrows representing salt bridge interactions. The figure was generated using the software Maestro.

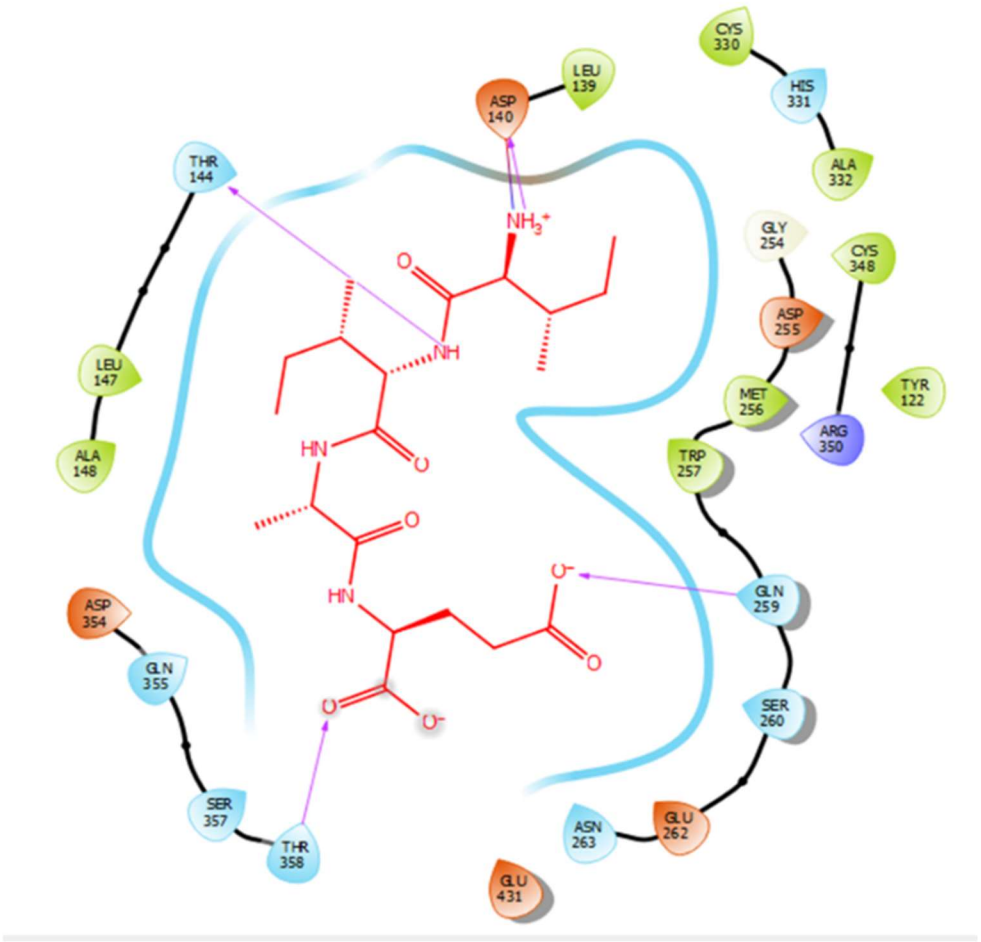

Figure 2.3. Docking results of the peptide IIAE in the active site human ACE. IIAE is represented in red, the interactions of human ACE residues with the peptide are indicated by arrows of different colours with purple representing hydrogen bond interactions, and blue arrows representing salt bridge interactions. The Figure was obtained using the software Maestro.

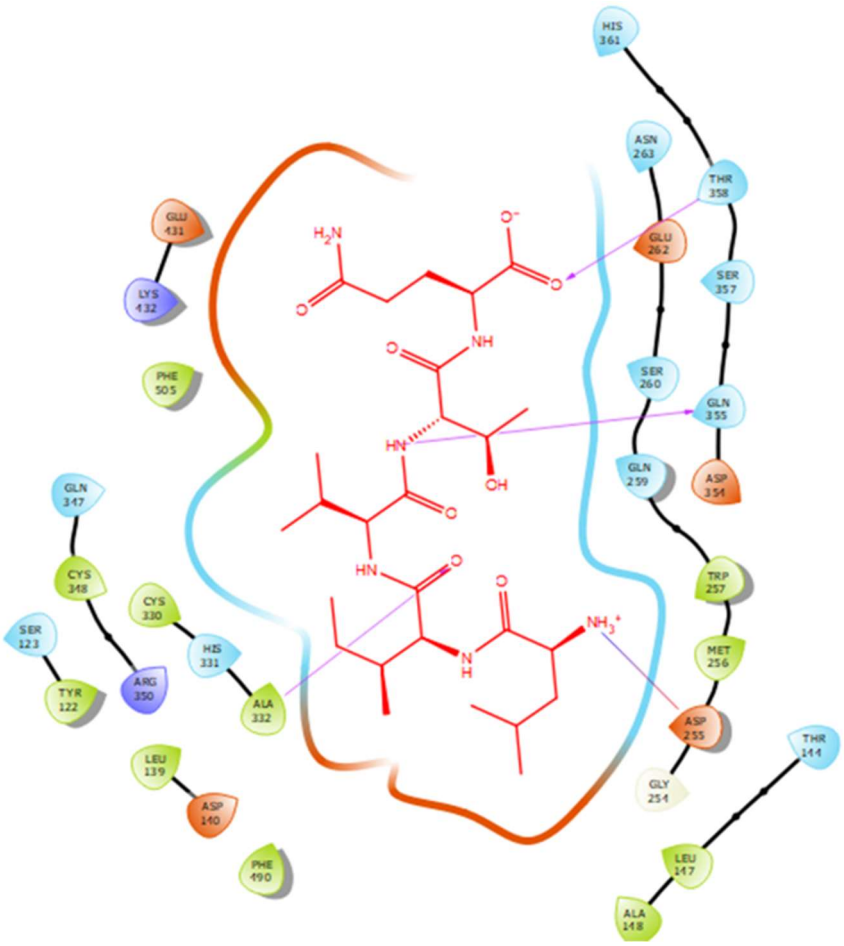

Figure 2.4. Docking results of the peptide LIVTQ in the human ACE active site. LIVTQ is represented in red, the interactions of human ACE residues with the peptide are indicated by arrows of different colours with purple representing hydrogen bond interactions, and blue arrows representing salt bridge interactions. The software Maestro was used for the generation of this figure.

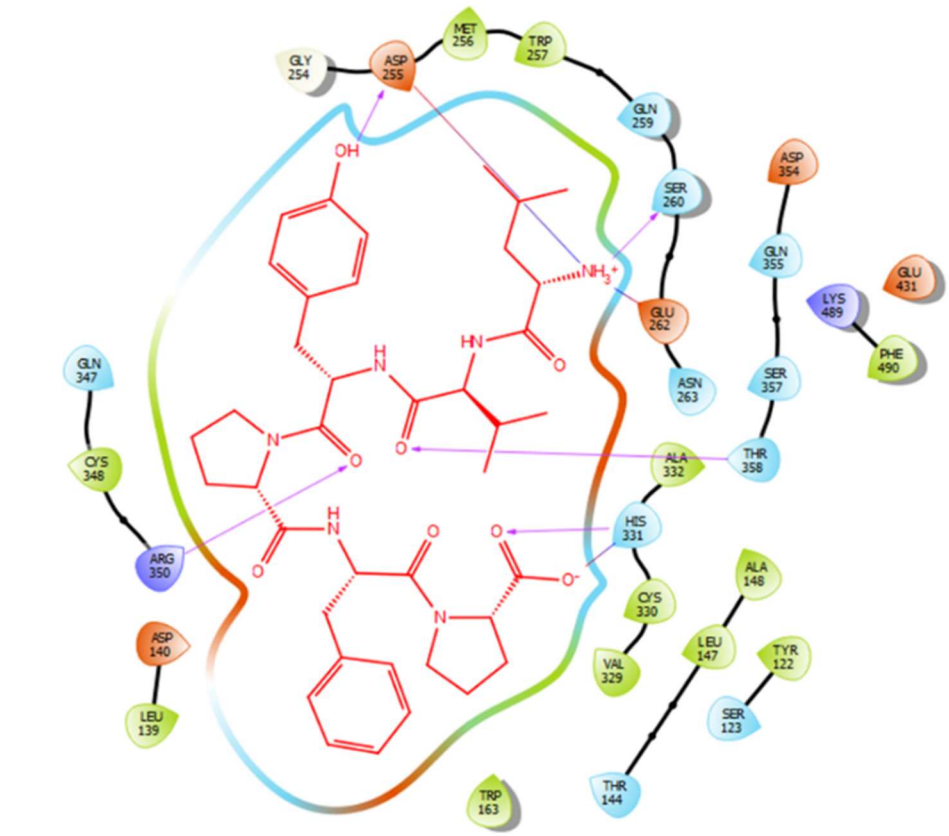

Figure 2.5. Docking results of the peptide LVYPFP in the human ACE active site. LVYPFP is represented in red, the interactions of human ACE residues with the peptide are indicated by arrows of different colours with purple representing hydrogen bond interactions, and blue arrows representing salt bridge interactions. The software Maestro was used for the generation of this figure.

Table 2.1. IPP docking results.

Protein 6F9V		**Ligand IPP**	
Residue	**Atom Name**	Interaction Type	Distance (Å)
NH_2 Arg 350	O^- (Pro)	Salt bridge	2.86
OD2 Asp 354	NH^{3+} (Ile)	Hydrogen bond	1.86
OE1 Gln 355	NH^{3+} (Ile)	Hydrogen bond	1.81
NE2 Gln 355	O- (Pro)	Hydrogen bond	2.04

Table 2.2. IIAE docking results.

Protein 6F9V		**Ligand IIAE**	
Residue	**Atom Name**	Interaction Type	Distance (Å)
OD2 Asp 140	NH (Ile)	Salt bridge	2.57
OG1 Thr 144	N (Ile)	Hydrogen bond	2.15
NE2 Gln 259	O (Glu)	Hydrogen bond	2.2
OG1 Thr 358	O (Glu)	Hydrogen bond	1.88

Table 2.3. LIVTQ docking results.

Protein 6F9V		**Ligand LIVTQ**	
Residue	**Atom name**	Interaction type	Distance (Å)
OD2 Asp 255	NH3+ (Leu)	Salt bridge	4.79

N Ala 332	O (Ile)	Hydrogen bond	2.56
OE1 Gln 355	N (Thr)	Hydrogen bond	1.85
OG1 Thr 358	O- (Gln)	Hydrogen bond	1.88

Table 2.4. LVYPFP docking results.

Protein 6F9V		**Ligand LVYPFP**	
Residue	**Atom Name**	Interaction Type	Distance (Å)
OD2 Asp 255	OH (Tyr)	Hydrogen bond	2.12
OG Ser 260	NH3+ (Leu)	Hydrogen bond	1.89
OE2 Glu 262	NH3+ (Leu)	Salt bridge	3.58
ND1 His 331	O (Pro)	Hydrogen bond	1.92
ND1 His 331	O (Pro)	Salt bridge	2.71
NH2 Arg 350	O (Tyr)	Hydrogen bond	2.61
OG1 Thr 358	O (Valine)	Hydrogen bond	1.75

2.2.3 Discussion

Hydrogen bonds interactions were demonstrated to play a crucial role in stabilizing the docked ligand complexes (Ling et al., 2018). The distance of hydrogen bond interactions between the whey derived peptides and ACE amino acid residues typically were short (< 3.0Å; Tables 2.1 – 2.4), indicating that the peptides' binding affinity to ACE was strong (Ling et al., 2018). In addition, these peptides formed a number of favorable salt bridge interactions with ACE residues, indicating that the ligands can pack tightly into the binding site and effectively inhibit ACE. Furthermore, it is interesting to note that hydrophobic amino acid residues such as proline, leucine, and isoleucine were mainly involved in establishing strong interactions with ACE, which goes in accordance with what is reported in SAR (Tables 2.1 – 2.4) (Pan et al., 2011).

Sampatrilat ((S, S, S)-*N*-{1-[2-carboxy-3-(N-mesyllysylamino) propyl]-1-cyclopentylcarbonyl}-tyrosine) (Figure 2.6) is a potent dual inhibitor of ACE and neutral endopeptidase. In the treatment of chronic heart failure, Sampatrilat could potentially provide a greater benefit than traditional ACE inhibitors (Venn et al., 2018; Wallis et al., 1998). In a recent study investigating the binding of Sampatrilat to the active site of ACE, the amino acid residues involved in the interactions with Sampatrilat were reported (Cozier et al., 2018). Interestingly, IIAE, LIVTQ, and LVYPFP interacted with three of these previously identified amino acid residues: IIAE interacted with residue Gln 259, LVYPFP interacted with residue

His 331 and IIAE, LIVTQ, and LVYPFP interacted with residue Thr 358. Furthermore, previous studies stated that the ACE-inhibitory drugs Captopril, Lisinopril, and Enalapril interact with ACE amino acid residues Gln281, His353, Glu384, Lys511, His 513, and Tyr520 1 (Natesh et al., 2004; Vercruysse et al., 2010; Wang et al., 2011). Apart from the amino acid residues in common, Lisinopril was reported to interact with Ala 354, Tyr 523, and Glu 162, and Enalapril to interact with Ala 354 and Tyr 523 (Natesh et al., 2004; Wang et al., 2011). According to the docking results, the peptides IIAE and LIVTQ interacted also with two of these residues: IIAE interacted with residue Asp 140 in common with Lisinopril, and LIVTQ interacted with Ala 332 in common with Lisinopril and Enalapril. (Amino acids residues are reported according to the Sampatrilat (PDB code 6F9V) amino acid sequence numbering, please see Table 2.A.1).

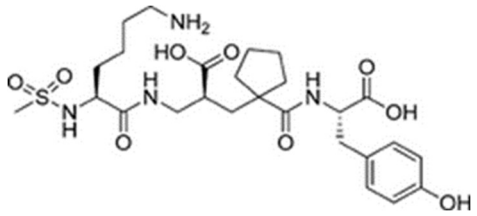

Figure 2.6. Chemical structure of Sampatrilat (Cozier et al., 2018).

Overall, the docking results together with comparisons with the ACE inhibitory drugs provide strong evidence for the ACE inhibitory activity of IIAE, LIVTQ, and LVYPFP. In our previous work (Welderufael et al., 2012), IIAE, LIVTQ, and LVYPFP were identified as major peptides within fractions of high ACE inhibitory activity. Additionally, based on known structure-activity relationships, it was assumed that these were the main contributors to the ACE inhibitory activity measured. The docking results herein corroborate these assumptions and suggest that most probably these are potent ACE inhibitors that will contribute to the ACE inhibitory and anti-hypertensive activity *in vivo*. Further work will be needed, using pure synthesized peptides, to confirm ACE inhibition and activity *in vivo*.

2.4. Materials and Methods

2.4.1. Whey-Protein Derived Peptides

In our previous work where we characterized angiotensin-converting enzyme (ACE) inhibitory peptides produced by enzymatic hydrolysis of whey proteins (Welderufael et al., 2012), peptide sequences were identified as major peptides in fractions from the enzymatic hydrolysates CDP (casein-derived peptides) and β-lactoglobulin. The well-known anti-hypertensive peptide IPP, along with some other novel peptide sequences that have structural similarities with reported ACE inhibitory peptides,

such as Leu-Val-Tyr-Pro-Phe-Pro (LVYPFP), Leu-Ile-Val-Thr-Gln (LIVTQ), and Ile-Ile-Ala-Glu (IIAE) were characterized and identified by a combination of chemical characterization (LC/MS; MS/MS) and SAR data. Their ACE inhibitory activity is summarized in Table 2.5; the IC_{50} is defined as the peptide concentration required to reduce the ACE activity by half.

Table 2.5. IC_{50} (µg/mL) value of the ACE-inhibitory peptide sequences.

Peptide	Protein Source	IC50 (µg/mL)	Reference
IPP	k-Casein	1.23	(Nakamura et al., 1995)
IIAE	β-Lg	128 *	(Welderufael et al., 2012)
LVYPFP	Casein	97	(Gonzalez-Gonzalez et al., 2013)
LIVTQ	β-Lg	113	(Welderufael et al., 2012)

* IC_{50} of β-Lg hydrolysate containing this peptide as one of the major peptides.

2.4.2. Homology between Human ACE and Rabbit ACE

EMBL-EBI (https://www.ebi.ac.uk/) was queried for human and rabbit ACE amino acid sequences, together with known three-dimensional protein structures. Reviewed sequences were selected and the protein sequence files were downloaded. The accession codes for the human ACE and the rabbit ACE used in this work are P12821 and P12822, respectively. The two sequences were then uploaded to Emboss Needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/) for multiple sequence alignment and comparison.

2.4.3. Molecular Docking

2.4.3.1. Docking Validation

In order to validate the accuracy and the reliability of the docking procedure to be used in this study, the original ligand (extracted from the coordinate files and taken from the Protein Data Bank; PDB code 6F9V was docked into the corresponding crystal structure of the receptor, using the automated docking procedure in the program Surflex-Dock (SFXC) (Jain, 2003), as provided by SYBYL-X2.1. The docked ligand mode and orientation from the docking procedure were compared to that found in the actual crystal structure of the complex using Pymol and PDBeFold (Schrodinger LLC, 2015; PBDeFold,

2019). Following the docking procedure, the root mean square deviation (RMSD) between the docked ligand and the ligand, as found in the crystal structure, was calculated. The success of the docking process depended on whether the value of RMSD between the real and best-scored docked conformations were within the 2 Å grid spacing, used in the docking procedure (Wang et al., 2003), and whether the molecular interactions were replicated. In this case, Sampatrilat was docked into the human ACE receptor as validation of the docking procedure.

2.4.3.2. Docking Procedure

Whey protein-derived peptides Ile-Pro-Pro (IPP), Leu-Ile-Val-Thr-Gln (LIVTQ), Ile-Ile-Ala-Glu (IIAE), and Leu-Val-Tyr-Pro-Phe-Pro (LVYPFP) were used as ligands in separate docking runs. Docking was performed using the docking algorithm Surflex-Dock, as provided in Sybyl-X 2.1. The X-ray crystallographic structure of sampatrilat-Asp in complex with Angiotensin-I-converting enzyme (PDB code 6F9V, 1.69 Å resolution) retrieved from the protein data bank (PDB) was chosen as the target protein for the docking studies, based on its high-resolution structure co-crystallized with sampatrilat-Asp (Cozier et al., 2018).

The Biopolymer Structure Preparation Tool, with the implemented default settings provided in the SYBYL programme suite, was used to prepare the protein structure for docking; hydrogens were added to the protein structure in idealised geometries, backbone and sidechains were repaired, residues were protonated, sidechain amides and sidechain bumps were fixed, stage minimization was performed, and all water and any ligand molecules were removed.

The three-dimensional (3D) structure of each ligand was constructed, using the “Build Protein” tool, as provided in Sybyl-X. Once constructed, charges were assigned to each atom of each molecule, using Merck Molecular Force Field (MMFF94) charges. Localized energy minimizations were then performed, and the final structure for each ligand in its lowest energy conformation was used for subsequent docking experiments. The resulting 3D coordinate files were converted to a MOL2 format for subsequent use in Surflex-Dock experiments, as provided in the SYBYL-X 2.0 software suite.

Surflex-Dock is a search algorithm that utilizes an empirically derived scoring function whose parameters are based on protein-ligand complexes of known affinities and structures. This method employs a “protomol”, which is an idealized active site, as a target to generate presumed poses of molecules or molecular fragments. The protomol is employed as a mimic of the ideal interactions

made by a perfect ligand to the active site of the protein. This molecular-similarity based alignment allows for optimization of potentially favorable molecular interactions, such as those defined by van der Waals forces and hydrogen bonds. In the present work, the protomol was defined by optimizing the threshold and bloat values to 0.5 and 0, respectively, to create a protomol that adequately described the binding pocket of interest. The extent of the protomol and its degree of coverage of an active site are controlled by these two parameters: the threshold value indicates the amount of buried-ness for the primary volume used to generate the protomol, and the bloat parameter determines the number of Ångstroms by which the search grid beyond that primary volume should be expanded. It is generally better to err on the side of a small protomol than on a protomol that is too large (Sharma et al., 2016). All parameters within the docking suite were left as the default values as established by the software (Ai et al., 2011; Lan et al., 2011). Each peptide was then individually docked into the protomol site, using the "Docking Suite" application, as provided in the SYBYL programme suite. The docking results were visualised using the programme Maestro.

Molecular interactions, for the docking results, are reported according to the Sampatrilat (PDB code 6F9V) amino acid sequence numbering; for comparisons between different sequence numbering in studies referred here (See supplemental section (Table 2.A.1). The software Maestro was used for the identification and characterisation of hydrogen bonds and salt-bridge interactions established between residues at the ACE active site and the peptides.

2.5. Conclusions

For the first time, reported herein, potential interactions between the naturally produced peptides from whey and ACE have been investigated, using a molecular docking approach. Peptides, IPP, IIAE, LIVTQ, and LVYPFP formed strong H bonds and salt bridge interactions with residues in the active site of human ACE. Moreover, a comparison with commercial ACE inhibitory drugs showed that the natural peptides interacted similarly to the drugs mimicking the same interactions with ACE active site residues. This study provides strong evidence for the ACE inhibitory activity of milk derived peptides, which have not been tested *in vivo* before. The results of this study, of novel milk derived whey peptides, could lead to the production of novel ACE inhibitors.

2.6. Appendix

Appendix A

Table 2.A.1. Comparisons between Captopril (PDB code 1O86) and Sampatrilat (PDB code 6F9V) sequence numbering.

Captopril (PDB Code 1O86) Amino Acid Sequence Numbering (Natesh et al., 2004)	Sampatrilat (PDB Code 6F9V) Amino Acid Sequence Numbering (Cozier et al., 2018)
Glu 162	Asp 140
Gln 281	Gln 259
His 353	His 331
Ala 354	Ala 332

2.7. References

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CHAPTER THREE Whey-derived peptides at the heart of the COVID-19 pandemic

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Contribution towards PhD thesis: Along with co-authors, I was involved in research conceptualisation. My responsibilities also included conducting the literature search, the molecular docking studies, and the formal data analysis and visualisation. Then, I wrote the initial draft of the manuscript for publication, and finally, I finalised the published manuscript presented below after including the comments and feedback received from co-authors Paula Jauregui, Kim Watson and Kim Jackson, as well as journal reviewers.

3.1. Introduction

Cardiovascular diseases (CVDs) are reported to be the leading cause of death globally (WHO, 2021). Elevated blood pressure (hypertension) is one of the most important and well-defined modifiable risk factors for the development of CVDs (Townsend et al., 2014). Thus, effective control of blood pressure plays a key role in preventing hypertension-related deaths (Mills et al., 2020; Borghi et al., 2015).

3.1.1. The classical and counter-regulatory renin–angiotensin system (RAS) pathways

Among different regulatory and contra-regulatory systems contributing to the pathogenesis of cardiovascular and renal diseases, the renin angiotensin system (RAS) is one of the main therapeutic targets for CVDs, and key players that regulate blood pressure. The RAS pathway includes a cascade of proteases generating some bioactive molecules (Chappell et al., 2016) (Figure 3.1). Renin is a glycoproteolytic enzyme secreted by the juxtaglomerular cells of the afferent arterioles of the kidney. The liver-derived angiotensinogen acts as the substrate for renin, which cleaves angiotensinogen to form the decapeptide angiotensin I (Ang I) (Griendling et al., 1993). Angiotensin converting enzyme I (ACE) (dipeptidyl carboxypeptidase, EC 3.4.15.1) is a zinc metallopeptidase that is found in male genital, vascular endothelial, neuro-epithelial, and absorptive epithelial cells (Acharya et al., 2003; Li et al., 2004; Wei et al., 1991). It displays both endopeptidase and exopeptidase activities, acting on a wide range of substrates (Sturrock et al., 2004). ACE hydrolyses the inactive decapeptide Ang I into the strong vasoconstrictor angiotensin II (Ang II). Additionally, ACE promotes the inactivation and degradation of the catalytic function of vasodilator bradykinin (BK) into inactive BK (1–7) and BK (1–

5) (Carey et al., 2003; Natesh et al., 2003; Tzakos et al., 2003). By promoting the production of the potent vasoconstrictor Ang II, as well as degrading the vasodilator BK, ACE plays a dual role in the RAS. In this respect, Ang II is a key component of the RAS pathway, exerting its effects via two G protein-coupled receptors angiotensin type 1 (AT1) and type 2 (AT2). Most of the pathophysiological effects of Ang II are mediated through AT1 receptors leading to vasoconstriction, cardiovascular inflammation, and aldosterone secretion (Cat et al., 2011). The AT2 receptor is associated with effects that counteract those of the AT1 receptor, however, many functions of the AT2 receptor are, as yet less clear, and studies reporting its importance are controversial (Padia et al., 2013).

In addition to the classical components of the RAS pathway (renin, ACE, Ang II, AT1 and AT2 receptors), novel peptides such as angiotensin 1–7 (Ang 1–7), and receptors such as angiotensin converting enzyme 2 (ACE2) appear to play a central role in the regulation of the system (Santos, 2014). ACE2 is an 805 amino-acid Type-I transmembrane protein that functions as a zinc metalloenzyme and monocarboxypeptidase (Tikellis et al., 2012). Its extracellular domain consists of a single catalytic metallopeptidase that shares 61% sequence similarity and 42% sequence identity with the catalytic domain of ACE. ACE2 is active and expressed in most tissues, however, the highest expression of ACE2 is mainly observed in vascular endothelial cells, and the renal tubular epithelium (Donoghue et al., 2000). Ang II appears to be the major substrate for ACE2 (Donoghue et al., 2000; Rice et al., 2004). Ang (1-7) is a vasodilator with anti-proliferative effects produced by the catalytic activity of ACE and ACE2, from Ang I or Ang II (Santos, 2014). The biologically active peptide Ang (1-9) is formed through the hydrolysis of the amino acid leucine from the C-terminus of Ang I by ACE2 (Donoghue et al., 2000) (Figure 3.1). Ang (1-9) is subsequently cleaved by ACE and the neutral endopeptidase 24.11 (NEP) to generate Ang (1-7) (Rice et al., 2004) which can also be generated directly through the cleavage of the amino acid phenylalanine at the C-terminal of Ang II (Vickers et al., 2022). ACE2 therefore plays a key role as a regulator of the RAS pathway, through degrading the vasoconstrictor/proliferative peptide Ang II and producing the vasodilator/antiproliferative peptide Ang (1-7) (Tzakos et al., 2003). Additionally, the identification of the G protein-coupled receptor Mas, as a receptor of Ang (1-7), was another pivotal step to establish the relevance of Ang (1-7) (Santos et al., 2003). The ACE2/Ang (1-7)/Mas axis is now accepted to counteract most of the harmful actions of the ACE/Ang II/AT1 receptor axis (Ferreira et al., 2002). ACE2 is thus a key counter regulatory enzyme and a potent modulator of blood pressure (D'Ardes et al., 2020).

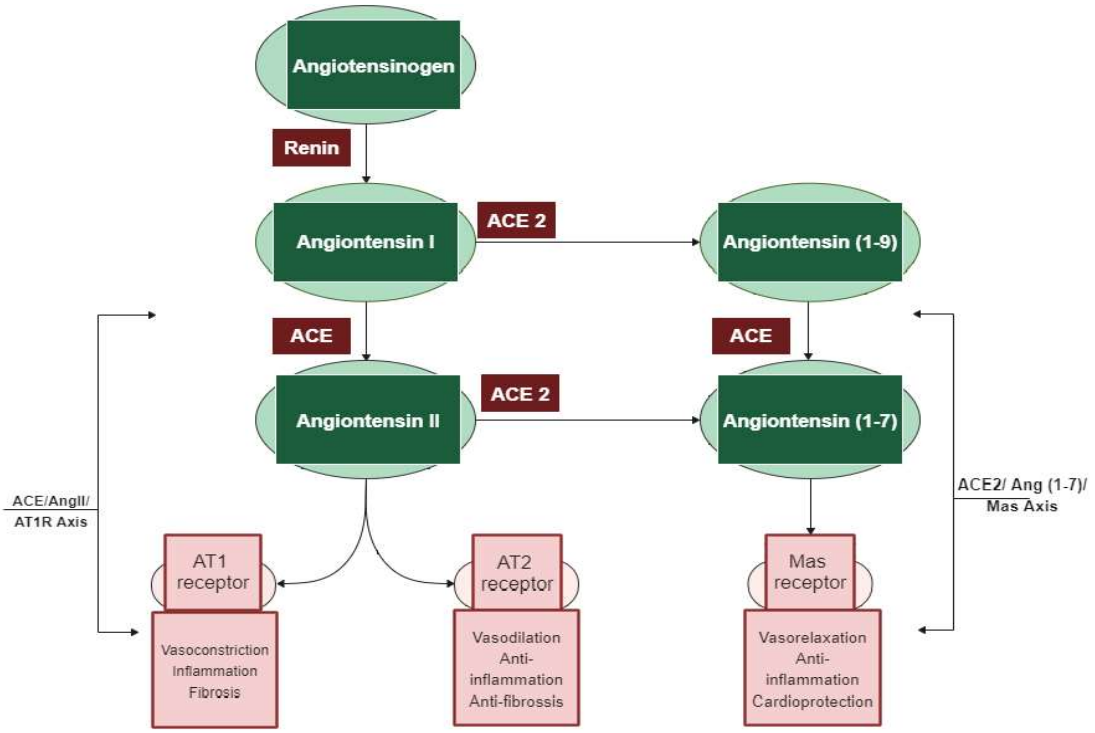

Figure 3.1. The two main axes of the renin–angiotensin system (RAS) cascade, and their opposing effects. Adapted from D'ardes et al. (2020).

Discovery of these novel components (ACE2, Ang (1–7), Mas receptor), which have been recently added to the RAS system, has completely altered our understanding of the regulatory mechanisms of this pathway. It is now widely accepted that the system is dual and consists of two axes: the primarily deleterious axis consisting of ACE/Ang II/AT1, and the beneficial axis consisting of ACE2/Ang-(1–7)/Mas. These novel RAS elements thus open new opportunities for interfering with the activity of the system and invigorating the development of new cardiovascular drugs targeting the beneficial and counter-regulatory axis of the RAS (Ferreira et al., 2002).

3.1.2. Characteristics of SARS-CoV-2

Novel coronavirus (COVID-19) is a pandemic that emerged in late 2019, and arguably became one of the greatest public health challenges of our time. COVID-19 is continuing to spread around the world, with the World Health Organization reporting more than 240 million confirmed cases and 4 million deaths globally, at the time of writing this article (October 2021) (WHO, 2021).

Coronaviruses belong to the subfamily Coronavirinae in the family Coronaviridae, of the order Nidovirales (International Committee on Taxonomy of Viruses, 2020). The subfamily Coronavirinae

can be divided into four genera: alpha-, beta-, gamma- and deltacoronavirus (Cui et al., 2019). According to Zhu et al. (2020), the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) belongs to the betacoronavirus genus. The alpha- and betacoronaviruses are mainly associated with infections in mammals, with severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV), and Middle East respiratory syndrome (MERS) coronavirus (MERS-CoV) providing two prominent examples of highly pathogenic coronaviruses causing severe respiratory disease in humans. Due to the greater resemblance of the novel virus with SARS-CoV, the Coronavirus Study Group (CSG) of International Committee on Taxonomy of Viruses (ICTV) named it "SARS-CoV-2" (the International Committee on Taxonomy of Viruses, 2020). Genome-wide phylogenetic analysis shows that SARS-CoV-2 shares 50% and 79.5% sequence identity to MERS-CoV, and SARS-CoV, respectively (Lu et al., 2020; Zhu et al., 2020). Like other betacoronaviruses, SARS-CoV-2 contains a positive-sense, single-stranded RNA genome of 29.9 kb in size (National Microbiology Data Center, 2020) encoding structural proteins which include spike (S), envelope (E), Nucleocapsid (N), and membrane (M) (Wu et al., 2020). Besides the genes encoding structural proteins, other genes encode non-structural proteins crucial for virus replication and translation of the viral genome in the host cells (Rothan & Byrareddy, 2020; Tortorici & Veesler, 2019).

ACE2 is a type I membrane protein. It consists of the C-terminal collectrin-like domain (CLD), and the N-terminal peptidase domain (PD) that provides a direct binding site for the coronavirus S protein (Yan et al.,2020). According to Wrapp et al. (2020), SARS-CoV-2 binds to ACE2 with much higher affinity (about 10-20 times higher) than the binding of SARS-CoV S protein to ACE2, which explains why SARS-CoV-2 is highly infectious. ACE2 is highly expressed in the mouth and tongue, which facilitates viral entry for SARS-CoV-2. It is also expressed highly throughout the gut (Lamers et al., 2020), and in the lower lungs on alveolar epithelial cells type I and type II (Wrapp et al., 2020). Post-infection, S protein expressed on the viral envelope attaches itself to ACE2 on the alveolar surface. This binding stimulates the clathrin-dependent endocytosis of the SARS-CoV-2 and ACE2 complex, which includes fusion at the cell membrane. Once SARS-CoV-2 is inside the cells, it exploits the alveolar cells' endogenous transcriptional machinery to replicate and spread through the entire lung (Perico et al., 2020).

3.1.3. Controversies regarding the role of ACE2 in COVID-19

Besides its role as SARS-CoV-2 receptor, ACE2 is established for its role in hypertension by negatively regulating the RAS through modulating blood pressure to maintain blood pressure homeostasis. The unique interaction of SARS-CoV-2 and host cell receptor ACE2 provides a critical link between COVID-

19 and, hypertension and CVDs (Hoffmann et al., 2020; Zhou et al., 2020). As ACE2 has been identified as the crucial receptor for SARS-CoV-2, the entire RAS should be evaluated when addressing the COVID-19 pandemic. According to Sriram and Insel (2020), the imbalance in the action of ACE2 and ACE is one of the main culprits of COVID-19 pathobiology. Hence, to treat ACE-2 mediated COVID-19, there are two primary approaches suggested to restore ACE/ACE2 balance in the literature (i) ACE inhibitors/increasing ACE2 or Ang (1-7) levels and/or (ii) seeking new molecules targeting the S protein or ACE2 receptor to prevent infection by SARS-CoV-2 (Saadah et al., 2020; Souza et al., 2020; Srivastava et al., 2022).

From a therapeutic perspective, activating the ACE2/Ang (1-7)/Mas axis or inhibiting the ACE/Ang II/AT1R axis could be promising (Ocaranza et al., 2006; Perico et al., 2020). Previous research has demonstrated that SARS-CoV infection significantly decreases ACE2 levels in infected mice (Kuba et al., 2005). The binding of SARS-CoV-2 to ACE2 also reduces levels of ACE2, thereby inhibiting the ACE2/Ang (1-7) pathway and shifting the balance of the RAS system, consequently, leading to exacerbation of acute severe pneumonia (Sun et al., 2020). By inhibiting conversion of Ang I to Ang II, ACE inhibitors reduce Ang II production and subsequently the effects triggered by its interaction with the receptor AT1R, namely vasoconstriction (D'ardes et al., 2020). The hypothetical association between treatment with ACE inhibitors and severe COVID-19 disease has been intensely debated in the literature (Kuster et al., 2020; Tomasoni et al., 2020; Vuille-dit-Bille et al., 2015; Zheng et al., 2020). One hypothesis suggests that use of these drugs could be harmful, in the sense that increased expression of ACE2 receptors may enhance viral binding and entry (Fang et al., 2020; Ferrario et al., 2005). The other hypothesis proposes that ACE inhibitors could be protective by decreasing production of Ang II and boosting the production of Ang (1–7), which attenuates inflammation and fibrosis and hence could attenuate lung injury (South et al., 2020; Zambelli et al., 2015). Various large cohort studies have suggested that the use of ACE inhibitors was not correlated with increased SARS-CoV-2 infection but was in fact linked to a reduced risk of mortality in hospitalized COVID-19 patients (Hippisley-Cox et al., 2020; Mehta et al., 2020; Zhang et al., 2020).

3.1.4. Whey-derived peptides as promising therapeutic candidates

Ever since the pandemic brought chaos to lives across the globe, scientists have been making extraordinary efforts to explore therapeutic candidates, such as developing effective vaccines and drugs against COVID-19 to reduce the severity of the outbreak. Given the significance of the ACE2 receptor, research groups have been seeking new molecules targeting this receptor to prevent

infection by SARS-CoV-2 and mitigate the development of COVID-19 disease (Hoffmann et al., 2020; Wang et al., 2021; Zhang et al., 2020). Many of the recent studies have investigated the potential of chemical compounds such as flavonols (Mouffouk et al., 2021), and peptides as novel therapeutic inhibitors against SARS-CoV-2, targeting the ACE2 receptor (Saadah et al., 2020; Souza et al., 2020; Srivastava et al., 2022). Peptide and peptide-base inhibitors represent attractive candidates that hold great promise for the development of ACE2 inhibitors due to their safety, specificity and efficacy compared to small molecule drugs. Antiviral peptides can also be rationally designed and optimised based on the known structures of viral proteins, as these can be developed to be highly specific for their respective targets (Brauer et al., 2013; Schütz et al., 2020). Strategies to interfere with the interaction of the S protein with the ACE2 receptor have been previously examined with SARS-CoV (Han et al., 2006). Hence, antagonist ACE2 proteins, or their derived peptides, may not only be a treatment for preventing the spread of SARS-CoV-2, but also for modulation of the RAS. These proteins and derived peptides could be used to both protect patients with COVID-19 disease and limit the spread of the current SARS-CoV-2 and other coronaviruses, by preventing replication of the virus and development of SARS in the lung (Schütz et al., 2020).

Computational approaches play a considerable role in the process of rapid drug development and discovery, in a cost- and time-efficient manner. In the literature, many researchers have aimed to identify novel ACE2 inhibitors utilising a molecular docking strategy (Saadah et al., 2020; Souza et al., 2020; Srivastava et al., 2022).

Given the high sequence similarity and sequence identity between ACE and ACE2 (Donoghue et al., 2000), and the reported reduced risk of mortality and disease associated with use of ACE inhibitors among COVID-19 patients (Hippisley-Cox et al., 2020; Mehta et al., 2020; Zhang et al., 2020), investigating the ability of ACE inhibitors to block ACE2 interaction with the SARS-CoV-2 S protein would be promising. Various animal and plant proteins have been used in the development of functional foods providing ACE inhibitory activity, however, milk is the main source of anti-hypertensive ACE inhibitory peptides reported to date (Giromini et al., 2017; Martínez-Maqueda et al., 2012). In fact, previous studies have demonstrated that milk-derived peptides are associated with lower blood pressure (Fekete et al., 2016), with some researchers generating evidence to support beneficial impact of milk proteins on vascular health (Fekete et al., 2013).

In our previous work, we characterized ACE inhibitory peptides produced by enzymatic hydrolysis of whey proteins (Welderufael et al., 2012). Peptide sequences were identified as major peptides in

fractions from the enzymatic hydrolysates CDP (casein-derived peptides) and β-lactoglobulin. The well-known anti-hypertensive peptide Ile-Pro-Pro (IPP), along with some other novel peptide sequences that have structural similarities with reported ACE inhibitory peptides, such as Leu-Val-Tyr-Pro-Phe-Pro (LVYPFP), Leu-Ile-Val-Thr-Gln (LIVTQ), and Ile-Ile-Ala-Glu (IIAE) were characterized and identified by a combination of chemical characterization (LC/MS; MS/MS) and structure-activity relationship data. These peptides produced naturally from whey by enzymatic hydrolysis, interacted with residues of human ACE in common with potent ACE-inhibitory drugs, such as Sampatrilat, Captopril, Lisinopril and Elanapril, which suggests that these natural peptides may be potent ACE inhibitors (Welderufael et al., 2012; Chamata et al., 2020). This present study aims to explore the efficacy of a natural therapeutic strategy that targets both RAS axes as potential treatment and/or prevention of COVID-19. Herein, we investigate the potential interactions between whey protein derived peptides with high ACE inhibitory activity (IPP, IIAE, LIVTQ, and LVYPFP) and human ACE2, employing a molecular docking approach. The overall aim is to obtain an improved understanding of such peptides' function, as RAS inhibitors, and to assess their potential therapeutic role at the heart of the COVID-19 pandemic.

3.2. Results and discussion

3.2.1. Molecular docking

In this study, molecular docking was conducted to elicit the potential molecular interactions between the specific amino acids at the binding site of human ACE2, and our previously identified whey-protein derived peptide sequences with high ACE inhibitory activities.

The peptide sequences were docked into the binding site of human ACE2, using the X ray crystallographic structure of the human ACE2 receptor (PDB code 6M0J). As 6M0J does not contain a co-crystallised ligand, to validate our docking approach, we used the co-crystallised MLN-4760 ACE2 receptor complex (PDB code 1R4L), whereby we extracted the co-crystallized ligand MLN-4760 and re-docked it into the prepared protein 1R4L. The root-mean-square deviation (RMSD) between the docked conformation (as generated by superimposition in the program PyMol), and the native co-crystallized ligand conformation was 0.3 Å, which is well within the 2 Å grid spacing used in the docking procedure, demonstrating that the docking method to be used was valid and reliable. Furthermore, the interactions between the docked ligand and the prepared target receptor mimicked those observed in the crystal structure of the same protein (PDB code 1R4L) (Towler et al., 2004).

To further validate our method, the ligand Carnosine was docked into the prepared X-ray crystal structure of human ACE2 receptor (PDB code 6M0J) to be used for subsequent docking runs. As Q9BYF1 is the UniPROT code for both 6M0J and 2AJF structures and given these are 100% identical ACE2 sequences in both X-ray crystal structures (Figures S1–S3), according to the EMBOSS needle results (Figure S4); the interactions between the docked ligand Carnosine and those observed in the crystal structure (PDB code 2AJF) were compared (Figure 3.2.a and S5) (Saadah et al., 2020). In our docking study presented herein, Carnosine interacted with key amino acid residues Glu 375, His 378, Glu 402, Tyr 515 in the active site of ACE2, in accordance with observations reported in the literature (Saadah et al., 2020; Teralı et al., 2020; Towler et al., 2004; Guy et al., 2003). In a study providing structural insights for the differences in inhibition pattern and substrate specificity for ACE and ACE2, amino acid residues His 374, His 378, Glu 375, Tyr 515, Glu 402, and Glu 406 were characterized in the active site of ACE2 (Guy et al., 2003). These observations were corroborated in the first reported crystal structure of ACE2 in its native and inhibitor-bound states, where key binding residues His 374, His 378, and Glu 402 were identified (Towler et al., 2004). According to Saadah et al. (2020), Carnosine interacted with amino acid residues His 378, Glu 402 and Tyr 515 at the active site of ACE2, also confirmed in our docking approach.

The synthetic ACE inhibitory drug Captopril was also docked into 6M0J. According to the docking results, Captopril did not interact with any key binding residues in the active site of ACE2 and formed only one potential hydrogen bond with the backbone of the amino acid residue Ala 348 (Figure 3.2.b and S6). These observations are in line with those reported by other researchers whereby ACE inhibitors such as Captopril cannot inhibit ACE2 (Guy et al., 2003; Guy et al., 2005).

Following validation, the human ACE2 receptor (PDB code 6M0J) was then used as the target molecule for docking the peptide sequences of interest into its active site.

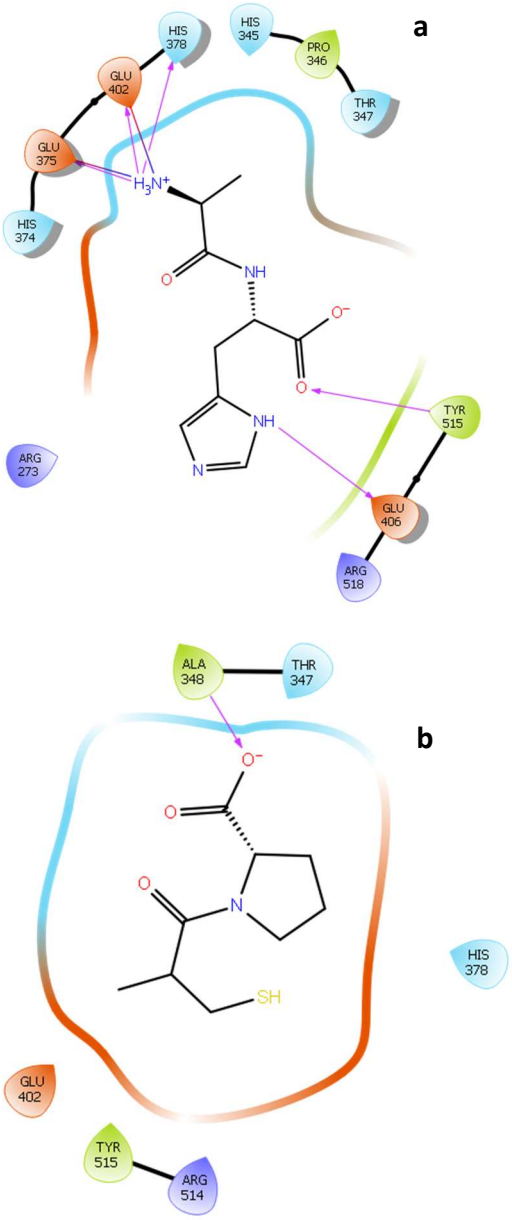

Figure 3.2. a) Docking results of the peptide Carnosine in the human angiotensin converting enzyme 2 (ACE2) active site. The interactions of human ACE2 residues with Carnosine (represented in black) are indicated by arrows of different colours with purple representing hydrogen bond interactions, and blue arrows representing salt bridge interactions. b) Docking results of synthetic drug Captopril in the human angiotensin converting enzyme 2 (ACE2) active site. The interaction of human ACE2 residues with Captopril (represented in black) is indicated by a purple arrow representing hydrogen bond interactions.

Hydrogen bond interactions play a crucial role in the specificity and stability of protein-ligand interactions. The results of ligand-driven docking into the binding site of ACE2 are summarised in Figures 2.3-2.6 and S7 – S10 and Table 3.1 and S1. It is known that His 374, His 378 and Glu 402 are important ligand binding residues in the zinc-binding site of ACE2 (Guy et al., 2003; Guy et al., 2005; Towler et al., 2004; Teralı et al., 2020). In the current study, IPP showed potential interactions with

the key residues His 378 and Glu 402 through hydrogen bond interactions at distances of 2.4 Å and 2.9 Å, respectively. Interestingly, IPP also interacted with these two amino acid residues His 378 and Glu 402 in common with Carnosine, the best-known drug candidate to match an ACE2 inhibitor structure (Saadah et al., 2020). Additionally, IPP formed a salt bridge and a hydrogen bond with amino acid residue Glu 375, another key active amino acid residue in ACE2 (Guy et al., 2003; Teralı et al., 2020) (Table 3.3, Figure 3.3). IIAE, LIVTQ and LVYPFP also interacted with residue Glu 402 in common with the potent ACE2 inhibitor Carnosine. This was via hydrogen bonding and salt bridge interaction at distances of 2.9 Å and 4.3 Å, respectively, for IIAE (Table 3.1, Figure 3.4), through two hydrogen bonds at distances of 2 Å and 2.6 Å, and one salt bridge interaction at a distance of 4.3 Å for LIVTQ (Table 3.1, Figure 3.5), and through hydrogen bonding and salt bridge interactions at distances of 2.9 and 3.8 Å, respectively, for LVYPFP (Table 3.1, Figure 3.6). Additionally, IIAE formed two hydrogen bonds and one salt bridge interaction with key binding amino acid residue Glu 375 (Table 3.1, Figure 3.4) (Guy et al., 2003; Teralı et al., 2020).

In another molecular docking study, conducted by Upreti et al. (2021), chloroquine phosphate, a commercial ACE2 inhibitor, exhibited well-established hydrogen bonds with amino acid residues Glu 406, Asp 367, Asp 269 and Phe 274. Peptides LIVTQ and LVYPFP also interacted with the amino acid residue Glu 406 through hydrogen bonds and salt bridge interactions (Table 3.1, Figures 3.5 and 3.6). Peptide LIVTQ additionally interacted with key residues His 374 and Glu 375 at distances of 2.8 Å and 3.5 Å, respectively (Table 3.1, Figure 3.5). Moreover, Arg 273 is a key amino acid residue for substrate binding in ACE2 that was found to form a salt-bridge with the C-terminus of potent and selective human ACE2 inhibitor, MLN-4760 (Guy et al., 2005; Teralı et al., 2020; Towler et al., 2004). Both peptides IPP and IIAE formed salt bridge interactions and hydrogen bonds with amino acid residue Arg 273 (Table 3.1, Figures 3.3 and 3.4).

Table 3.1. Docking results of IPP, IIAE, LIVTQ, and LVYPFP.

Protein 6MOJ		**Ligand IPP**	
Residue	Atom Name	Interaction Type	Distance (Å)
NH1 Arg 273	O- (Pro2)	Salt bridge	3.0
OE1 Glu 375	NH3+ (Ile)	Salt bridge	4.1
OE2 Glu 375	NH3+ (Ile)	Hydrogen bond	2.0
NE2 His 378	NH3+ (Ile)	Hydrogen bond	2.4
OE1 Glu 402	NH3+ (Ile)	Hydrogen bond	2.9
OE2 Glu 402	NH3+ (Ile)	Salt bridge	3.1
		Ligand IIAE	
NH2 Arg 273	O1 (Glu)	Salt bridge	3.0
NH2 Arg 273	OE1 (Glu)	Hydrogen bond	2.9
NH1 Arg 273	OE2 (Glu)	Salt bridge	3.0
OE1 Glu 375	NH3+ (Ile)	Salt bridge	3.0
OE2 Glu 375	NH3+ (Ile)	Hydrogen bond	2.6
OE2 Glu 375	NH (Ile)	Hydrogen bond	2.4
OE1 Glu 402	NH3+ (Ile)	Hydrogen bond	2.9
OE2 Glu 402	NH (Ala)	Hydrogen bond	2.9
CG Glu 402	NH3 + (Ile)	Salt bridge	4.3
		Ligand LIVTQ	
ND1 His 374	O (Gln)	Hydrogen bond	2.8
OE1 Glu 375	NH3 + (Leu)	Salt bridge	3.5
OE2 Glu 375	NH3 + (Leu)	Hydrogen bond	2.9
OE2 Glu 375	NH (Ile)	Hydrogen bond	2.3
OE1 Glu 402	NH3+ (Leu)	Hydrogen bond	2.0
OE2 Glu 402	NH3+ (Leu)	Salt bridge	4.3
OE2 Glu 402	NH (Val)	Hydrogen bond	2.6
OE1 Glu 406	OH (Thr)	Hydrogen bond	2.9
OE1 Glu 406	NH2 (Gln)	Hydrogen bond	2.9
OE1 Glu 406	NH (Gln)	Hydrogen bond	2.9
OE2 Glu 406	NH (Thr)	Hydrogen bond	2.9
OE2 Glu 406	NH (Val)	Hydrogen bond	2.3
NE2 Gln 442	O (Gln)	Hydrogen bond	2.8

NH2 Arg 518	OH (Thr)	Hydrogen bond	2.1
		Ligand LVYPFP	
CG2 Thr 276	O- (Pro)	Hydrogen bond	2.6
OE1 Glu 402	NH3+ (Leu)	Salt bridge	3.8
OE2 Glu 402	NH3+ (Leu)	Hydrogen bond	2.9
CO Glu 406	NH3+ (Leu)	Salt bridge	4.0
OE1 Glu 406	NH (Val)	Hydrogen bond	2.8
OE1 Glu 406	NH (Tyr)	Hydrogen bond	2.4
OE2 Glu 406	NH3+ (Leu)	Hydrogen bond	2.8

3. 2.2. ACE and ACE2

Sequence alignment of ACE2 with ACE revealed the high conservation between these two enzymes. Analysis of critical active site residues between ACE and ACE2 has demonstrated that these two proteins are structurally well conserved. Since their active site structures are highly conserved and there exists a strong similarity between the catalytic domains of ACE and ACE2, consequently, the catalytic mechanism of ACE2 closely resembles that of ACE. However, due to differences in substrate specificity, distinctive key differences are present between the active site pockets of ACE and ACE2. Indeed, these differences occur in the ligand-binding pockets, particularly in the binding of the peptide C-terminus and at the S2' subsite. The cavity in ACE2 is smaller than that of ACE, which allows an extra amino acid to bind in the specificity pocket. Additionally, the S1 subsite of ACE is larger than that of ACE2 (Guy et al., 2003). In our previous work, we provided strong *in vitro* evidence for the ACE inhibitory activity of peptide sequences IPP, IIAE, LIVTQ, and LVYPFP (Chamata et al., 2020; Welderufael et al., 2012). Although all 4 peptides have been found to exhibit high ACE inhibitory activity, peptide IIAE formed strong hydrogen bonds with the amino acid residues Gln 259, and Thr 358 in the active site of ACE, in common with the ACE inhibitory drug Sampatrilat. IIAE also interacted with the amino acid residues Gln 259, and His 331, in common with other ACE inhibitory drugs such as Captopril, Lisinopril and Elanapril, and with the amino acid residue Asp 140, in common with Lisinopril. Additionally, IPP has been identified as the most potent ACE inhibitor from milk protein (Nakamura et al., 1995). When compared to the other peptides' interactions with ACE, IIAE and IPP seem to display the highest ACE inhibitory activities. Notably, according to the ACE2 docking results, both peptides IPP and IIAE also seem to display the highest ACE2 inhibitory activity.

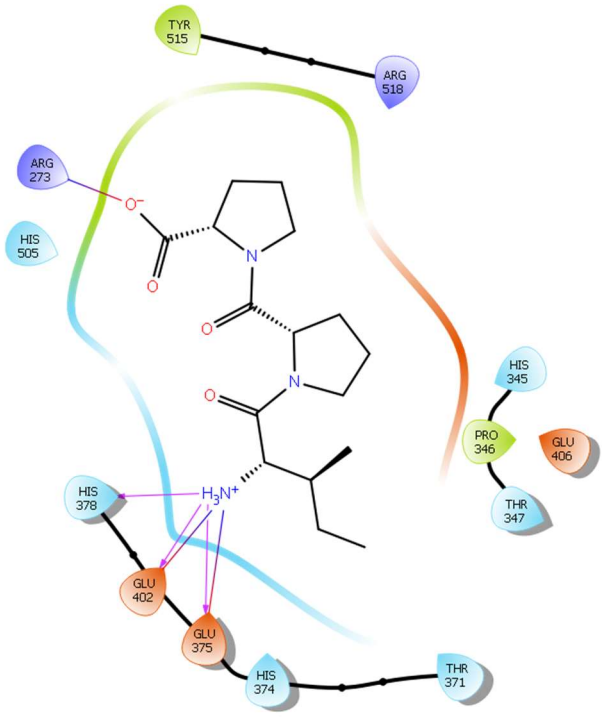

Figure 3.3. Docking results of the peptide IPP in the active site of human angiotensin 2-converting enzyme (ACE2). Interactions of human ACE2 residues with the peptide IPP (represented in black) are indicated by arrows of different colours, with purple representing hydrogen bond interactions and blue arrows representing salt bridge interactions.

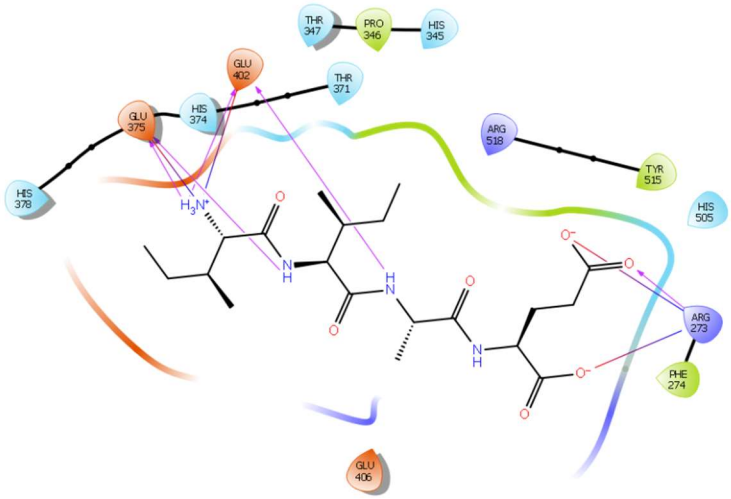

Figure 3.4. Docking results of the peptide IIAE in the active site human ACE2. The interactions of human ACE2 residues with the peptide IIAE (represented in black) are indicated by arrows of different colours with purple representing hydrogen bond interactions, and blue arrows representing salt bridge interactions.

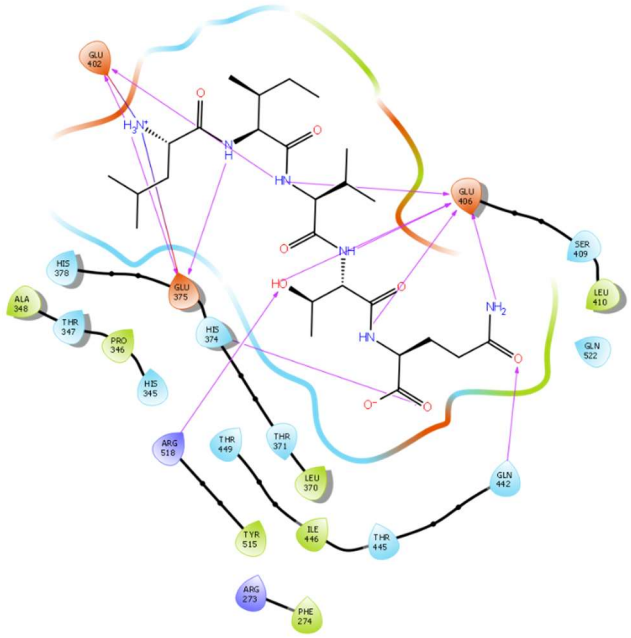

Figure 3.5. Docking results of the peptide LIVTQ in the human ACE2 active site. The interactions of human ACE2 residues with the peptide LIVTQ (represented in black) are indicated by arrows of different colours with purple representing hydrogen bond interactions, and blue arrows representing salt bridge interactions.

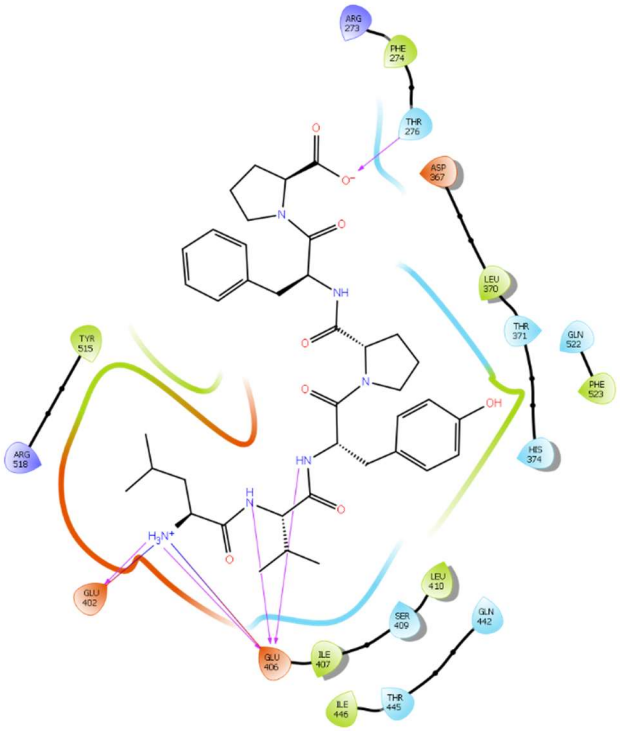

Figure 3.6. Docking results of the peptide LVYPFP in the human ACE2 active site. The interactions of human ACE2 residues with the peptide (represented in black) are indicated by arrows of different colours with purple representing hydrogen bond interactions, and blue arrows representing salt bridge interactions.

3. 2.3. Potential use of ACE inhibitors in the treatment of COVID-19

To date, there is no effective drug available to treat COVID-19 patients (Bimonte et al., 2020). Although COVID-19 vaccines were shown to be closely associated with a significant reduction in symptomatic infections (Centers for Disease Control and Protection et al., 2021), vaccine hesitancy is widespread worldwide, which could hinder populations achieving herd immunity (Dror et al., 2020). The rapid global emergence of novel SARS-CoV-2 variants, unequal international distribution of COVID-19 vaccines, and slow vaccine rollouts, especially in developed countries, could also be significant factors obstructing the achievement of herd immunity and bringing an end of the pandemic. Although antimalarial drugs chloroquine and hydroxychloroquine, and some synthetic drugs such as remdesivir (Bimonte et al., 2020) and ritonavir/lopinavir (Arabi et al., 2018; Bimonte et al., 2020) are currently used to treat COVID-19 patients, as yet, there remains no effective and approved drug available against COVID-19 (WHO, 2021). Various side effects associated with the drugs were also observed among treated patients (Arabi et al., 2018l; Bimonte et al., 2020), delaying widespread acceptance and administration.

Consequently, identifying safe and effective compounds that can restrain entry of SARS-CoV-2 into host cells via ACE2 is a priority for the scientific community. In this respect, an active area of research is the impact of milk/whey-derived bioactive peptides and their potential health benefits, as ingredients of health-promoting functional foods (Mohanty et al., 2016). Peptide sequences from whey proteins exhibit different bioactivities, including ACE inhibitory activity. In fact, milk is the main source of anti-hypertensive ACE inhibitory peptides reported to date (Pihlanto-Leppälä, 2020; Martínez-Maqueda et al., 2012, Mohanty et al., 2016).

In the scientific community, controversy has arisen over whether the use of ACE inhibitors would be harmful or beneficial in the context of the COVID-19 pandemic (Barochiner et al., 2020; Kuster et al., 2020; Tomasoni et al., 2020; Vaduganathan et al., 2020; Wu et al., 2020; Zheng et al., 2020). Although increased COVID-19 disease severity seems to manifest in people with cardiovascular comorbidities (Sommerstein et al., 2020; Zhou et al., 2020), it is suggested that this association could be related to advanced age and obesity (Sommerstein et al., 2020). Moreover, there seems to be growing evidence that use of ACE inhibitors does not worsen the prognosis of COVID-19 (He et al., 2007). In fact, in a cohort study, including 8.3 million people, ACE inhibitors were not found to be significantly associated with increased risks of COVID-19 disease, nor of requiring ICU care (Hippisley-Cox et al., 2020). In agreement, another meta-analysis study also reported that the use of ACE inhibitors was not associated with requiring intensive care, mechanical ventilation, progression to severe disease, and

increased risk of death. However, some researchers have reported a 16% reduction in the risk of COVID-related mortality with the use of ACE inhibitors (Barochiner et al., 2020).

Some studies suggest that ACE inhibitors could even play a protective role in hypertensive patients by averting organ injury (Danser et al., 2020). Indeed, *in vivo* models support the role of ACE inhibitors in blunting lung injury and exerting health benefits in both human and animal trials (Caldeira et al., 2012; Cohen et al., 2012; Lukkarinen et al., 2005; Medhora et al., 2012). Data from human studies also revealed that ACE inhibitors can reduce or prevent pneumonia (Mortensen et al., 2012; Shrikrishna et al., 2012), namely, (i) Treatment of chronic obstructive pulmonary disease with ACE inhibitors was found to reduce disease complications, and (ii) Treatment with ACE inhibitors was shown to mitigate the effects of radiation pneumonitis (Harder et al., 2015). In short, there is consistent evidence indicating that ACE inhibitors seem to have beneficial effects in modulating lung damage, including in the context of pulmonary injury caused by viral infection. Due to insufficient evidence of the potentially harmful effects of ACE inhibitors, and overwhelming evidence supporting their benefits, multiple scientific societies rejected the recommendation to discontinue ACE inhibitors, in the context of the COVID-19 pandemic (Bozkurt et al., 2020; European Society of Hypertension, 2021; ESC Council on Hypertension on ACE-Inhibitors and Angiotensin Receptor Blockers. 2020). Interestingly, ACE inhibitors were reported to be associated with significant pulmonary inflammatory response reduction in patients admitted with viral pneumonia (Henry et al., 2018) and attenuated inflammatory response in COVID-19-infected patients (Meng et al., 2020; Yan, 2020). This emerging evidence prompted many researchers to advocate for the use of RAS inhibitors in the therapeutic management of COVID-19 infection (Barochiner et al., 2020).

Regarding the role of the RAS pathway in the pathophysiology of COVID-19 and SARS-CoV-2 infection, there are two primary theories. First, data from the literature have shown that Ang II mediated inflammation is a main mediator of acute lung injury and fibrosis (Gu et al., 2016; Kuba et al., 2005; Li et al., 2008). Similar to SARS-CoV, loss of ACE2 activity and expression could lead to an increase in Ang II levels in the lungs and consequently drive COVID-19 acute lung injury. One study reported significantly higher Ang II levels in COVID-19 patients that correlated with viral load and indicators of lung injury. However, this study had considerable methodological limits: only 12 patients took part in the clinical study and circulating levels of ACE and/or ACE2 were not determined (Chappell et al., 2015; Lui et al., 2020; South et al., 2020). Furthermore, data from the original SARS-CoV epidemic indicated that infection with SARS-CoV-2 may lead to ACE-2 dependent myocardium infection, which results in

decreased cardiac ACE2 expression, accelerating acute heart injury (Oudit et al., 2009). However, it is important to note that there is no clinical data to confirm this.

Second, there is concern that ACE inhibitors may potentially increase the expression and levels of ACE2 in the lungs, which facilitates SARS-CoV-2 infection such that administering ACE inhibitors may increase the risk of severe and fatal disease (Fang et al., 2020; Watkins, 2020). In select animal models, ACE inhibitors were able to increase heart and kidney ACE2 expression (Ferrario et al., 2005; Ishiyama et al., 2004). However, there are no data proving that these compounds can increase lung ACE2 expression in both animal models and human trials. Likewise, there are no available data demonstrating that the increased expression of ACE2 would necessarily indicate increased risk of disease severity or infection, or that the use of these agents is correlated with increased virulence or viral infectivity. In fact, it does not appear to be any consistent association between increased ACE2/Ang (1–7)/Mas pathway activity and expression and use of ACE inhibitors in the few clinical studies assessing the effect of ACE inhibitors on the ACE2/Ang (1–7) pathway (Epelman et al., 2008; Furuhashi et al., 2015; Lely et al., 2004; Park et al., 2009). Although there is lack of evidence to demonstrate the effect of ACE inhibitors on ACE2 expression and thus SARS-CoV-2 infectivity, the bulk of experimental evidence indicates that ACE inhibitors may reduce the action of Ang II, and consequently attenuate Ang II-driven acute lung injury (South et al., 2020; Zambelli et al., 2015). ACE inhibitors therefore offer promise as potential novel therapeutics to treat COVID-19 disease (Sun et al., 2020).

Intriguingly, based solely on experimental studies in which RAS inhibitors were administered *in vivo* (Burrell, 2005; Hampl et al., 2015; Liao et al., 2011; Wood et al., 1990; Yamaguchi et al., 1998), Zamai (2020) highlighted a reasonable hypothesis that using inhibitors that block both ACE2 and ACE pathways in COVID-19 patients could be very beneficial in the treatment of COVID-19. In short, observations from these studies indicate that hypoxia/hypercapnia, a condition that occurs in SARS patients, is highly likely to upregulate the activity of both arms of the RAS. A strong correlation was also observed between the gene expression of ACE2 and that of ACE (Wakahara et al., 2007). Another observation suggested the possibility of a positive feedback induced by SARS-CoV infection, leading to surface expression of both ACE and ACE2 (Clarke et al., 2013; Haga et al., 2008; Jia et al., 2009). Altogether these observations indicate that RAS-mediated positive feedback loops can be induced by SARS-CoV-2 at different organ levels. Consequently, in order to block these feedback loops, Zamai (2020) suggested that different compounds can be produced to inhibit both RAS pathways and subsequently prevent critical, advanced and untreatable stages of the COVID-19 disease.

IPP, IIAE, LIVTQ, LVYPFP, bioactive peptides derived from whey proteins, were initially characterized as ACE inhibitors through *in vitro* and *in silico* assays in our previous works (Chamata et al., 2020; Welderufael et al., 2013). Findings from the current study demonstrate additional novel effects for these bioactive whey-derived peptides, as potential ACE2 inhibitors. These results strongly support our hypothesis that these whey-derived peptides not only could exhibit ACE inhibitory activity but also could bind to ACE2 and as such, could have a potential effect to intervene in the interaction between ACE2 and SARS-CoV-2 S protein. Additionally, compared to synthetic ACE-inhibitory drugs, these peptides are from a natural source and do not exhibit toxic side effects, which might also help to reduce the risks associated with traditional drugs in the treatment of COVID-19 infection.

3.3. Materials and methods

3.3.1 Structure similarity between 1RL4 and 6M0J

EMBL-EBI (https://www.ebi.ac.uk/) was queried for 1RL4, 6M0J and 2AJF amino acid sequences, together with known three-dimensional (3D) protein structures. Reviewed sequences were selected, and the protein sequence files were downloaded. The three sequences were then uploaded to Emboss Needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/) for pairwise sequence alignment and comparison.

3.3.2. Docking procedure

The X-ray crystallographic structure of ACE2 bound with CoV-2 S protein (PDB code 6M0J, 2.45 Å resolution) retrieved from the Protein Data Bank (PDB) was chosen as the target protein for the docking studies, based on its high-resolution structure co-crystallized with SARS-CoV-2 S protein. In this crystal structure, the interaction between SARS-CoV-2 S protein and cell receptor ACE2 is mediated by a defined receptor-binding domain (RBD) (Figure 3.7.a) (Lan et al., 2020). The binding site cleft of ACE2 (Figure 3.7.b) and details of the active site residues (Figure 3.7.c) have been previously characterized (Guy et al., 2003; Guy et al., 2005; Teralı et al., 2020; Towler et al., 2004) and were used in this work to guide the docking procedure.

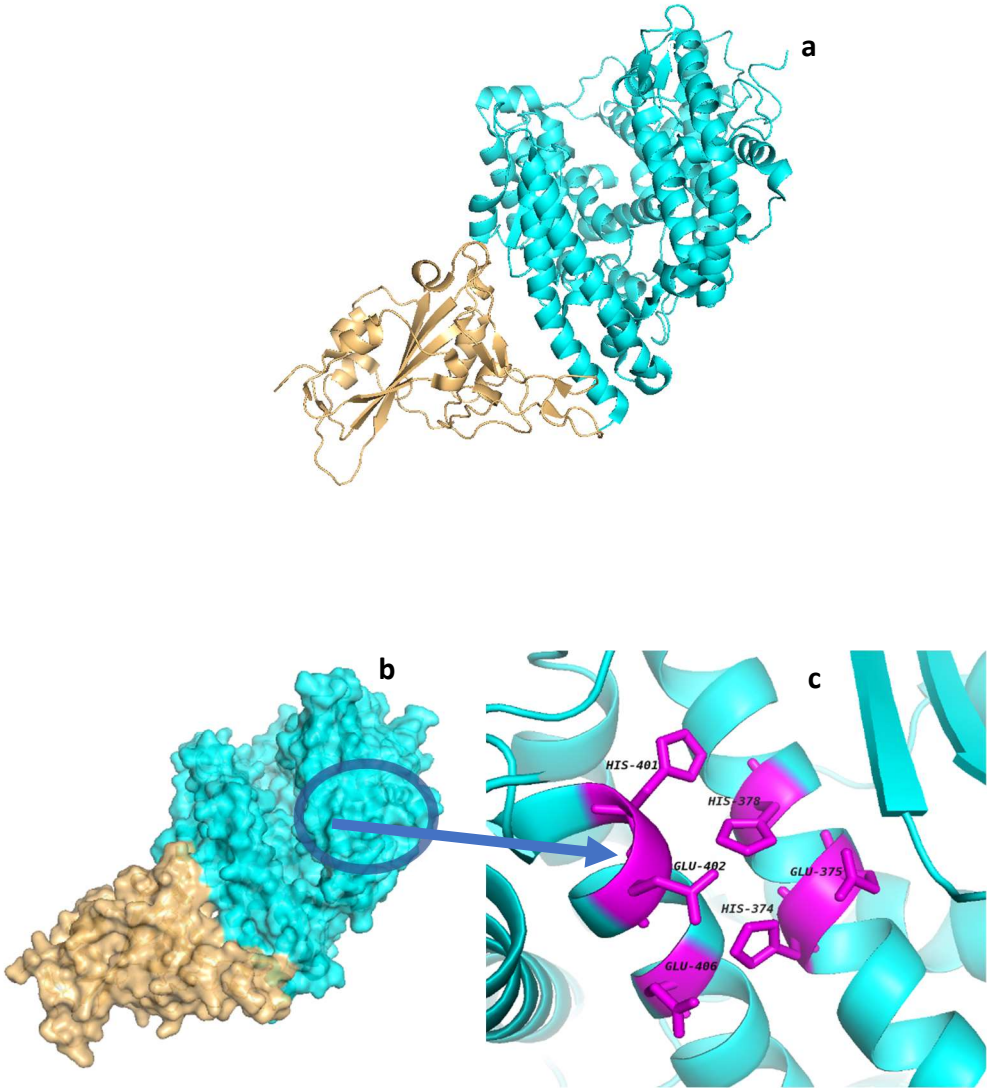

Figure 3.8. Structure of the SARS-CoV-2 receptor-binding domain (RBD) complexed with ACE2.

(a) Crystal structure of the SARS-CoV-2 RBD complexed with ACE2. ACE2 is shown in cyan. RBD of SARS-CoV-2 is shown in gold. b) Surface representation of protein 6M0J showing the binding site cleft. c) Amino acid residues in the active site of ACE2 as highlighted in the literature (Guy et al., 2003; Guy et al., 2005; Teralı et al., 2020; Towler et al., 2004; Upreti et al., 2021). The figures were generated using the software PyMol.

Whey protein-derived peptides IPP, IIAE, LIVTQ, and LVYPFP were employed as ligands in separate docking runs. The docking methodology has been validated, as previously described, and docking was performed following the same methodology used in our previous work (Chamata et al., 2020), using the docking algorithm Surflex-Dock, as supplied by SYBYL-X 2.1.

To prepare the protein structure for docking, the Biopolymer Structure Preparation Tool, with the implemented default settings provided in the SYBYL programme suite was used. Hydrogens were added to the protein structure in idealised geometries, backbone and sidechains were repaired, residues were protonated, sidechain amides and sidechain bumps were fixed, stage minimization was performed, and all water and any ligand molecules were removed. Using the “Build Protein” tool provided in Sybyl-X, the three-dimensional (3D) structure of each ligand was constructed. Once formed, charges were assigned to each atom of each ligand, using Merck Molecular Force Field (MMFF94) charges. Localised energy minimizations were then performed, and the final structure for each ligand in its lowest energy conformation was used for successive docking runs. The resulting 3D coordinate files were converted to a MOL2 format for subsequent use in Surflex-Dock experiments, as provided in the SYBYL-X 2.0 software suite.

Surflex-Dock is a search algorithm that employs an empirically derived scoring function whose parameters are based on protein-ligand complexes of established structures and affinities. This procedure uses a “protomol”, which is an idealised active site, as a target to produce presumed poses of molecules. The protomol is utilised as a mimic of the ideal interactions made by a perfect ligand to the active site of the protein. This alignment is based on molecular similarity, which allows for optimisation of theoretically favourable molecular interactions, such as those specified by hydrogen bonds and van der Waals forces. To create a protomol that effectively depicted the binding pocket of interest, the protomol was defined by optimizing the bloat values and threshold to 0 and 0.5, respectively. The bloat values and threshold are two parameters that control the extent of the protomol and its degree of coverage of an active site. The threshold parameter shows the amount of buriedness for the primary volume used to generate the protomol, and the bloat value indicates the number of Ångströms by which the search grid beyond that volume should be expanded. In general, it is better to err on the side of a small protomol than on a protomol that is too large (Sharma et al., 2016). All other parameters within the docking suite were left as the default values as determined by the software (Ai et al., 2011; Lan et al., 2011). Using the "Docking Suite" application, provided in the SYBYL programme suite, each peptide was then individually docked into the protomol site. For further visualisation and analysis, Maestro (Schrödinger Release 2021-2: Maestro, Schrödinger, LLC, New York, NY, 2021) was used for the characterisation and identification of hydrogen bonds and salt-bridge interactions established between the peptides and residues at the ACE2 active site. Figures were also generated using the software Maestro (Figures 3.2-3.6).

3.3.3. Docking validation

In order to validate the accuracy and the reliability of the docking procedure to be used in this study, the original ligand (extracted from the coordinate files and taken from the Protein Data Bank; PDB code 1R4L was docked into the corresponding crystal structure of the receptor, using the automated docking procedure in the program Surflex-Dock (SFXC) (Jain, 2003), as provided by SYBYL-X2.1 (Towler et al., 2004). The docked ligand mode and orientation from the docking procedure were compared to that found in the actual crystal structure of the complex using PyMol and PDBeFold (PDBeFold, 2021; Schrödinger, 2015). Following the docking procedure, the RMSD value between the docked ligand and the ligand, as found in the crystal structure, was calculated. The success of the docking process depended on whether the value of RMSD between the real and best-scored docked conformations were within the 2 Å grid spacing, used in the docking procedure (Wang et al., 2003), and whether the molecular interactions were replicated. In this case, MLN-4760 was docked into the human ACE2 receptor as validation of the docking procedure.

For further validation, the ligand Carnosine, the best-known drug candidate to match an ACE2 inhibitor structure, was docked into the known binding site of ACE2 (PDB code 6M0J), according to the methodology applied by Saadah et al. (2020). The docking results of Carnosine were then compared to the published docking results of the ligand into the active site of ACE2 (PDB code 2AJF) (Saadah et al., 2020). The ligand Captopril was also prepared and docked into the binding site of ACE2, according to the same methodology.

3. 4. Conclusions

For the first time, presented herein, potential interactions between the naturally produced peptides from whey proteins and ACE2, the host cell receptor of SARS-CoV-2, have been examined, using a molecular docking approach. Peptides IPP, IIAE, LIVTQ, and LVYPFP all formed strong hydrogen bonds and salt bridge interactions with key residues in the active site of human ACE2. Among the four peptides examined, IPP and IIAE are the most promising candidates to exert an antiviral activity on SARS-CoV-2, through inhibiting ACE2 via specific molecular interactions with key residues of ACE2. IIAE and IPP also formed strong interactions at the active site of ACE2, in common with known potent pharmaceutical ACE2 inhibitors. According to the results of this study, whey-derived peptides IPP, IIAE, LIVTQ, and LVYPFP are suggested as potential candidates to be used in the treatment of SARS-CoV-2 via inhibition of the host cell receptor ACE2. Moreover, in comparison with well-known ACE inhibitory drugs such as Captopril and Lisinopril, the natural peptides produced from whey have a dual inhibitory

action against both ACE and ACE2, and may be associated with fewer side-effects, which may represent advantages in the treatment of COVID-19.

The structural insights provided by this molecular docking study are valuable in understanding and manipulating the regulation of ACE2. These peptides could also provide important scaffolds for further insight into the design of novel potent therapeutic inhibitors against SARS-CoV-2, based on ACE2 inhibition. Further *in vitro* and *in vivo* studies, however, are needed to further substantiate these whey-derived peptides' underlying inhibitory mechanisms against ACE2. It also remains unknown whether inhibiting ACE2 would be efficient in attenuating infections by SARS-CoV-2, and, likewise, further research is urgently needed to understand the underlying molecular mechanisms related to these inhibitory mechanisms.

3.5 Supplementary data

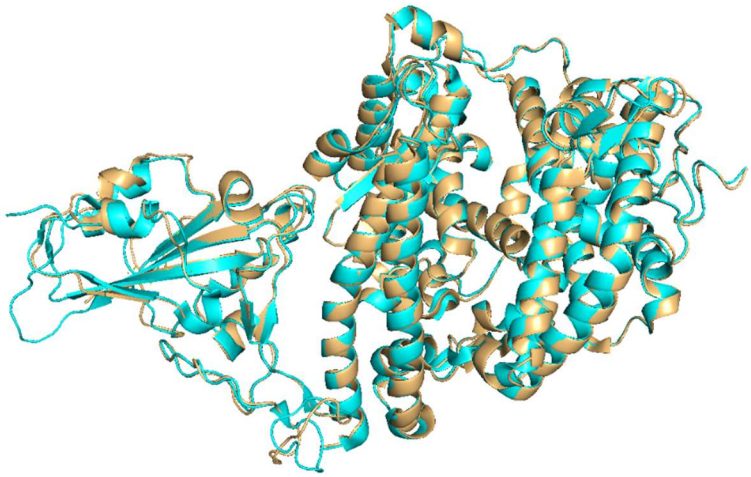

Figure S.1 Superimposition of 6M0J and 2AJF crystal structures. RMSD value calculated = 0.46.

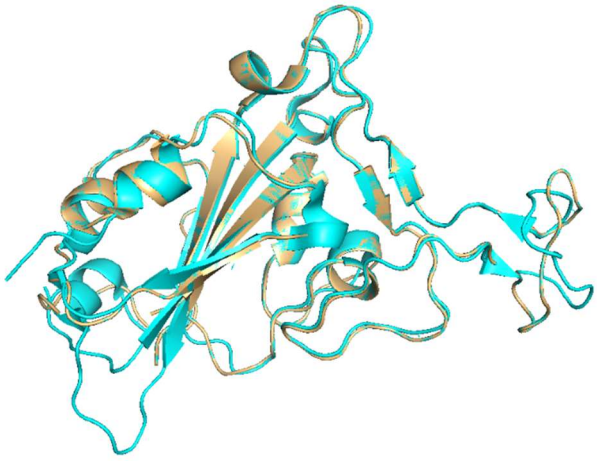

Figure S.2 Superimposition of the SARS-CoV 2 S protein receptor-binding domain (Residues 337-515) of 6M0J and SARS-CoV S protein receptor-binding domain (Residues 323-501) of 2AJF. RMSD value calculated = 0.41.

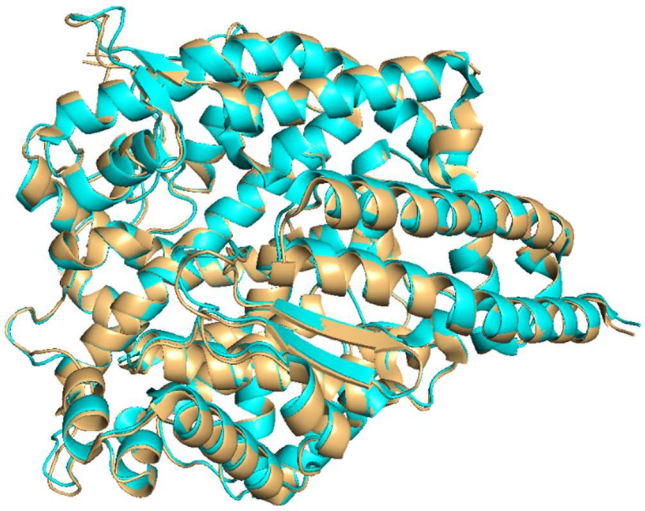

Figure S.3 Superimposition of the ACE2 sequence (Residues 21- 615) of 6M0J and the ACE2 sequence (Residues 21- 615) of 2AJF. RMSD value calculated = 0.43.

```
6M0J              1 CPFGEVFNATRFASVYAWNRKRISNCVADYSVLYNSASFSTFKCYGVSPT     50
                    ||||||||||:|.|||||.||:|||||||||||||..||||||||||.|
2AJF              1 CPFGEVFNATKFPSVYAWERKKISNCVADYSVLYNSTFFSTFKCYGVSAT     50

6M0J             51 KLNDLCFTNVYADSFVIRGDEVRQIAPGQTGKIADYNYKLPDDFTGCVIA    100
                    |||||||:||||||||::||:||||||||||.||||||||||||.|||:|
2AJF             51 KLNDLCFSNVYADSFVVKGDDVRQIAPGQTGVIADYNYKLPDDFMGCVLA    100

6M0J            101 WNSNNLDSKVGGNYNYLYRLFRKSNLKPFERDISTEIYQAGSTPCNGVEG    150
                    ||:.|:|:...|||||.||..|...|:|||||||...:.....||. ...
2AJF            101 WNTRNIDATSTGNYNYKYRYLRHGKLRPFERDISNVPFSPDGKPCT-PPA    149

6M0J            151 FNCYFPLQSYGFQPTNGVGYQPYRVVVLSFE    181
                    .|||:||..|||..|.|:||||||||||||
2AJF            150 LNCYWPLNDYGFYTTTGIGYQPYRVVVLSFE    180
```

(I) residues are identical
(.) conserved change
(:) part of the residue is similar but not that conserved
% similarity = 80.2
% identity = 72.4

Figure S.4 EMBOSS NEEDLE pairwise sequence alignment results between SARS CoV-2 S protein receptor-binding site of 6M0J and SARS CoV S protein receptor-binding site of 2AJF.

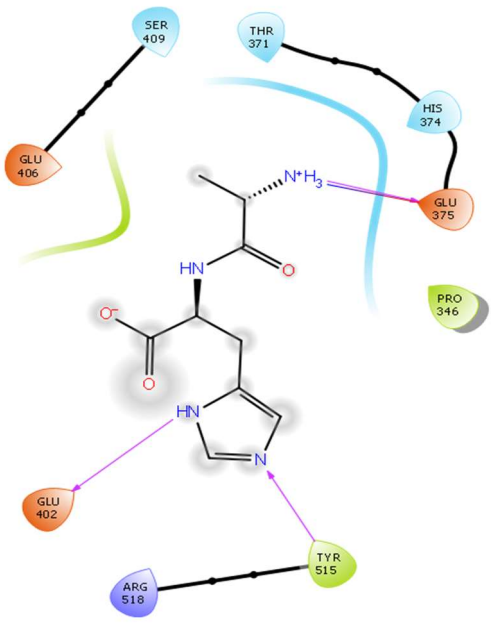

Figure S.5 Docking results of the peptide Carnosine in the human angiotensin converting enzyme 2 (ACE2) active site (PDB code 2AJF). The interactions of human ACE2 residues with the peptide (represented in black) are indicated by arrows of different colours with purple representing hydrogen bond interactions, and blue arrows representing salt bridge interactions.

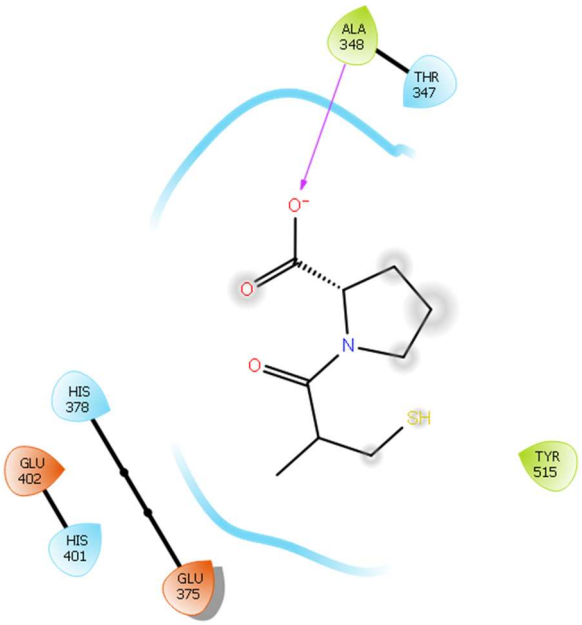

Figure S.6 Docking results of Captopril in the human angiotensin converting enzyme 2 (ACE2) active site (PDB code 2AJF). The interaction of human ACE2 residues with Captopril (represented in black) is indicated by a purple arrow representing hydrogen bond interactions.

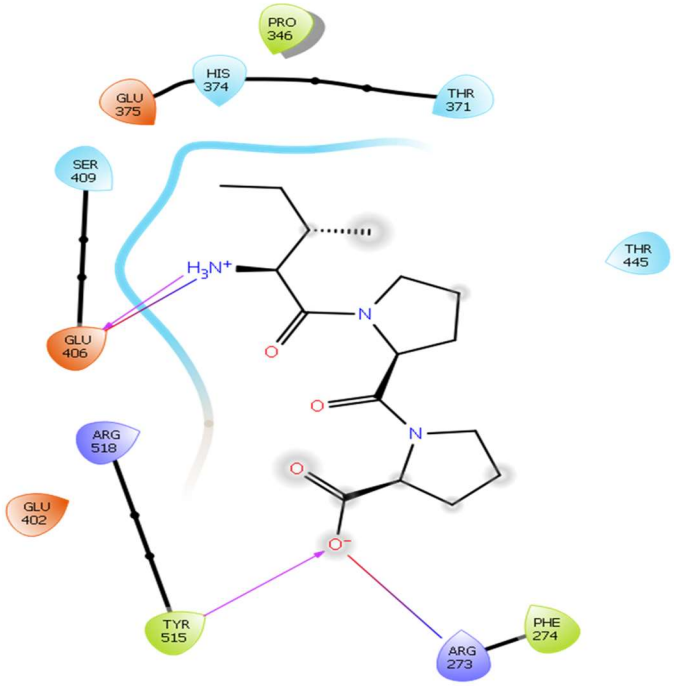

Figure S.7 Docking results of the peptide IPP in the active site of human angiotensin 2-converting enzyme (ACE2) (PDB code 2AJF). Interactions of human ACE2 residues with the peptide IPP (represented in black) are indicated by arrows of different colours, with purple representing hydrogen bond interactions and blue arrows representing salt bridge interactions.

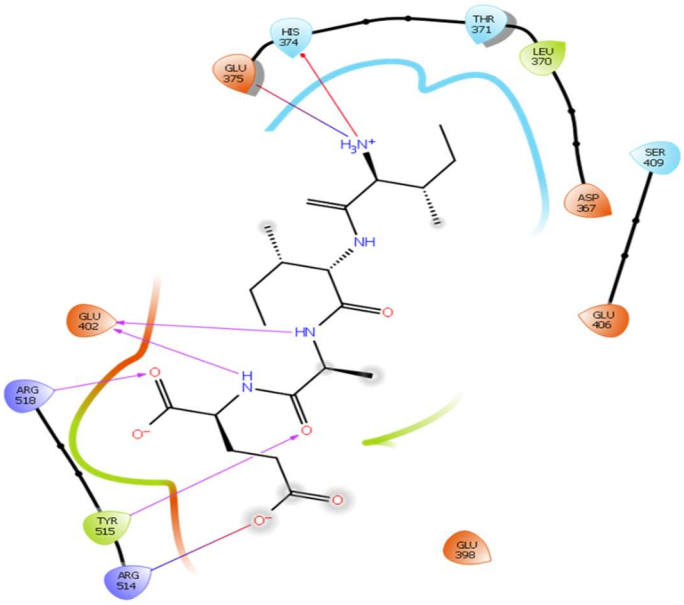

Figure S.8 Docking results of the peptide IIAE in the active site human ACE2 (PDB code 2AJF). The interactions of human ACE2 residues with the peptide IIAE (represented in black) are indicated by arrows of different colours with purple representing hydrogen bond interactions, and blue arrows representing salt bridge interactions.

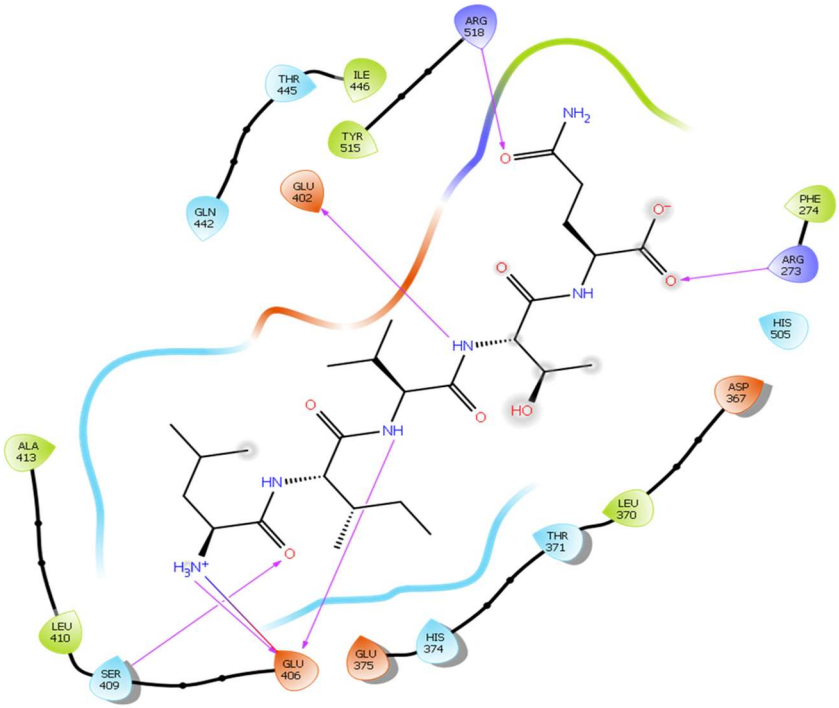

Figure S.9 Docking results of the peptide LIVTQ in the human ACE2 active site (PDB code 2AJF). The interactions of human ACE2 residues with the peptide LIVTQ (represented in black) are indicated by arrows of different colours with purple representing hydrogen bond interactions, and blue arrows rep-resenting salt bridge interactions.

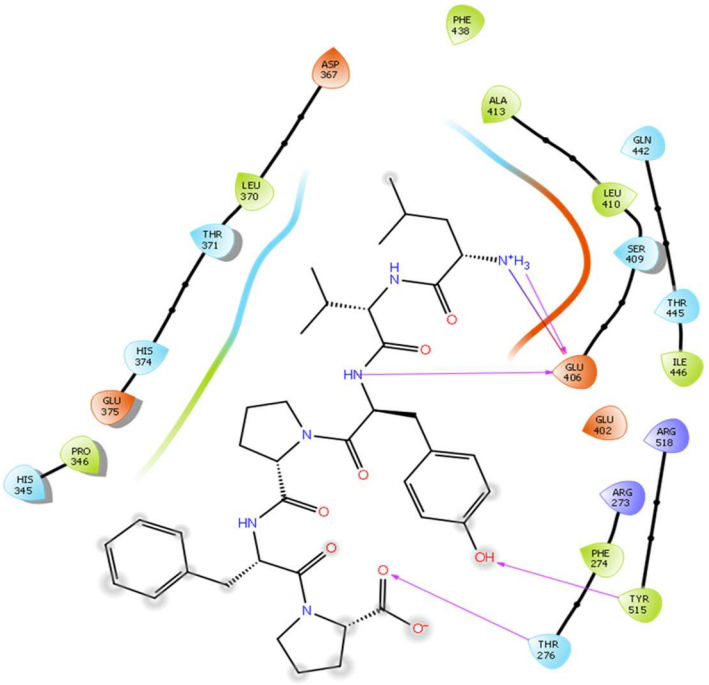

Figure S.10 Docking results of the peptide LVYPFP in the human ACE2 active site (PDB code 2AJF). The interactions of human ACE2 residues with the peptide (represented in black) are indicated by arrows of different colours with purple representing hydrogen bond interactions, and blue arrows representing salt bridge interactions.

Table S.1 Docking results of IPP, IIAE, LIVTQ, and LVYPFP.

Protein 2AJF		**Ligand IPP**	
Residue	Atom Name	Interaction Type	Distance (Å)
OE2 Glu 406	NH3+ (Ile)	Hydrogen bond	2.9
OE2 Glu 406	NH3+ (Ile)	Salt bridge	3.3
NH1 Arg273	O- (Pro)	Salt bridge	4.6
OH Tyr 515	O- (Pro)	Hydrogen bond	3.0
		Ligand IIAE	
OH Tyr 515	O (Ala)	Hydrogen bond	2.8
NH2 Arg 518	O2 (Glu)	Hydrogen bond	2.9
OE1 Glu 402	NH (Glu)	Hydrogen bond	3.0
OE1 Glu 402	NH (Ala)	Hydrogen bond	2.7
OE2 Glu 375	NH_3^+ (Ile)	Salt bridge	3.2
NH1 Arg 514	O- (Glu)	Salt bridge	3.7
		Ligand LIVTQ	
NH2 Arg273	O- (Gln)	Hydrogen bond	2.9
NH2 Arg 518	O (Gln)	Hydrogen bond	3.0
OE1 Glu 406	NH3+ (Leu)	Hydrogen bond	2.8
OE2 Glu 406	NH (Ile)	Hydrogen bond	2.9
CD Glu 406	NH3+ (Leu)	Salt bridge	3.5
OG Ser 409	O (Leu)	Hydrogen bond	3.0
OE1 Glu 402	NH (Thr)	Hydrogen bond	3.0
		Ligand LVYPFP	
OE1 Glu 406	NH3+(Leu)	Hydrogen bond	3.0
OE2 Glu 406	NH (Val)	Hydrogen bond	2.8
CD Glu 406	NH3+ (Leu)	Salt bridge	4.1
OH Tyr 515	OH (Tyr)	Hydrogen bond	2.8
OG1 Thr 276	O (Pro)	Hydrogen bond	2.9

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CHAPTER FOUR Angiotensin-converting enzyme inhibitory peptides produced from simulated gastrointestinal digestion of milk whey proteins

Contribution towards PhD thesis: The *in vitro* simulated gastrointestinal digestion methodology was developed by Alexis Poitinier, a visiting researcher from France. Additionally, performance of Mass Spectrometry analysis was performed by Nicholas Michael (CAF, Department of Chemistry, University of Reading). My responsibilities included conducting the literature search, method development and conduction of *in vitro* Angiotensin-converting enzyme (ACE) inhibitory assay and all other assays, performance of molecular docking studies, and the formal data analysis and visualisation. Then, I wrote the initial draft of the manuscript, and lastly, I finalised the manuscript presented below after including the comments and feedback received from my supervisors Paula Jauregui, Kim Jackson, and Kim Watson.

4.1. Introduction

Food-derived peptides have been attracting widespread attention from scientists because of their low cost and health benefits. Bioactive peptides (BAPs) are reported to have a positive effect on the major systems of the human body, most notably the cardiovascular system, by reducing the risks of developing chronic diseases (He et al., 2018). Consequently, food-derived peptides have been widely pursued as promising ingredients for functional foods (Udenigwe, 2014; Udenigwe et al., 2012), and the identification and characterisation of food-derived BAPs have become a significant emerging research topic. However, the ability to translate these novel research findings into practical applications is not developing at the same pace. Among the main factors behind this delay is the peptides' low bioavailability, following oral administration, resulting in a lack of correlation between the *in vitro* bioactivities and *in vivo* functions (Wang et al., 2020), as the health benefits of bioactive peptides are dependent upon the amount of these compounds that become bioavailable and bioaccessible following digestion (Santos et al., 2019). To reach the target organs where bioactive peptides can exert their beneficial effects, peptides need to resist the low pH in the stomach as well as the digestive enzymes' action during the transport of these peptides through the gastrointestinal tract and across the intestinal epithelial barrier (FitzGerald et al., 2004; Roufik et al., 2006; Quirós et al., 2009). Therefore, to evaluate the bioaccessibility and bioavailability, and subsequently the peptides' effects *in vivo*, the identification of BAPs directly in food is insufficient, and it is of utmost

importance to take into consideration their efficacy following digestion (Sánchez-Rivera et al., 2014; Santos et al., 2019; Zhang et al., 2017).

In vitro methodologies that simulate gastrointestinal procedures are commonly applied to determine food-derived bioactive compounds' bioaccessibility, through simulation of gastric digestion and absorption by intestinal epithelium (Santos et al., 2019). Compared to clinical studies, *in vitro* mechanisms have the advantages of being less costly and labour-intensive, more rapid, and they do not have ethical limitations. Additionally, *in vitro* simulations of human digestion are more suitable for mechanistic studies (Brodkorb et al., 2019). To this end, an extensive range of models, simulating human digestion, and thus capable of predicting bioaccessibility, have been developed (Lucas-González et al., 2018; McClements et al., 2020). *In vitro* simulated gastrointestinal digestion (SGID) methods typically consist of oral, gastric, and small intestinal phases. Taking into account pH, digestion time, digestive enzymes and their concentrations and salt concentrations, these approaches try to mimic *in vivo* physiological conditions (Mackie et al., 2020). Within the field of *in vitro* digestion in food research, a standardised protocol, based on an international consensus, was developed by the COST INFOGEST in 2014 (Minekus et al., 2014). Further, studies in the literature have reported the correlation of the results obtained using the INFOGEST *in vitro* protocol to those obtained using an *in vivo* approach (Bohn et al. 2018, Dupont et al. 2019, Egger et al. 2016).

Significant health benefits of milk are associated with the biologically active peptides encrypted in the sequences of milk proteins (Sánchez & Vázquez, 2017). The main protein fractions found in bovine milk consist of caseins, β-lactoglobulin (β-Lg), α-lactalbumin (α-La), and immunoglobulins (Schlimme & Meisel,1995; de Wit, 1998; Kelly & McDonagh, 2000). Milk proteins, subjected to *in vivo* digestion within the gastrointestinal tract, are hydrolysed into peptides exhibiting novel biological properties, in comparison with the parental, intact protein (Chatterton et al., 2004). However, a variety of the peptides' bioactivities are only exerted following their release from the precursor protein, which is induced by the action of gastrointestinal digestive enzymes or microorganisms (Kekkonen 2009; Korhonen 2009; Pihlanto, 2006). These BAPs have been identified in milk of several species and can be released from both whey proteins and caseins (Korhonen & Pihlanto, 2006). Once they are liberated, their displayed bioactivities include anti-hypertensive, opioid, immunomodulation, and antimicrobial activities (Mohanty et al., 2016; Price et al., 2022).

Milk is the main source of BAPs with angiotensin-converting enzyme (ACE) inhibitory activity reported to date (Martínez-Maqueda, 2012). ACE hydrolyses the inactive peptide angiotensin I into the potent vasoconstrictor angiotensin II. In this way, ACE inhibition plays a key role in blood pressure homeostasis, and ACE inhibitory drugs are prescribed to treat hypertension or other

cardiovascular-related diseases (Acharya et al., 2003). ACE possesses two biologically active substrates: angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) and bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg). The significance of the C-terminal sequences of ACE inhibitory peptides was reported in a study investigating the binding of peptides hippuryl-histidyl-leucine (Hip-His-Leu) and hippuryl-phenylalanine-arginine (Hip-Phe-Arg) whose C-terminal dipeptides are similar to that of angiotensin I and bradykinin, respectively (Hong et al., 1980). These two peptides were demonstrated to have similar binding affinity trends as angiotensin I and bradykinin, showing substrate specificity for ACE. Moreover, the enzyme has specificity for smaller peptides made of up to 12 amino acids and containing positively charged hydrophobic amino acids at the C-terminal end (Hong et al., 1980; Mullally, Meisel, & FitzGerald, 1997; Nakamura et al., 1995). The most favourable structure–function relationship of potent ACE inhibitory di- and tri-peptide sequences also was reported in the literature and includes dipeptides having amino acids with hydrophobic and bulky side chains and tripeptides containing hydrophobic amino acids at the N-terminus, aromatic amino acids at the C-terminus, and positively charged amino acids in the middle (Wu et al., 2006).

Various ACE inhibitory peptides were identified, following enzymatic hydrolysis of milk proteins and/or fermentation with *Lactobacillus* (Hernandez-Ledesma et al., 2014). BAPs from several milk proteins have been successfully generated using digestive enzymes and combinations of different proteinases. Examples include casein-derived peptides RYLGY and AYFYPEL that were isolated after hydrolysis with pepsin. When administered *in vivo*, these peptides were able to reduce blood pressure in spontaneously hypertensive rats (del Mar Contreras et al., 2009). LLF, LVRT, and LQKW also are examples of potent β-Lg derived ACE inhibitory peptides that were hydrolysed with thermolysin (Hernandez-Ledesma et al., 2002). In a series of two papers, the *in vitro* release of BAPs after digestion of bovine whey proteins and milk caseins was investigated by Picariello et al. (2010, 2013). Their research findings indicate that, under gastrointestinal conditions, some ACE inhibitory peptides were released, and were even able to translocate across monolayers of Caco-2 cells. Additionally, other potent ACE inhibitory peptides were derived from human milk following *in vitro* SGID (Hernandez-Ledesma et al., 2007), and from *in vitro* gastro-pancreatic digestion of β-casein from donkey milk (Bidasolo et al., 2012). Furthermore, compared to synthetic drugs, ACE inhibitory peptides derived from whey proteins are believed to have fewer adverse effects, and thus they are safer for consumption (Lee et al., 2010).

In this work, whey was subjected to an *in vitro* SGID procedure that mimics the chemical and physiological conditions of the gastrointestinal tract to process whey proteins, according to the method described by Minekus et al., (2014). A simulation of the absorption in the intestine was then performed, and the peptide fractions were pre-purified by solid phase extraction. The different

fractions were then characterised in terms of their ACE inhibitory activities, and the fractions with the highest activities were analysed with mass spectrometry (MS), then a database search was used to identify the peptide sequences present in the different whey protein hydrolysates. Finally, a molecular docking approach was employed to predict the ability of the newly identified peptide sequences to interact with ACE. The main aim of the work presented in this chapter was to explore whether the combination of the chemical characterization, quantitative structure-activity relationship (QSAR) data and molecular docking will help to identify the BAPs that are potentially responsible for the ACE inhibitory activity measured in the separated milk hydrolysate fractions, without having to apply cumbersome purification methods to isolate and identify such peptides.

4.2 Materials and methods

4.2.1 Materials

Pepsin from porcine gastric mucosa (P 7000), pancreatin from porcine pancreas (P3292), α-Amylase from porcine pancreas (A3176), bile salts (B8756), angiotensin I-converting enzyme from rabbit lung tissue (ACE, A6778), N-[3-(2-Furyl) acryloyl]-Phe-Gly-Gly (FAPGG, F7131) and Captopril (C4042) were obtained from Merck Life Science UK Limited (Dorset, UK). All other chemicals used were obtained from Fisher Scientific UK Ltd (Loughborough, Leicestershire, UK) and were of analytical grade. The ultrafiltration membrane with a molecular weight cut off (MWCO) of 3 kDa (Vivaspin20) was purchased from Sartorius AG (Goettingen, Germany).

4.2.2 Simulated gastrointestinal digestion (SGID)

Whey protein isolate (Volactive® UltraWhey 90) obtained from Volac International Limited (Royston, UK) constituted of 93% proteins was subjected to a SGID procedure according to the method described by Minekus et al., (2014). Briefly, a simulated gastric fluid (SGF) and a simulated intestinal fluid (SIF) were freshly prepared daily from stock solutions and kept at 37°C before their use. These two solutions consisted of salts and simulated digestion fluids. Simulated fluids and stock solutions were prepared with KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2$ and $(NH_4)_2CO_3$ at the same concentrations specified in Minekus et al. (2014). Table 4.1 shows the final concentrations of salts in the digestion mixtures, and Figure 4.1 represents a summary of the digestion method.

Whey protein isolate was directly subjected to the gastric phase, which consists of five parts of whey mixed with four parts of SGF stock electrolyte solution to obtain a final ratio of whey to SGF of 50:50 (v/v), after addition of water and other components of the SGF such as pepsin, $CaCl_2$, and HCl. Ten mL of whey solution (7 mg/mL) was mixed with 7.5 mL of SGF followed by the addition of 1.6 mL of Pepsin (25 000 U/mL) and 5 µL of a stock solution of $CaCl_2(H_2O)$ (44.1 g/L). The pH was adjusted to 3 with 1 M of HCl. Finally, the sample was incubated for 2 hours at 37°C with continuous agitation. The intestinal phase consisted of four parts of SIF electrolyte stock solution mixed with five parts of gastric chyme at a final ratio of gastric chyme to SIF of 50:50 (v/v) after addition of water and other constituents of SIF such as pancreatin, bile salts, $CaCl_2$ and NaOH. Five mL of the digestive enzyme pancreatin (100 U/mL) was added, followed by 2.5 mL of a bile solution (160 mM). Finally, a concentration of 0.33 mM of SIF in the final digestion mixture was achieved with addition of 40 µL of a stock solution of $CaCl_2(H_2O)$ (44.1 g/L). The pH was adjusted to 7 with 1 M of NaOH and the sample was incubated for 2 hours at 37°C with constant agitation. The samples were immediately frozen at −40°C. Three replicate digestions of each whey sample were performed. Table 4.1 summarises the composition of the SGF, and SIF used in the digestion method.

Table 4.1: Preparation of stock solutions of simulated digestion fluids (Minekus et al., 2014).

	Simulated Gastric Fluid (SGF)	**Simulated Intestinal Fluid (SIF)**
Constituent	Concentration ($mmol.L^{-1}$)	Concentration ($mmol.L^{-1}$)
KCl	6.9	6.8
KH_2PO_4	0.9	0.8
$NaHCO_3$	25	85
NaCl	47.2	38.4
$MgCl_2$	0.1	0.33
$(NH_4)_2CO_3$	0.5	-

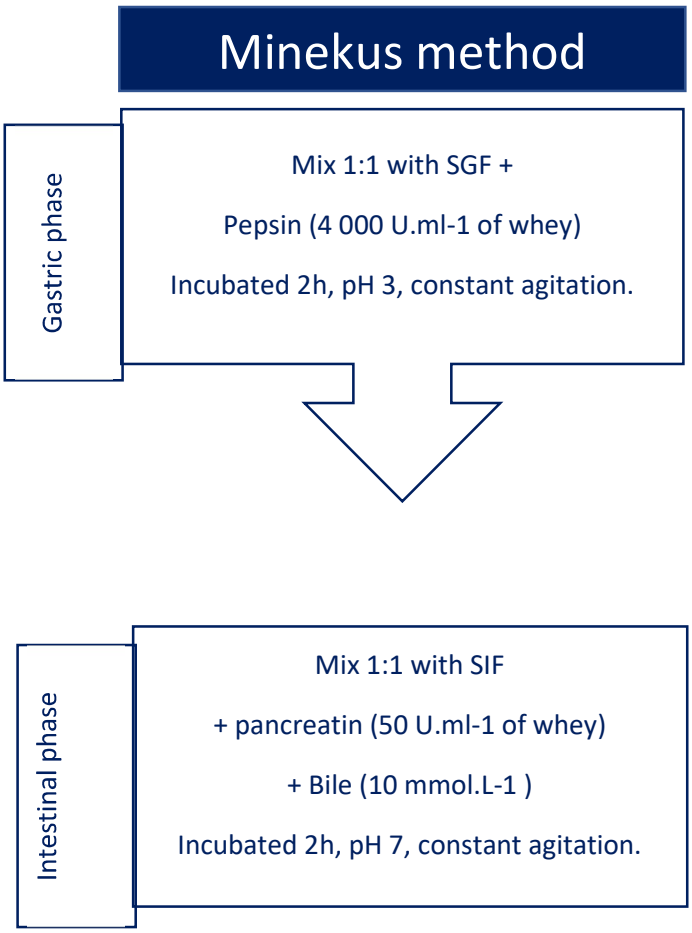


Figure 4.1. Summary of Minekus method (Minekus et al., 2014).

4.2.3 Simulation of intestinal absorption

Membrane technology (ultrafiltration, UF) has been commonly utilised to purify proteins. This method has been demonstrated to successfully fractionate proteins via UF membrane, with various reported advantages, in comparison with the conventional protein separation methods (Emin et al., 2018; Ishak et al., 2017; Jana et al., 2011). The key advantage of UF being the production of a product with low toxicity and a high level of purity (Mayani et al., 2010). To simulate the *in vivo* digestion of whey, a simulation of the absorption in the intestine was performed prior to separation using ultrafiltration with a molecular weight cut off (MWCO) membrane of 3 kDa (Vivaspin20, Sartorius, Goettingen, Germany) (Soleymanzadeh et al., 2019).

4.2.4 Fractionation of crude hydrolysates by solid phase extraction

Solid-phase extraction (SPE) is a commonly used procedure to isolate and chemically separate different compounds in a liquid matrix. This technique utilises solid particles (stationary phase) to separate analytes from a liquid matrix (mobile phase). When the sample is eluted through with SPE columns, non-polar compounds will be adsorbed onto the column. These adsorbed compounds present on the solid phase can be subsequently separated by selective elution using a solvent with a greater affinity for the analytes (Pochiraju, 2018; Lehotay et al., 2000). By providing a high-surface-area active substrate, Octadecyl bonded silica gel (referred to as C18) functions as a reversed-phase adsorbent (Dallas et al., 2015; Meisel et al., 2003).

To perform a pre-purification step of the most active whey-derived ACE inhibitory peptides, fractionation of the whey hydrolysates was carried out by SPE. Sep-Pak Plus C18 cartridges (820 mg of sorbent, 80 µm, 125 Å, 12% carbon loading, end-capping; Waters Corp., Milford, MA, USA) were used with a stepwise gradient of 1% acetic acid in acetonitrile for the selective elution of peptides. Prior to sample loading, cartridges were conditioned with 10 mL of 1% acetic acid in acetonitrile followed by 10 mL of 1% acetic acid in HPLC grade water. One mL of the digested whey samples was then loaded onto the conditioned cartridge and separated into 7 different fractions. Peptides were eluted with a stepwise gradient of 1 mL volumes of 1% acetic acid in 16, 18, 22, 26, 30, 40 and 100% acetonitrile (Ortiz Chao, 2008).

4.2.5 Total protein determination by the bicinchoninic acid assay (BCA) assay

Fractions obtained were further analysed for total protein content, using the BCA method adapted from Smith et al. (1985). This method is sensitive, simple, and tolerant to many detergents and substances known to interfere with the Lowry method, such as buffer salts and ionic detergents. It is based on the Biuret reaction, and it consists of two steps. First, when a protein is in the presence of an alkaline medium containing cupric ions (Cu^{2+}), the blue-coloured copper II ion can form a complex with the peptide bonds of the protein, and Cu^{2+} is reduced to cuprous ion (Cu^{+}). The second step involves the chelation of this complex with two molecules of BCA, and the subsequent formation of a stable purple chromophore with a strong absorbance at 562 nm.

The working reagent was made by mixing 50 parts of Reagent A (a carbonate buffer containing BCA reagent) in 0.1 M NaOH solution with one part of Reagent B (a cupric sulfate solution). Two mL of the BCA working reagent were then added to 0.1 mL of protein sample (fractions obtained from hydrolysates). The samples were then vortexed and incubated for 30 min at 37°C. Finally, the absorbance readings were taken at 562 nm using an Ultrospec 1100 pro UV/Visible spectrophotometer (Amersham Biosciences, Little Chalfont, UK), and a calibration curve of BSA (0.1 – 1 mg/mL) was generated (Figure 4.2). Serial dilutions of BSA were used as standard. Samples were analysed in triplicates, and the average protein concentration is reported.

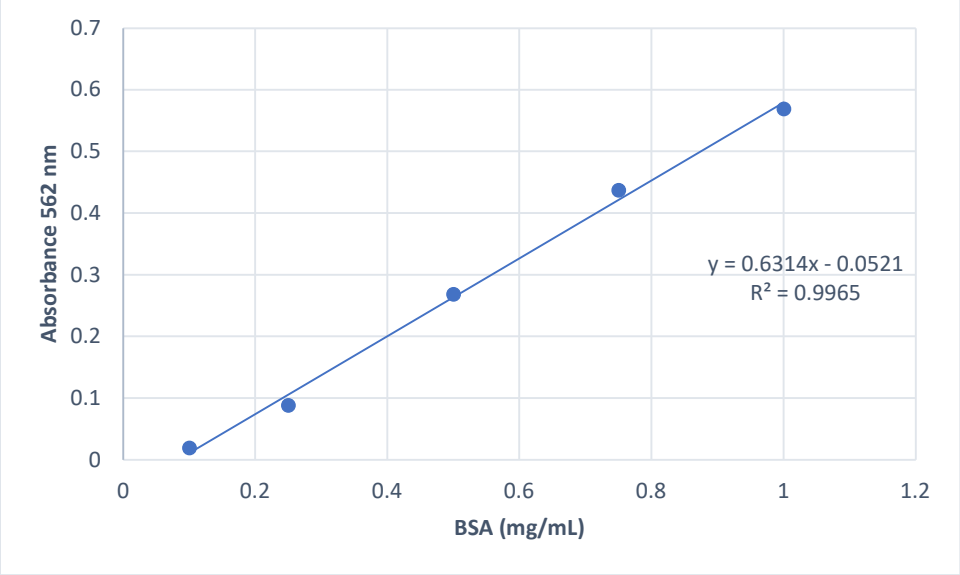


Figure 4.2: BSA standard curve for total protein determination by BCA method.

4.2.6 ACE inhibitory activity assay

To identify the peptide fractions with the highest % of ACE inhibition, assessment of their inhibitory activity was performed using a method originally developed by Holmquist et al. (1979), based on the hydrolysis of the substrate furanacryloyl–prolyl-glycyl glycine (FAPGG), with some modifications (Murray et al., 2004; Lahogue et al., 2010; Vermeirssen et al., 2002; Henda et al., 2013). Volumes in the assay were scaled down to perform the assay using microtiter plates. Briefly, 150 µL of FAPGG (0.88 mM in 50 mM Tris HCl (pH 7.5) containing 300 mM NaCl (assay buffer) was pipetted into a 96 well plate and incubated for 1 minute at 37°C within a Tecan SPARK microplate reader. The substrate solution was then mixed with 10 µL of inhibitor aliquot (peptide fractions, or Captopril in the case of the positive control assay), and the reaction was started by the addition of 10 µL freshly prepared ACE (14.6 mU) in buffer Tris 0.05 M • HCl 5 M • NaCl 0.3 M in 50% glycerol solution. A blank sample was prepared by replacing the inhibitor solution with the assay buffer. The absorbance at 340 nm was recorded every 1 min for 30 minutes, and the slope averaged over a linear interval of 30 minutes was taken as a measurement of the ACE inhibitory activity.

The percentage of ACE inhibition (ACEi) was calculated according to the equation below:

$$ACEi\% = \left(1 - \frac{\rho A_{inhibitor}}{\rho A_{control}}\right) \times 100$$

$ACE_i\%$= Percentage of ACE inhibition

$\rho A_{inhibitor}$ = slope with inhibitor

$\rho A_{control}$= slope with control

The IC_{50} value (protein concentration needed to inhibit the ACE activity by 50%) was determined by linear regression analysis plotting the inverse ACEi% against the inverse concentration of whey protein hydrolysates (mg/mL) in samples.

Captopril, a synthetic ACE-inhibitor, was used as a positive control. A stock solution of Captopril 1mM was prepared in Tris buffer 50 mM • HCl (pH 7.5) with 300 mM of NaCl, and final concentrations of Captopril in this assay were 0.7, 3, 6, 10, and 20 nM (Figure 4.3) (Henda et al., 2013; Vermeirssen et al., 2002). Each sample was tested in triplicate. Additionally, as a measurement of precision, the coefficient variations were calculated. All analyses were performed in triplicate.

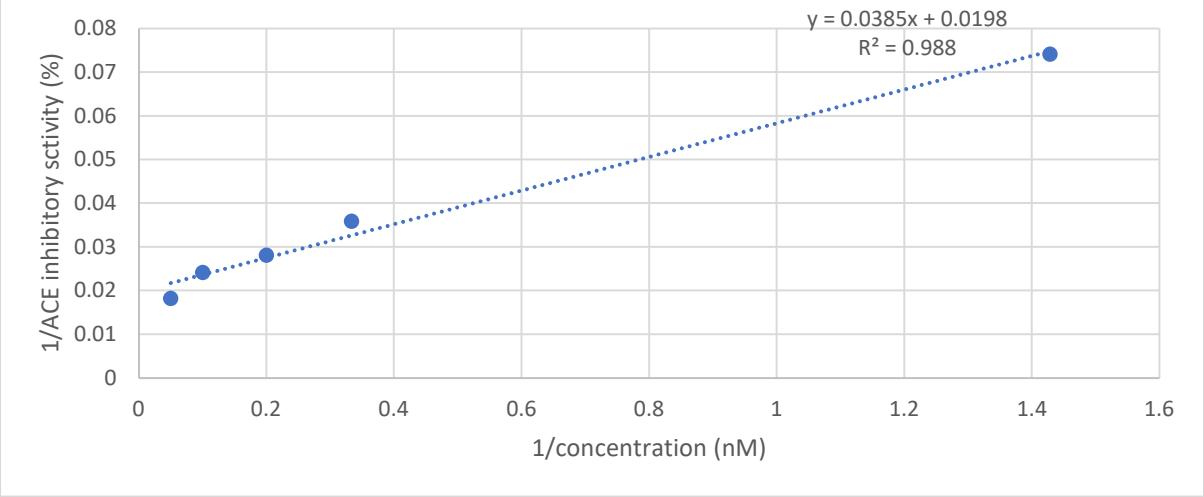


Figure 4.3: Inverse angiotensin-converting enzyme inhibitory (ACEi) activity against the inverse of a range of captopril concentrations.

4.2.7 Mass spectrometry analysis

The mass spectrometry analysis of the different ACE inhibitory peptide fractions obtained after the SPE fractionation was then carried out as described below.

The presented samples (10 µL) were injected onto a column (Ace5 C18 150 x 2.1 mm, 5 µm particles with 100 Å pores). The HPLC equipment was an Accela, and the mobile phases were (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile (all LCMS grade). A gradient was employed; time 0 = 5%B, 5 mins = 10%B, 10 mins = 40%B, 15 mins = 80%B, 15.5 mins = 5%B, 25 mins = 5%B, flow rate 200 ul/min. Column oven was set at 40 °C. The HPLC was directed into a Thermo Scientific LTQ Orbitrap XL. A valve was used so that the first 3 mins of flow was directed to waste, as was the last 7 mins. The instrument was operating a data-dependent acquisition in positive ion mode where scan 1 was a full scan from 85 - 1600 m/z at a resolution of 15,000 and scan 2 was fragmenting the top 3 ions from scan 1, at a resolution of 7,500. A lock mass of 214.089626 (n-butyl benzenesulfonamide, plasticizer) was employed.

Data analysis was performed in BioPharma Finder v5.0 (Thermo Fisher Scientific). BioPharma Finder provides automated tools for the identification of chromatographic components within a protein sample digest. Peptide sequence identification using this data analysis software is based on the evaluation of mass accuracy and the comparison of experimental and predicted MS/MS fragmentation spectra (Vanhinsbergh et al., 2022). The employment of the software Biopharma Finder™ allows for a fast identification of cleavage sites of the whey proteins derived peptides by the digestive enzymes Pepsin and Pancreatin. The software searches for the best identification for each

detected analyte. If found, the application provides the data in a Results table in the following columns: Peptide Sequence, Protein, Delta (ppm), RT (min), and m/z, as well as other parameters. Delta (ppm) displays the difference between the theoretical mass of the identified peptide and the experimental measured mass. The Mass Accuracy (ppm) parameter specifies the maximum mass deviation (ppm) when the theoretical peptide mass is compared to the calculated mass of a particular ion (Thermo Fisher Scientific, 2015). In this study, the value of the Mass Accuracy parameter was selected at 5 ppm to make the results readily interpretable (Table 4.2). Retention time (RT) shows the time after injection at which a compound elutes, and m/z parameter provides the mass-to-charge ratio of ions obtained from molecules (Thermo Fisher Scientific, 2015). The peptide sequences were then uploaded to the Peptide Analyzing Tool (Thermo Fisher Scientific, https://www.thermofisher.com/uk/en/home/life-science/protein-biology/peptides-proteins/custom-peptide-synthesis-services/peptide-analyzing-tool.html) to calculate their hydrophobicity values. Main settings for Biopharma finder processing methods are shown in Table 4.2.

Table 4.2. Main settings for Thermo BioPharma Finder processing method.

Analysis method	**Peptide mapping**
MS Signal Threshold	6
MS Noise	1000000
Signal threshold	6000000
Typical peak width	0.25 min
Maximum peak width	0.5 min
Maximum RT shift	1 min
Mass accuracy	5 ppm
Selected protease	Non-specific
Protease specificity	Low

4.2.8 Molecular docking

4.2.8.1 Docking Validation

To validate the accuracy and the reliability of the docking procedure to be used in this study, the original ligand (extracted from the coordinate files and taken from the Protein Data Bank; PDB code 6F9V was docked into the corresponding crystal structure of the receptor, using the automated docking procedure in the program Surflex-Dock (SFXC) (Jain, 2003), as provided by SYBYL-X2.1. The docked ligand mode and orientation from the docking procedure were compared to that found in the actual crystal structure of the complex using PyMOL (Schrodinger LLC, Version 1.8) and PDBeFold (Accessed: http://www.ebi.ac.uk/msd-srv/ssm/cgi-bin/ssmserver). Following the docking procedure, the root mean square deviation (RMSD) between the docked ligand and the ligand, as found in the crystal structure, was calculated. The success of the docking process depended on whether the value of RMSD between the real and best-scored docked conformations were within the 2 Å grid spacing, used in the docking procedure (Wang et al., 2003), and whether the molecular interactions were replicated. In this case, Sampatrilat, which is a potent dual inhibitor of ACE and neutral endopeptidase, was docked into the human ACE receptor as validation of the docking procedure.

4.2.8.2 Docking procedure

The whey protein-derived peptides Ala-Ile-Pro-Pro-Lys (AIPPK), Ile-Glu-Ser-Pro-Pro-Glu-Ile (IESPPEI), Val-Glu-Leu-Leu-Lys-His-Lys-Pro (VELLKHKP), Thr-Pro-Glu-Val-Asp-Asp-Glu (TPEVDDE), and Pro-Phe-Pro-Gly-Pro-Ile (PFPGPI), identified via MS analysis in section 4.2.7, were used as ligands in separate docking runs. Docking was performed using the docking algorithm Surflex-Dock, as provided in Sybyl-X 2.1. The X-ray crystallographic structure of Sampatrilat-Asp in complex with ACE (PDB code 6F9V, 1.69 Å resolution) retrieved from the protein data bank (PDB) was chosen as the target protein for the docking studies, based on its high-resolution structure co-crystallized with sampatrilat-Asp (Cozier et al., 2018).

The Biopolymer Structure Preparation Tool, with the implemented default settings provided in the SYBYL programme suite, was used to prepare the protein structure for docking; hydrogens were added to the protein structure in idealised geometries, backbone and sidechains were repaired, residues were protonated, sidechain amides and sidechain bumps were fixed, stage minimization was performed, and all water and any ligand molecules were removed.

The three-dimensional (3D) structure of each peptide ligand was constructed, using the "Build Protein" tool, as provided in Sybyl-X. Once constructed, partial charges were assigned to each atom of each molecule, using the Merck Molecular Force Field (MMFF94). Localized energy minimizations were then performed, and the final structure for each ligand in its lowest energy conformation was used for subsequent docking experiments. The resulting 3D coordinate files were converted to a MOL2 format for subsequent use in Surflex-Dock experiments, as provided in the SYBYL-X 2.0 software suite.

Surflex-Dock is a search algorithm that utilizes an empirically derived scoring function whose parameters are based on protein-ligand complexes of known affinities and structures. This method employs a "protomol", which is an idealized active site, as a target to generate presumed poses of molecules or molecular fragments. The protomol is employed as a mimic of the ideal interactions made by a perfect ligand to the active site of the protein. This molecular similarity-based alignment allows for optimization of potentially favorable molecular interactions, such as those defined by van der Waals forces and hydrogen bonds. In the present work, the protomol was defined by optimizing the threshold and bloat values to 0.5 and 0, respectively, to create a protomol that adequately described the binding pocket of interest. The extent of the protomol and its degree of coverage of an active site are controlled by these two parameters: the threshold value indicates the amount of buried-ness for the primary volume used to generate the protomol, and the bloat parameter determines the number of Ångstroms by which the search grid, beyond that primary volume, should be expanded. It is generally better to err on the side of a small protomol than on a protomol that is too large (Sharma et al., 2016). All parameters within the docking suite were left as the default values as established by the software (Ai et al., 2011; Lan et al., 2011). Each peptide was then individually docked into the protomol site, using the "Docking Suite" application, as provided in the SYBYL programme suite. For further visualization and analysis, Maestro (Schrödinger Release 2021-2: Maestro, Schrödinger, LLC, New York, NY, USA, 2021) was used for the characterization and identification of the hydrogen bonds and salt bridge interactions established between the peptides and residues at the ACE active site. Figures were also generated using the software Maestro (Figures 4.5– 4.9). Molecular interactions, for the docking results, were reported according to the Sampatrilat (PDB code 6F9V) amino acid sequence numbering; for comparisons between different sequence numbering in studies referred here (Table 4.3).

Table 4.3. Comparisons between Captopril (PDB code 1O86) and Sampatrilat (PDB code 6F9V) sequence numbering.

Captopril (PDB Code 1O86) Amino Acid Sequence Numbering (Natesh et al., 2004)	Sampatrilat (PDB Code 6F9V) Amino Acid Sequence Numbering (Cozier et al., 2018)
Glu 162	Asp 140
Gln 281	Gln 259
His 353	His 331
Ala 354	Ala 332
Val 380	Thr 358
Glu 384	Glu 362
His 387	His 365
Phe 391	Tyr 369
Glu 403	Arg 381
Glu 411	Glu 389
Lys 454	Lys 432
Lys 511	Lys 489
His 513	His 491
Tyr 520	Tyr 498
Arg 522	Arg 500
Tyr 523	Tyr 501

4.3 Results and discussion

Whey was subjected to SGID according to the methodology developed by Minekus et al., (2014). Whey protein hydrolysates were then subjected to an ultrafiltration membrane with MWCO of 3 kDa, since it has been demonstrated that the most potent ACE inhibitors from digested milk are frequently short peptides (Gómez-Ruiz et al., 2004; Hernández-Ledesma et al., 2007, Tagliazucchi et al., 2016). Additionally, peptides with higher ACE inhibitory activities are commonly found in samples containing peptides of masses <3 kDa and are most likely to display *in vivo* anti-hypertensive activities (Gómez-Ruiz et al, 2007). Other researchers have also reported that the highest ACE inhibitory activity of milk bioactive peptides was found in the post-pancreatic <3 kDa digest (Tagliazucchi et al., 2016).

4.3.1 Identification and selection of peptides with high ACE inhibitory activity

4.3.1.1 Fractionation of digested whey and ACE inhibitory activity determination

Following ultrafiltration, whey-derived peptides with a molecular weight lower than 3 kDa were fractionated based on their polarity through SPE. Seven fractions (numbered 1–7) were then collected and were further analysed in terms of protein content and ACE inhibitory activity. To identify the peptide fractions with the highest % ACE inhibition, assessment of their inhibitory activity was performed as described in section 4.2.6. The ACE inhibitory IC_{50} value reported for Captopril ranges from 0.025 – 0.0015 µM when using FAPGG for assay of ACE activity (Shalaby et al. 2006). In our hands, the IC_{50} for Captopril was 1.91 nM (Figure 4.3, Also see Appendix, Table 4.A.1). This value corresponds well with the range of values reported in the literature.

The results show that Fraction 4 (F4) and Fraction 7 (F7), eluted with 1% acetic acid in 26% acetonitrile, and 1% acetic acid in 100% of acetonitrile, had the highest ACE inhibitory activities (%) of 53.3, and 73.3 %, with a 50% inhibition concentration (IC_{50}) of 0.09 and 0.14 mg/mL, respectively (Table 4.4). The coefficient of variations (CV) of 2.7%, and 2.3 %, for F4 and F7, respectively, were also calculated (See Appendix, Table 4.A.2.). The IC_{50} value of these two fractions was determined by the regression equation fitting from the ACE inhibitory activities at different peptide concentrations. The ACE inhibitory potency of BAPs derived from whey hydrolysates has been reported previously (Ibrahim et al., 2017; Raikos et al., 2019). However, the use of various methods and their subsequent modifications to assess ACE inhibitory activity makes it quite difficult to directly compare IC_{50} values obtained from different studies; as some reports do not calculate the IC_{50} value of Captoptil, or other ACE inhibitory standards, nor do they include how many enzyme units were utilised in the ACE inhibition assay (Murray et al., 2004). In this respect, the spectrophotometric assay developed by Cushman and Cheung (1971), and which is commonly used to assess ACE inhibitory activity of hydrolysates, was shown to overestimate the amount of substrate released during ACE-catalysed reactions (Wu et al., 2002). Further, previous studies have shown that the IC_{50} values of ACE inhibitory peptides with the same amino acid sequences were significantly different, and contingent upon experimental conditions and/or different methods employed (Iroyukifujita et al., 2000; Nakano et al., 2006; Wu et al., 2006). Although the IC_{50} values of both fractions F4 and F7 were higher than those obtained for anti-hypertensive drugs, such as Captopril (Table 4.4, Walstra, 1999), the whey-derived ACE inhibitory peptides present in F4 and F7 are naturally occurring, compared to synthetic ACE inhibitory drugs which were reported to cause adverse side effects (Tabacova & Kimmel, 2001). Additionally, some ACE inhibitors were shown to exhibit better anti-hypertensive properties *in vivo*

in comparison with their *in vitro* inhibitory activities, due to the natural inhibitors' slower degradation, in contrast with drug compounds (Vermeissen et al., 2004).

Additionally, Fractions F1, F2, and F3 eluted with 1% acetic acid in 16% of acetonitrile, 1% acetic acid in 18% acetonitrile, and 1% acetic acid in 22% of acetonitrile had ACE inhibitory activities of 44.8, 38.1, and 34.4%, at concentrations of 0.11, 0.14, and 0.14 mg/mL, respectively. Fractions 5 and 6 eluted with 1% acetic acid in 30% acetonitrile, and 1% acetic acid 40% acetonitrile, respectively, exerted a weak or non-detectable ACE inhibitory activity, with F6 having 6.7%, and F5 showing no ACE inhibitory activity. These fractions might contain a mix of unhydrolyzed proteins, consequently leading to no BAP being released, which has also been reported by Yea et al., (2014).

Table 4.4. ACE inhibitory activity (%) of whey-derived peptide fractions, with their corresponding acetonitrile (%) and protein concentration (mg/mL) values.

Peptide fraction	*Acetonitrile gradient (%)	ACE inihibitory activity (%)	Protein Concentration (mg/mL)
F1	16	44.8	0.11
F2	18	38.1	0.14
F3	22	34.4	0.14
F4	26	53.3	0.12 (**IC_{50}= 0.09)
F5	30	-6.7	0.14
F6	40	6.7	0.21
F7	100	73.3	1.21 (**IC_{50}= 0.14)

*For elution

**IC_{50} is defined as the concentration of inhibitory peptide required to inhibit 50% of the ACE activity.

The fractionation of crude hydrolysates was carried out utilising a six-step acetonitrile gradient to perform selective elution of analytes present in the samples. Reverse phase SPE separates compounds based on their hydrophobicity, with the most polar compounds eluting first, and most hydrophobic compounds eluting last. The activity of the whey-derived peptides in fractions 1, 2, 3, 4, 7 depends on their inherent amino acid composition and sequence (Dallas et al., 2015; Meisel et al., 2003). It is accepted that the presence of hydrophobic amino acids in BAPs is of great significance in terms of inhibiting ACE activity. This is due to the stable interaction between ACE and the peptides (Acquah et

al., 2018; Siow & Gan 2013). Properties of BAPs derived from milk protein hydrolysates were reviewed and compiled by Fitzgerald & Meisel (2003). Previous data on the relationship between ACE inhibitory peptides and their structure-activity relationship have shown that increased side chain hydrophobicity of amino acid residues enhances inhibitory potential against ACE (Martens & Martens, 2000). Another group of researchers employed quantitative QSAR modelling on ACE inhibitory peptides derived from milk proteins. In this study, a correlation between structural properties such as increased hydrophobicity and ACE inhibition of peptides containing up to six amino acids was demonstrated (Pripp, et al., 2004). This correlation has also been reported in other more recent studies where the presence of hydrophobic amino acids at the C-terminus enhanced ACE inhibition ability of BAPs (Fu et al., 2016; Xu et al., 2021). According to the results of the *in vitro* ACE inhibitory activity assay performed in this study, F7 had the highest ACE inhibitory activity (%) of 73.3% (Table 4.4). These results are notable since F7 is the fraction that was eluted last, and thus it is the most hydrophobic fraction. Thus, this corroborates the observations regarding the impact of the amino acid residues' hydrophobicity on the inhibitory potential of peptides against ACE.

Furthermore, in the work conducted by Xu et al., (2021), among the 11 peptides screened according to hydrophobicity values, two BAPs having the same ratio of hydrophobic amino acid residues were found to have different IC_{50} values. This shows that, in addition to the proportion of hydrophobic amino acid residues, there are other factors that impact ACE inhibitory activity of BAPs (Katayama et al. 2007; Vasquez-Villanueva et al. 2019), which might explain the relatively high ACE inhibitory activities in F1 - F4 of 44.8, 38.1, 34.3, and 53.3%, respectively, when compared to F5 and F6, both of which possess a higher quantity of hydrophobic amino acids. In terms of favourable structure–function relationships for high ACE inhibitory activity, dipeptides including bulky and hydrophobic amino acids are more potent whereas tripeptides, having aromatic amino acids at the C-terminus end, positively charged amino acids in the middle and hydrophobic amino acids at the N-terminus end, are most potent. Additionally, shorter peptides, up to 12 amino acids at the carboxyl end, are more likely to interact with ACE (Welderufael et al., 2012). Kobayashi et al. (2008) investigated the effects of aromatic amino acids in the third position of a tripeptide on ACE inhibitory activity. They found that the difference in the ACE inhibitory activity between the bioactive peptides (Ile-Lys-Trp, Leu-Lys-Trp, Ile-Lys-Tyr, and Leu-Lys-Phe) resulted from the aromatic amino acids W (tryptophan), Y (tyrosine), and F (phenylalanine). The highest inhibitory activity was presented by Leu-Lys-Trp, with the largest amino acid in the C-terminal. Accordingly, ACE inhibitory activity is affected by the size of the amino acid, as well as its hydrophobicity (Kobayashi et al., 2008).

The authors also examined the effects of the charged amino acid in the second position of a tripeptide and reported that to obtain a high inhibitory activity, it was essential to have a positively charged

residue next to an aromatic residue (Kobayashi et al. 2008). They also highlighted that a tripeptide sequence consisting of either I or L, positively charged amino acids, and aromatic amino acids is likely to have high ACE inhibitory activity. The charged amino acid takes part in binding to ACE while the bulky aromatic amino acid prevents access between substrates and the active site of ACE (Kobayshi et al., 2008). Some studies also have indicated that tripeptides show higher ACE inhibitory activity, and the C terminus end of the tripeptides substantially affects binding to ACE (Pan et al., 2011). In the current study, F7 had the highest ratio of hydrophobic amino acids resulting in the highest ACE inhibitory activity (73.3%), compared to other fractions. However, other structural differences of whey-derived peptides may have affected the ACE inhibitory activities of the first four fractions, despite containing smaller proportions of hydrophobic amino acids. Hence why, it was of utmost importance to identify the BAP sequences present in the fractions.

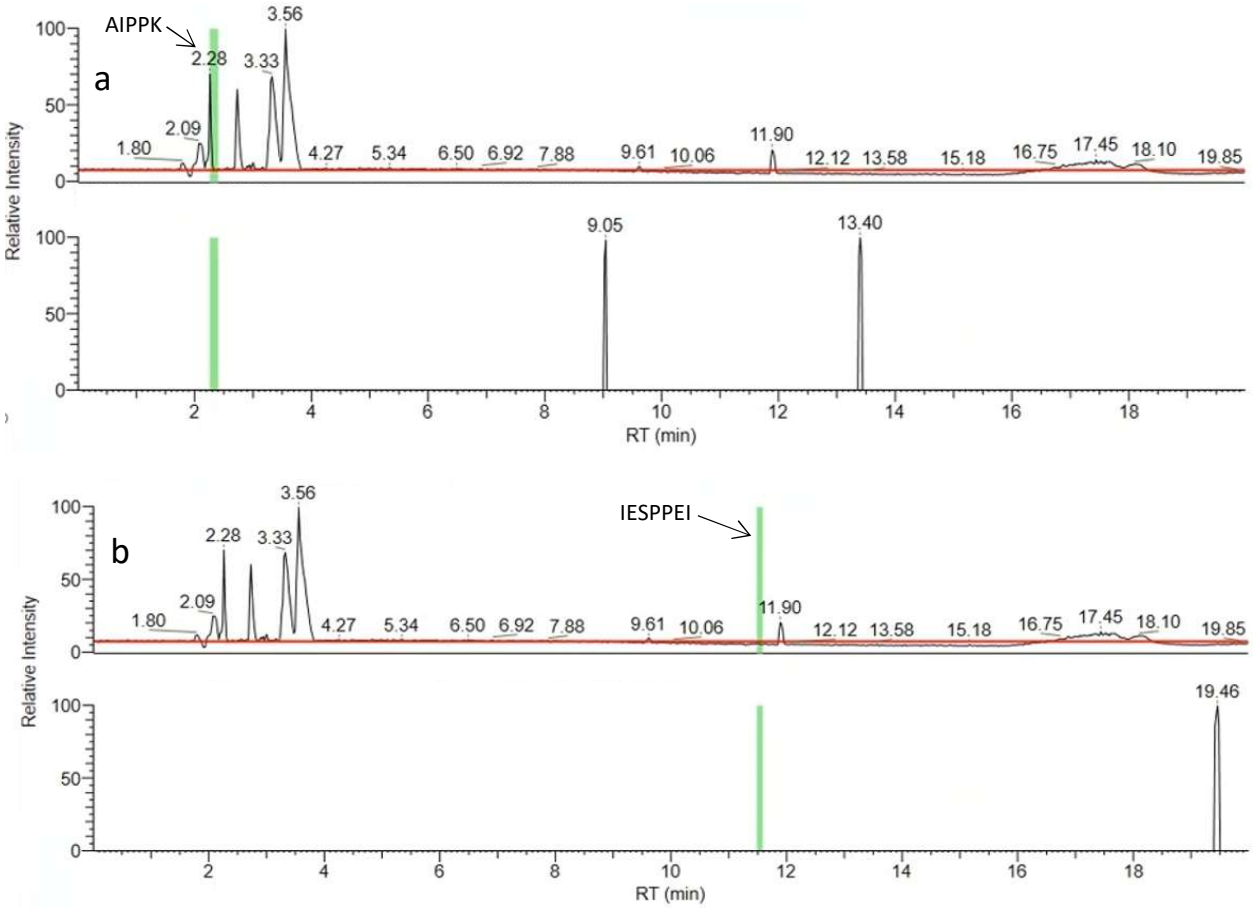
4.3.1.2. MS determination of peptide sequences

Fractions (1–7) were subsequently subjected to LC-MS analysis under the conditions outlined in Section 4.2.7 for peptide sequence identification. The software Biopharma Finder acquisition conditions (Table 4.2) have been applied to the whey-derived peptides in the various fractions with MW up to 3 kDa and allowed for identification of parent proteins and peptide sequences (Table 4.5). The base peak chromatograms obtained for the various fractions are also shown in Figure 4.4.

Table 4.5. List of whey-derived peptide sequences identified using the software Biopharma Finder™, including their corresponding parent protein, their hydrophobicity, retention time (RT), m/z, accuracy expressed as parts per million, and the fractions in which the BAPs were found.

Peptide sequence	Protein	Hydrophobicity*	Delta (ppm)	RT (min)	*m/z*	Fractions**
AIPPK	κ-Casein	6.71	-0.21	2.34	263.17	4,7
IESPPEI	κ-Casein	16.36	0.61	11.55	784.4	4,5,7
VELLKHKP	Bovine Serum Albumin (BSA)	18.05	-0.31	14.82	963.9	7
TPEVDDE	β-Lactoglobulin (β-LG)	6.16	0.2	9.16	804.32	2,3
PFPGPI	β-casein	22.60	0.25	12.47	627.35	7

*Value obtained using Peptide Analyzing Tool (Thermo Fisher Scientific, https://www.thermofisher.com/uk/en/home/life-science/protein-biology/peptides-proteins/custom-peptide-synthesis-services/peptide-analyzing-tool.html)
** Peptide fractions obtained from SPE.



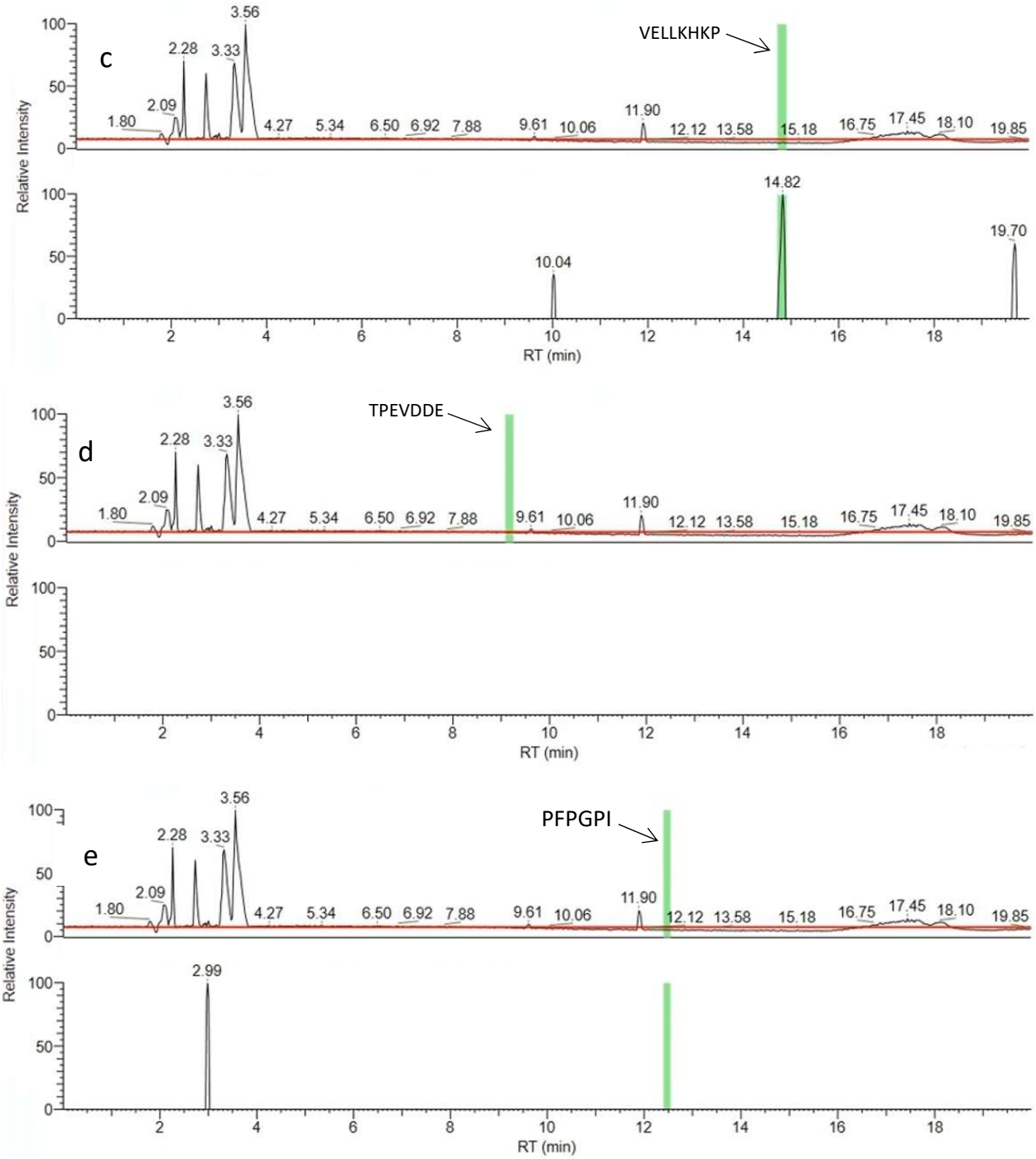


Figure 4.4 Base peak chromatograms obtained for the whey-derived peptides. a) Peptide AIPPK b) Peptide IESPPEI c) Peptide VELLKHKP d) Peptide TPEVDDE e) Peptide PFPGPI

In this work, F (1–7) were obtained from hydrolysates produced from β-Lg, BSA, and casein-derived peptides in sweet whey (Table 4.5). The chemical characterisation of each fraction was carried out using MS, to identify the main peptides contributing to the ACE inhibitory activity measured in these fractions. In this study, as in other reported works, a predictable overlap of peptide sequences present in the fractions was found. This is probably due to the small differences in the percentage of

acetonitrile in the gradient solutions (Minervini et al., 2003) in F(1-4). MS analysis of whey-derived peptides in each fraction confirmed the presence of five peptide sequences identified: i) AIPPK eluted at 2.34 min (Figure 4.4.a), with a higher proportion found in F7 compared with F(4-6) ii) IESPPEI eluted at 11.55 min (Figure 4.4.b), with a higher proportion found in F5 compared with F4 and F7 iii) VELLKHKP eluted at 14.82 min (Figure 4.4.c), largely present in F7 iv) TPEVDDE eluted at 9.16 min (Figure 4.4.d), a higher proportion found in F2, compared with F3 and F4 (Figure 4.4.e), v) PFPGPI eluted at 12.47 min (Figure 4.4.e), with a higher proportion found in F7, compared with F6.

4.3.1.3. Structure-function relationship of ACE inhibitory peptides

Although QSARs have been established for ACE inhibitory peptides, these could not solely be used to predict the ACE inhibitory activity of peptide sequences (Pripp et al., 2005; Wu et al., 2006). Pripp et al., (2004) developed a QSAR modelling for ACE inhibitory peptides derived from milk proteins formed by up to six amino acids. In this work, they demonstrated that increased side chain hydrophobicity at the C-terminal enhanced the peptides' ACE inhibitory activities. The hexapeptide PFPGPI, derived from β-casein, and present in F7, the fraction with the highest ACE inhibitory activity of 73.3% (Tables 4.4 and 4.5, Figure 4.4), has three hydrophobic amino acid residues (Isoleucine, Proline, Glycine) at the C-Terminal, as well as the highest estimated hydrophobicity value of 22.60 (Table 4.5). These results are in agreement with the observations by Pripp et al., 2004. Additionally, PFPGPI has a very similar peptide sequence to another casein-derived hexapeptide LVYPFP reported by Welderufael et al. (2012), and also was found to be a potent ACE inhibitor with IC_{50}= 97 µg/mL (Gonzalez-Gonzalez et al., 2013), a value slightly higher than the IC_{50} of 140 µg/mL measured for F7 in the current study. PFPGPI also has a very similar peptide sequence to LVYPFPGP, an ACE inhibitory peptide derived from β-casein and reported in goat sodium caseinate hydrolysates (Minervini et al., 2003), as well as to the peptide sequence VYPFPGPIPN, a potent ACE inhibitor, with an IC_{50} value of 14.8 µM (Saito et al., 2000). Various studies have been published regarding the ACE inhibitory potential of casein-derived peptides (FitzGerald and Meisel, 2000). VPP and IPP, the two most potent ACE inhibitors found in digested milk, were generated from casein (Nakamura et al., 1995). Further, Gobbetti et al (2000) established that the most potent ACE inhibitory peptides were derived from β-casein fragments.

Peptide AIPPK, present in F4 and F7, possesses a charged amino acid (Lysine, K) at the C-Terminal (Tables 4.4 and 4.5), which suggests that there might be other structural factors contributing to the ACE inhibitory potential of peptides, apart from hydrophobicity at the C-Terminal. In fact, positively charged amino acids, such as K at the C-terminal sequences were shown to contribute towards

inhibition of ACE activity (Bidasolo et al., 2012; Cheung et al., 1980). When caseinomacropeptide Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn (MAIPPKKN) loses the amino acid asparagine from the C-terminal sequence, this results in Met-Ala-Ile-Pro-Pro-Lys-Lys (MAIPPKK), a peptide known for its vasodilatory effect, leading to a significant blood pressure lowering effect at a dose level of 10 mg/kg (Miguel et al., 2007). κ-Casein derived AIPPK could represent a fragment of vasodilatory MAIPPKK. Also, like other well-characterised ACE inhibitory peptides, peptide AIPPK has other structural features of ACE inhibitors, for example, the presence of charged amino acids followed by hydrophobic amino acid residues at the C-terminal (Vermeirssen et al., 2004). Additionally, casein-derived peptide IPP has been recognized as the most potent ACE inhibitor from milk proteins with an IC_{50} of 5 μM (1.6 μg/mL) (Nakamura et al.,1995). As IPP could be a fragment of the casein-derived peptide AIPPK present in F4 and F7, this could also be one of the main contributors to the highest ACE inhibitory activities measured in F4 and F7.

All whey-derived peptides identified have some structural features of ACE inhibitors. AIPPK, IESPPEI, VELKHKP, TPEVDDE, PFPGPI all contain charged or hydrophobic amino acids at the two penultimate sequences at the end of the C-terminal (Gobbetti et al., 2000). Almost all these major whey-derived peptides also contain either hydrophobic or charged amino acids at one of the three C-terminal sequences (AIPKK, IESPPEI, VELKHKP, and PFPGPI). F7 is the fraction with the highest estimated hydrophobicity value of 22.60 (Table 4.5), and which was eluted with 100% acetonitrile, also making it the fraction most likely to have the highest amount of hydrophobic amino acids. Interestingly, F7 appears to contain the highest amount of BAPs identified: AIPPK, IESPPEI, VELLKHKP, and PFPGPI, which could all be contributing to its high ACE inhibitory activity. These results corroborate observations reported in the literature, whereby an increased ratio of hydrophobic amino acid residues enhances ACE inhibitory activity of anti-hypertensive peptides (Katayama et al. 2007; Vasquez-Villanueva et al. 2019).

Furthermore, the three amino acids at the N-terminal sequence of β-Lg derived TPEVDDE were similar to the ACE inhibitory peptide KPTPEGDLEI, also derived from β-Lg (Table 4.5) (Welderufael et al., 2012). Additionally, both peptides TPEVDDE and KPTPEGDLEI possess further structural similarities which include the presence of a hydrophobic amino acid at the 5th position from the N-terminal, and both possess an aspartic acid at the 6th position from the N-terminal. As TPEVDDE was the only peptide found in F2 and F3, it is likely to be the main contributor towards the ACE inhibitory activities observed in these two fractions, which is in agreement with these findings.

Sagardia et al., (2013) developed a new QSAR model for ACE inhibitory oligopeptides. They reported that the tripeptide residues at the carboxyl end dominate in competitive binding to the active site of

ACE (Hernández-Ledesma et al., 2011). Proline residues, aromatic amino acids (W, F), and branched-chain aliphatic amino acids (L, I, V) at the C-terminus were shown to contribute to the increase in the peptides' ACE inhibitory potential (Aluko 2015; Hernández-Ledesma et al., 2011; Kapel et al. 2006; Pan et al., 2011). Due to proline's rigid ring structure, which may lock the carboxyl group into a conformation that is favourable for interaction with the active site of ACE, the presence of the amino acid proline at the C-terminal of a peptide is particularly important for ACE inhibitory potency (Cushman et al., 1977). The peptide VELKHKP contains a proline residue at the C-terminal. VELKHKP was only found in F7, suggesting that the peptide is likely to be contributing to the ACE inhibitory activity measured in F7.

Furthermore, studies showed that amino acid residues at the N-terminal also had a significant effect on ACE inhibitory activity (García-Tejedor et al. 2015; Ishiguro et al. 2012; Li et al. 2016). In fact, the ACE inhibitory activity of peptides was found to be positively correlated with the presence of aliphatic amino acids (V, L, G, I, A) at the N-terminal. Such observations are consistent with the results obtained in this work, whereby peptides AIPPK and VELKHKP all contain aliphatic amino acid residues at their N-terminal and were present in F4 and F7, the fractions with the highest ACE inhibitory activities. Based on this consideration, this consolidates our hypothesis regarding the contribution of

i) Peptide AIPPK towards the ACE inhibitory activity measured in F4 and F7
ii) Peptide VELKHKP towards the ACE inhibitory activity of F7.

As IESPPEI was found in F4, F5 and F7, and since the ACE inhibitory activity measured in F5 was -6.7%, there are two hypotheses: either IESPPEI is not exhibiting any ACE inhibitory activity, or it could be acting synergistically with other peptides present in the fraction.

4.3.2. Molecular docking of selected peptide sequences in fractions with high ACE inhibitory activity

In this present work, molecular docking was conducted to elucidate the potential molecular interactions between the whey-protein derived peptide sequences, identified via MS analysis, and specific amino acids at the binding site of human ACE. The peptide sequences were docked into the binding site of the human ACE, using the X ray crystallographic structure of the human ACE receptor (PDB code 6F9V). The extracted co-crystallized ligand, Sampatrilat (Cozier et al., 2018), was first re-docked into the prepared protein to be used for docking to validate the docking procedure. The

RMSD between the docked conformation, as generated by the program PyMol, and the native co-crystallized ligand conformation was 0.7 Å, which was well within the 2 Å grid spacing used in the docking procedure, demonstrating that the docking method to be used was valid and reliable. Additionally, the interactions between the docked ligand and the prepared target receptor mimicked those observed in the protein crystal structure (PDB code 6F9V). Following validation, the human ACE receptor (PDB code 6F9V) was used as the target molecule for docking the peptide sequences of interest into its active site.

Hydrogen bonds are a significant factor that contribute to the specificity and stability of protein-ligand interactions. Figures 4.5 – 4.9 and Table 4.6 show the hydrogen bond interactions associated with each ligand and the surrounding ACE active site residues. Peptide AIPPK formed 5 hydrogen bonds with the ACE residues: His 331, Glu 389, Lys 489, Tyr 498, Tyr 501 (Figure 4.5, Table 4.6). VELLKHKP formed two hydrogen bonds with residues His 331 and Glu 362 (Figure 4.7, Table 4.6). TPEVDDE formed four hydrogen bonds with: Thr 358, Tyr 369, His 491, and Tyr 498 (Figure 4.8, Table 4.6). As for the peptide PFPGPI, four hydrogen bonds were formed with residues Ala 332, His 365, Lys 489, and His 491 (Figure 4.9, Table 4.6). It is interesting to note that several peptides had some H bonds in common: His 331 formed H bonds with two of the peptides, AIPPK and VELLKHKP; Lys 489 with AIPPK and PFPGPI; Tyr 498 with AIPPK and TPEVDDE; His 491 with TPEVDDE and PFPGPI.

Additionally, peptides AIPPK, TPEVDDE and PFPGPI formed one salt-bridge interaction with Glu 389 (Figure 4.5, 4.8, 4.9 and Table 4.6), whereas IESPPEI formed only salt bridge interactions with Arg 383, Lys 432, and Arg 500 (Figure 4.6, Table 4.6). As for VELLKHKP, two salt-bridge interactions were formed with Glu 362, and Lys 489, in common with TPEVDDE (Figures 4.7 and 4.8, Table 4.6).

Table 4.6. Docking results of AIPPK, IESPPEI, VELLKHKP, TPEVDDE, and PFPGPI.

Protein 69FV		**Ligand AIPPK**	
Residue	**Atom Name**	**Interaction Type**	**Distance (Å)**
CD2 His 331	O (Pro)	Hydrogen bond	2.5
OE1 Glu 389	NH3+ (Ala)	Hydrogen bond	1.5
OE2 Glu 389	NH3+ (Ala)	Salt bridge	3.3
NZ Lys 489	O (Lys)	Hydrogen bond	1.9
OH Tyr 498	O (Lys)	Hydrogen bond	1.8
OH Tyr 501	O (Pro)	Hydrogen bond	1.9
		Ligand IESPPEI	
O Arg 383	O- (Glu)	Salt bridge	4.9
NZ Lys 432	O- (Glu)	Salt bridge	4.2
NH1 Arg 500	O- (Glu)	Salt bridge	2.9
		Ligand VELLKHKP	
NE2 His 331	O (Glu)	Hydrogen bond	2.2
OE2 Glu 362	NH (Leu)	Hydrogen bond	2.0
OE2 Glu 362	NH3+ (Val)	Salt bridge	4.9
NZ Lys 489	O- (Glu)	Salt bridge	4.4
		Ligand TPEVDDE	
CG2 Thr 358	O- (Asp)	Hydrogen bond	1.9
OH Tyr 369	OH (Thr)	Hydrogen bond	1.9
OE2 Glu 389	NH3+ (Thr)	Salt bridge	3.4
NZ Lys 489	O- (Asp)	Salt bridge	2.8
NE2 His 491	O (Glu)	Hydrogen bond	2.0

OH Tyr 498	O- (Asp)	Hydrogen bond	1.7
		Ligand PFPGPI	
O Ala 332	NH (Gly)	Hydrogen bond	1.9
NE2 His 365	NH (Phe)	Hydrogen bond	1.6
OE1 Glu 389	N+H2 (Pro)	Salt bridge	3.7
NZ Lys 489	O (Ile)	Hydrogen bond	1.9
NE2 His 491	O (Pro)	Hydrogen bond	2.5

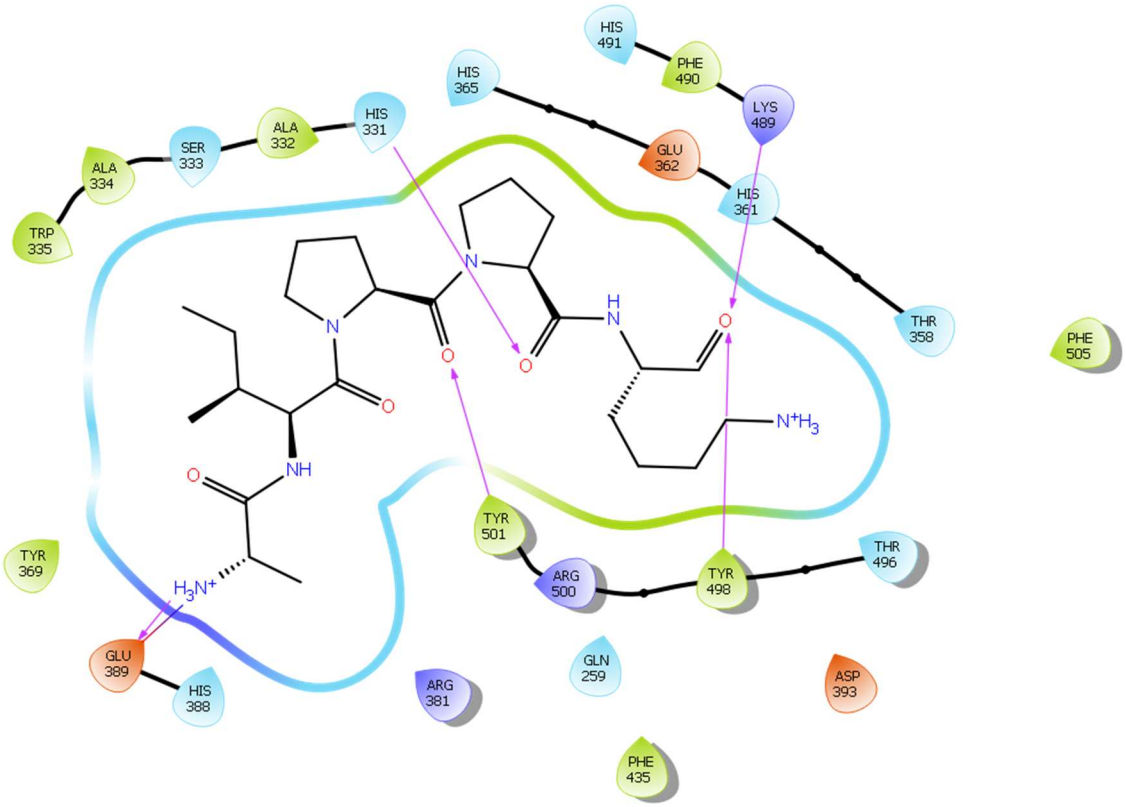


Figure 4.5. Docking results of the peptide AIPPK in the active site of human angiotensin I-converting enzyme (ACE). AIPPK is represented in black, interactions of human ACE active site residues with the peptide are indicated by arrows of different colours, with purple representing hydrogen bond interactions and blue arrows representing salt bridge interactions.

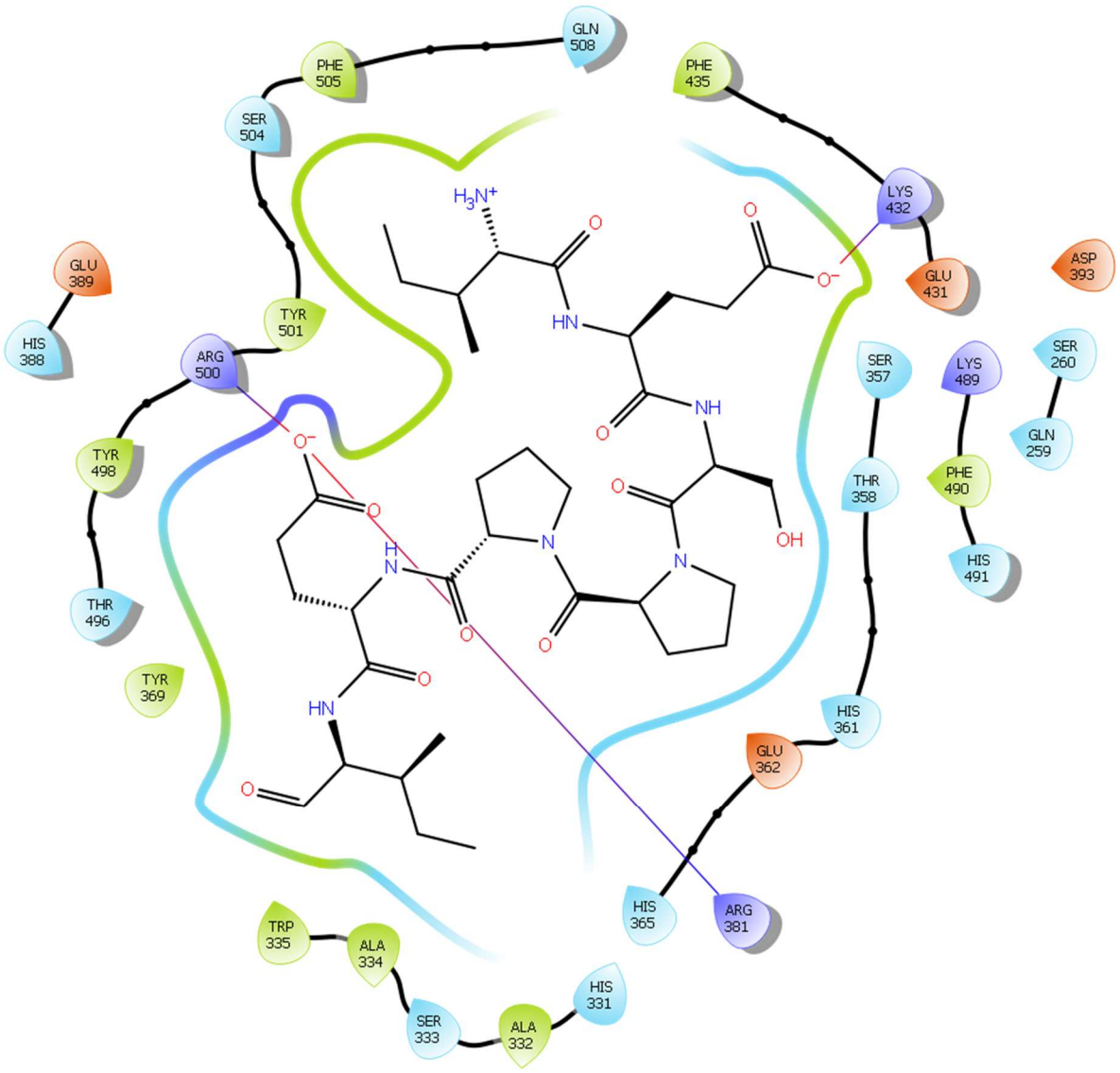


Figure 4.6. Docking results of the peptide IESPPEI in the active site of human angiotensin I-converting enzyme (ACE). IESPPEI is represented in black, interactions of human ACE active site residues with the peptide are indicated by arrows of different colours, with purple representing hydrogen bond interactions and blue arrows representing salt bridge interactions.

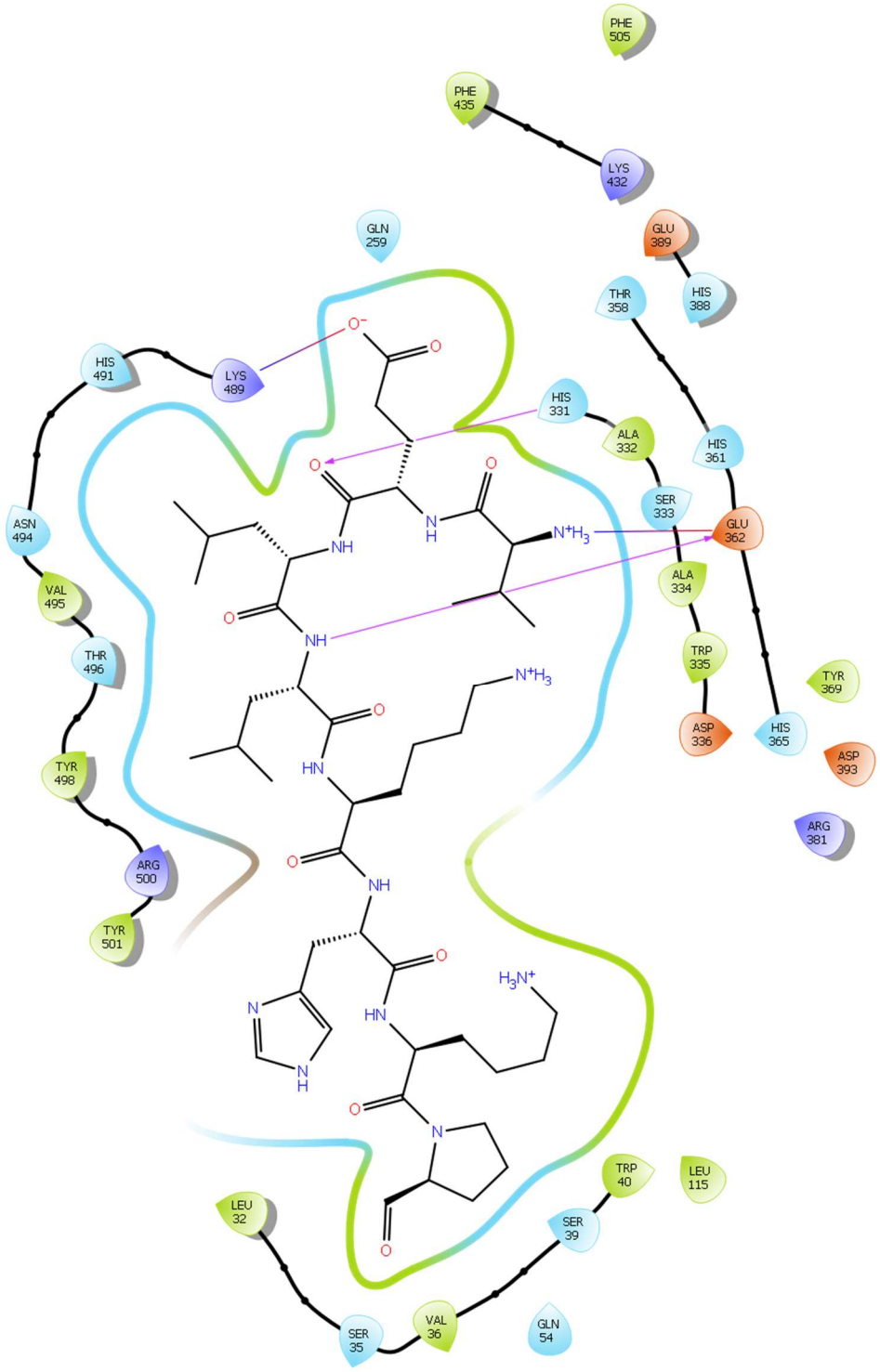


Figure 4.7. Docking results of the peptide VELLKHKP in the active site of human angiotensin I-converting enzyme (ACE). VELLKHKP is represented in black, interactions of human ACE active site residues with the peptide are indicated by arrows of different colours, with purple representing hydrogen bond interactions and blue arrows representing salt bridge interactions.

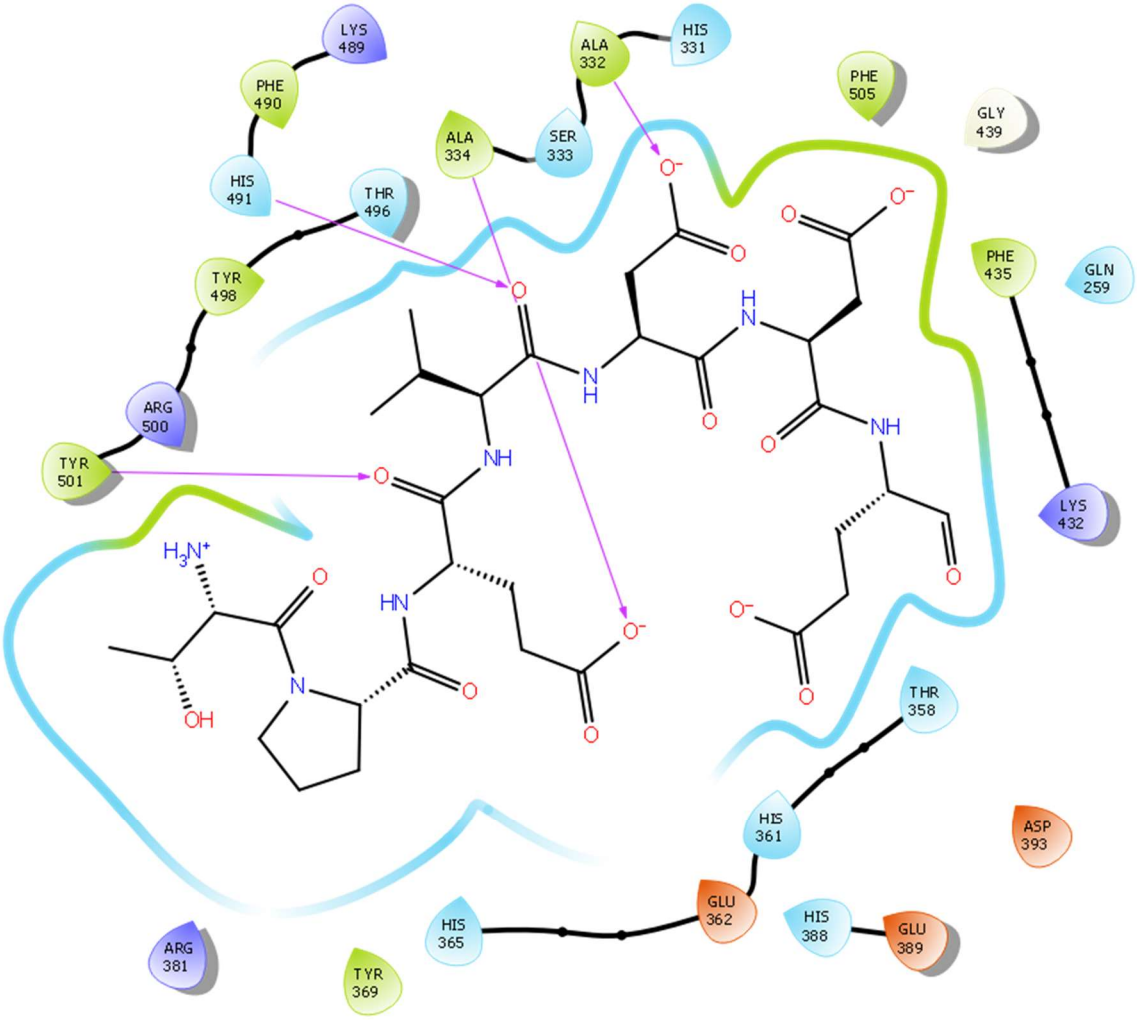


Figure 4.8 Docking results of the peptide TPEVDDE in the active site of human angiotensin I-converting enzyme (ACE). TPEVDDE is represented in black, interactions of human ACE active site residues with the peptide are indicated by arrows of different colours, with purple representing hydrogen bond interactions and blue arrows representing salt bridge interactions.

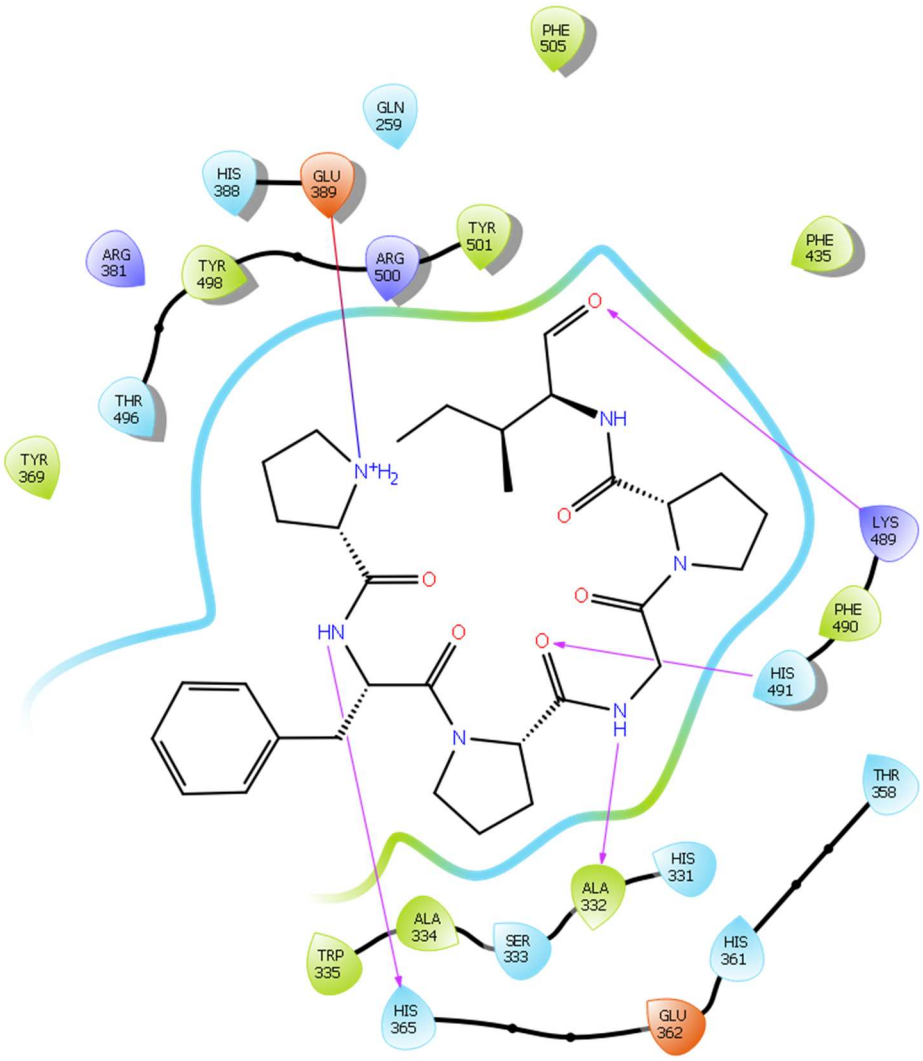


Figure 4.9 Docking results of the peptide PFPGPI in the active site of human angiotensin I-converting enzyme (ACE). PFPGPI is represented in black, interactions of human ACE active site residues with the peptide are indicated by arrows of different colours, with purple representing hydrogen bond interactions and blue arrows representing salt bridge interactions.

Hydrogen bond interactions have been shown to play a crucial role in stabilizing the docked ligand complexes (Tu et al., 2018). The distance of hydrogen bond interactions between the ACE amino acid residues and the whey-derived peptide sequences typically were short (<2.5 Å; Table 4.6), demonstrating that the peptides' binding affinity to ACE was strong (Ling et al., 2018). In addition, these peptides formed several favourable salt bridge interactions with ACE residues, showing that the ligands can pack tightly into the binding site and effectively inhibit ACE. Furthermore, it is interesting to note that hydrophobic amino acid residues such as proline, alanine, phenylalanine, leucine, and isoleucine were mainly involved in establishing strong interactions with ACE, which is

supported by observations reported in structure-activity relationship (SAR) (Table 4.6) (Pan et al., 2011).

The amino acid residues involved in the binding of Sampatrilat to the active site of ACE have been detailed using high resolution crystal structures elucidated by X-ray crystallography (Cozier et al., 2018). Interestingly, TPEVDDE, PFPGPI and AIPPK appear to interact with two of these previously identified amino acid residues: TPEVDDE, AIPPK and PFPGPI interact with Glu 389, and PFPGPI interacts with His 365. Furthermore, previous studies stated that the ACE inhibitory drugs Captopril, Lisinopril, and Enalapril interact with ACE amino acid residues Gln281, His353, Glu384, Lys511, His 513, and Tyr520 (Natesh et al., 2004; Vercruysse et al., 2010; Wang et al., 2011). Apart from the amino acid residues in common, Lisinopril was reported to interact with Glu 162, Ala 354, His 383, Glu 387, Arg 522, and Tyr 523 (Natesh et al., 2004). Captopril was also shown to interact with His 337, Gln 265, Lys 495, His 497, and Tyr 504 (Akif et al., 2010), and Enalapril appears to interact with Ala 354 and Tyr 523 (Natesh et al., 2004; Vercruysse et al., 2010; Wang et al., 2011). According to the docking results, all whey-derived peptides except for IESPPEI appear to interact with amino acid residues in common with the anti-hypertensive drugs Captopril, Lisinopril and Elanapril (Figures 4.5, 4.7, 4.8. 4.9 and Table 4.6).

In our previous studies, ACE inhibitory peptides produced by enzymatic hydrolysis of whey proteins were isolated and characterized (Welderufael et al., 2012). Peptide sequences were identified as major peptides in fractions from the enzymatic hydrolysates of β-Lg and CDP. The well-known anti-hypertensive peptide Ile-Pro-Pro (IPP), together with other novel peptide sequences that have structural similarities with reported ACE inhibitory peptides, such as Leu-Val-Tyr-Pro-Phe-Pro (LVYPFP), Leu-Ile-Val-Thr-Gln (LIVTQ), and Ile-Ile-Ala-Glu (IIAE), were characterised and identified by a combination of chemical characterization (LC/MS; MS/MS) and SAR data. In a following molecular docking study, these peptides produced naturally from whey by enzymatic hydrolysis interacted with residues of human ACE, in common with potent ACE inhibitory drugs, such as Sampatrilat, Captopril, Lisinopril, and Elanapril, which suggests that these natural peptides may be potent ACE inhibitors (Chamata et al., 2020; Welderufael et al., 2012). Interestingly, all the whey-derived peptide sequences identified via MS analysis in the present work, except for IESPPEI, interacted with amino acid residues in common with IIAE, LIVTQ and LVYPFP (Figures 4.5, 4.7 – 4.9 and Table 4.6).

Overall, the docking results together with comparisons with the ACE inhibitory drugs provide strong evidence for the ACE inhibitory activity of AIPPK, PFPGPI, TPEVDDE and VELLKHKP. IESPPEI – identified in F4, F5, and F7 – did not interact with any key amino acid residues at the binding site of ACE, which suggests that IESPPEI is very unlikely to be inhibiting ACE effectively. The ACE inhibitory

activity measured in F5, where IESPPEI was the only peptide identified, was -6.9% (Table 4.4), which further confirms the lack of ACE inhibitory potency of IESPPEI. Hydrogen bond interactions were demonstrated to play the most important role in stabilizing the peptides' pose, docking complex as well as enzyme catalytic reactions (Chaudhary et al., 2009). Accordingly, when compared to the other peptide interactions with ACE; AIPPK, PFPGPI, and VELLKHKP, in this particular order, seem to display the highest ACE inhibitory activities, suggesting that these peptide sequences are majorly contributing to the ACE inhibitory activities observed in F7, and also confirming the *in vitro* ACE inhibitory results and observations obtained from SAR (Bidasolo et al., 2012; Cheung et al., 1980; FitzGerald and Meisel, 2000; Gobbetti et al., 2000; Hernández-Ledesma et al., 2011; Pripp et al., 2004; Vermeirssen et al., 2004). Also, AIPPK seems to be the only contributor to the ACE inhibitory activity reported in F4 (Tables 4.4 and 4.5), and TPEVDDE appears to be the main contributor to the ACE inhibition measured in F2 and F3 (Tables 4.4 – 4.6).

Results from molecular docking showed that peptides AIPPK, TPEVDDE, VELLKHKP, PFPGPI can bind favourably to the active site of ACE by interacting with critical amino acids in the catalytic binding pocket of ACE. The docking results herein corroborate the assumptions made in Section 4.3.1.3 regarding the main contributors to the ACE inhibitory activities observed in F1, F2, F3, F4 and F7, and suggest that most probably AIPPK, TPEVDDE, VELLKHKP and PFPGPI are potent ACE inhibitors that appear to contribute to the *in vitro* ACE inhibitory activity.

4.4 Conclusions

Our results have shown that Minekus method can be used for the preparation of BAPs, and analysis of potent ACE inhibitory peptides derived from whey proteins. The main peptide sequences identified using MS analysis were κ-Casein derived AIPPK, BSA derived VELLKHKP, β-LG derived TPEVDDE, and β-casein derived PFPGPI. The molecular docking results revealed that the ACE inhibition of peptides AIPPK, VELLKHKP, TPEVDDE, and PFPGPI is mainly attributed to forming strong hydrogen bonds and salt bridge interactions with key residues in the active site of human ACE.

Additionally, a comparison with commercial ACE inhibitory drugs such as Sampatrilat, Captopril, Lisinopril, and Elanapril showed that the natural peptides interacted similarly to the drugs mimicking the same interactions with ACE active site residues. These results indicate that AIPPK, VELLKHKP, TPEVDDE, and PFPGPI exhibit potent *in vitro* and *in silico* ACE inhibitory activities, which makes these peptides promising candidates for the development of functional foods that can be used in the effective prevention and treatment of hypertension. Moreover, the correlation observed between

the experimental results and the molecular docking results, show the reliability of molecular docking as a pre-screening tool to identify potential protein-derived BAPs from natural sources in a timely and efficient manner. The method developed in this study could therefore be applied to predict which peptide sequences are likely to be responsible for the ACE inhibitory activity *in vitro* without having to purify such peptides first.

4.5 Appendix

Table 4.A.1: ACE inhibitory (ACE_i) activity assay: Positive control results.

Concentration of Captopril (nM)	ACEi(%) 1st trial	ACEi(%) 2nd trial	ACEi(%) 3rd trial	Average	Std	CV
0	0.00	0.00	0.00	0.00	0.00	
0.7	13.33	13.11	14.04	13.49	0.48	3.59
3	27.12	27.64	28.90	27.89	0.92	3.28
5	31.67	34.43	34.82	35.62	1.72	4.82
10	45.00	44.26	35.09	41.45	5.52	13.32
20	53.33	55.38	55.88	54.86	1.35	2.46

Table 4.A.2. ACE inhibitory activities (ACE_i) of whey-derived peptides.

Peptide fraction	Acetronitrile%	ACEi%	Average	Std	CV
F1(1)	16	42.9	44.8	3.3	7.4
F1(2)		48.6			
F1(3)		42.9			
F2(1)	18	42.9	38.1	2.6	4.1
F2(2)		34.3			
F2(3)		37.1			
F3(1)	22	34.3	34.3	2.9	8.3
F3(2)		37.1			
F3(3)		31.4			
F4(1)	26	51.4	53.3	1.9	2.7
F4(2)		51.4			
F4(3)		57.1			
F5(1)	30	-5.7	-6.7	1.7	-24.7
F5(2)		-5.7			
F5(3)		-8.6			
F6(1)	40	5.7	6.7	1.7	24.7
F6(2)		8.6			
F6(3)		5.7			
F7(1)	100	74.3	73.3	1.7	2.3
F7(2)		74.3			
F7(3)		71.4			

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CHAPTER FIVE General discussion and perspectives

5.1 Angiotensin I-converting enzyme (ACE) and blood pressure

The trend towards healthy living has become more pronounced worldwide in recent years, due to a growing incidence of chronic diseases. Numerous efforts are being made to investigate potential food-derived functional ingredients that may be utilised to generate health-promoting foods. Over the past few years, bioactive peptides (BAPs) from various food sources have been identified within these functional ingredients. Furthermore, these peptides have attracted a lot of attention due to several reports demonstrating the peptides' enormous potential in exhibiting health-promoting effects. This could aid in the prevention and treatment of many lifestyle-related diseases, such as cardiovascular diseases.

Many food-derived proteins contain different peptide sequences encrypted within their primary structures, and which exert beneficial effects upon human health, such as the blood pressure-lowering effect. Indeed, one of the most extensively studied activity is the anti-hypertensive activity with particular focus on the *in vitro* Angiotensin I-converting enzyme (ACE) inhibitory activity. Furthermore, food-derived peptides are believed to represent a healthier and more natural alternative source for chronic treatment of hypertension. Although the inhibitory capacity of food-derived peptides is lower than that of chemically designed drugs, it is thought that food-derived peptides are safer than pharmaceutical drugs due to their lack of some drug-associated adverse side effects, such as angioedema, skin rashes and dry cough. However, considering the lack of consensus in their physiological anti-hypertensive effects in different human populations, the role of food peptides in regulating blood pressure is still a subject of ongoing debate.

Although milk proteins are the richest source of ACE inhibitory peptides, several studies have reported that high *in vitro* ACE inhibitory activities of milk protein derived BAPs are not necessarily associated with observed *in vivo* anti-hypertensive activities. Also, the mechanisms underlying the action of ACE inhibitors remain unclear, and some limitations of the classical approaches to produce ACE inhibitory peptides from food proteins have been discussed by researchers, i.e. uncertainty regarding the bioactivity of the milk protein hydrolysates. Accordingly, the structure–activity relationship and interaction between ACE and whey protein derived peptides with previously reported high *in vitro* ACE inhibitory activities were examined employing a molecular docking

approach (Chapter two). The developed docking methodology to be used was also demonstrated to be valid and reliable, with an RMSD value between the docked conformation, and the native co-crystallized ligand conformation, of 0.1 Å (Wang et al., 2003). The molecular docking results obtained show that whey-derived peptides IPP, LIVTQ, IIAE, LVYPFP formed strong hydrogen bonds with amino acid residues in common with the ACE inhibitory drugs Sampatrilat, Captopril, Lisinopril and Elanapril. These results provide strong evidence for the ACE inhibitory activity of these natural peptides.

The next step after validating and showing the reliability of the docking procedure developed in Chapter 2 was to evaluate more closely the structure-activity relationship of milk protein-derived BAPs. Many proteolytic enzymes have been used to generate BAPs, and intense research has been carried out to investigate the various enzymes' abilities to produce BAPs from whey hydrolysates. In this context, whey was subjected to a simulated gastric digestion according to the method described by Minekus et al., (2014) (Chapter four). Studies have shown that large polypeptides are unable to enter the active site of ACE (Natesh et al., 2003). Therefore, it was of interest to fractionate the whey protein-derived hydrolysates. The fractionation of whey proteins and use of MS techniques helped to identify major BAPs in whey protein hydrolysates.

The most potent whey-derived ACE inhibitory fractions were also characterised in terms of *in vitro* ACE inhibitory activity. The results obtained in chapter four show that BAPs can be generated from hydrolysis of whey proteins during gastrointestinal digestion. Among these potent peptides, some novel sequences were identified, such as AIPPK, TPEVDDE, PFPGPI, and VELLKHKP. The molecular docking methodology developed earlier enabled us to identify some potent peptides, among which some novel sequences were identified, such as AIPPK, TPEVDDE, PFPGPI, and VELLKHKP. The molecular docking results showed that peptides AIPPK, VELLKHKP, and PFPGPI exhibited high affinity for ACE and interacted with ACE in the key active site residues to form short hydrogen bonds and salt bridge interactions. Furthermore, these peptides showed marked structural similarities with previously described ACE inhibitors and interacted with residues of human ACE, in common with potent ACE inhibitory drugs, such as Sampatrilat, Captopril, Lisinopril, and Elanapril. It is also interesting to note that these peptides interacted with similar amino acids residues at the ACE active site to those reported for whey-derived peptides IIAE, LIVTQ, and LVYPFP, which were identified in Chapter two. These observations provide further strong evidence for the ACE inhibitory activity of peptides AIPPK, PFPGPI, and VELLKHKP.

In addition, the identified peptides IPP, IIAE, LIVTQ, and LVYPFP (Chapter two), and AIPPK, TPEVDDE, PFPGPI, and VELLKHKP (Chapter four) were 8 or less amino acids in length, which agrees with

previously published data that define most ACE inhibitory peptides to contain between 2 and 12 amino acids and molecular weights less than 3 kDa (Ryan et al., 2011). The small size of the whey-derived peptides presented in Chapters 2 and 4, could offer inherent advantages, such as low production costs and good membrane penetration ability (Waldamnn, 2014). Potent ACE inhibitory peptides are characterised by small size, which enhances their chance of crossing the intestinal epithelial barrier, to exert their biological activity in target organs (Segura et al., 2013). In fact, small sized peptides were shown to exhibit higher anti-hypertensive effects than long-chain peptides, because they may more easily fit in the active site of ACE, and subsequently assert inhibitory activity (Li et al., 2018). Oral administration of naturally extracted small molecule inhibitors in clinical studies was also not associated with adverse side effects, as reported in the literature (Li et al., 2021; Mora et al., 2014; Waldamnn, 2014).

Furthermore, the majority of the identified peptides contain hydrophobic and aromatic amino acid residues at their C-terminal. This finding is interesting because the presence of hydrophobic and branched-chain amino acids in the peptide structure was found to highly impact the ACE inhibitory activity of BAPs (Girgih et al., 2014; Hernández-Ledesma et al.,2011; Lassoued et al., 2015). Bulky aromatic amino acids were also reported to increase hydrophobic interactions with enzymes (Girgih et al., 2014). Also, the three amino acid residues located at the C-terminal sequence are a determining factor for competitive binding to the active site of ACE, and thus for exerting ACE inhibitory activity (Hernández-Ledesma et al., 2011; Lassoued et al., 2015). Based on these observations, peptide PFPGPI was found to have the highest ACE inhibitory activity of all the peptides studied and deemed to be the most promising for inhibiting ACE activity. Interestingly, based on the molecular docking approach in Chapter four, PFPGPI also seems to be the most promising peptide for inhibiting ACE activity, when compared to the other identified peptides' interactions with ACE.

Similarly, peptide AIPPK seems to display a high ACE inhibitory activity. AIPPK could represent a fragment of vasodilatory MAIPPKK, a peptide known for exerting significant blood pressure lowering effects (Miguel et al., 2007). Some researchers have demonstrated the promoting role of positively charged and basic amino acids (Lysine) at the C-terminal sequence on peptides' ACE inhibitory activity (Zheng et al., 2017). Also, similarly to other well-characterised ACE inhibitory peptides, peptide AIPPK has other structural features of ACE inhibitors, such as the presence of charged amino acid residues followed by hydrophobic amino acids at the C-terminal sequence (Vermeirssen et al., 2004). Additionally, peptide IPP, known as the most potent ACE inhibitor from milk proteins (Ehlers et al., 2011; Nakamura et al.,1995) could be a fragment of AIPPK, which could also be one of the significant contributors to the highest ACE inhibitory activities measured in whey protein

hydrolysates. This situation also occurs with peptide IIAE, a potent ACE inhibitor, as demonstrated in our earlier works (Chapter 2, Welderufael et al., 2012).

These outcomes indicate that experimental measurement of ACE inhibitory activities of hydrolysates fractions and docking results of the identified peptide sequences within these fractions seem to be in agreement. This finding demonstrates the potential effectiveness of molecular docking as a pre-screening tool to identify potential milk protein-derived BAPs. This methodology could also be an invaluable tool to obtain an improved insight on the structure-activity relationship of ACE inhibitory peptides. This finding is of great importance, because the methodology proposed in this work could be used to predict which peptide sequences are likely to be responsible for the ACE inhibitory activity *in vitro* in milk/food-derived protein hydrolysates, without some challenges of the classical approaches, such as having to apply cumbersome purification processes to isolate and characterise BAPs. Further work will, however, be needed, using pure synthesised peptides, to confirm the *in vitro* and *in vivo* ACE inhibitory activity of these whey-protein derived hydrolysates.

5.2 Angiotensin II-converting enzyme (ACE2) and coronavirus disease 2019 (COVID-19)

The COVID-19 pandemic has triggered an imperative search for antiviral drugs with proven efficacy for the prevention and treatment of the COVID-19 disease, with no clear success at the time the manuscript in Chapter three was written (May 2021, Chapter three). Therefore, there is currently an urgent need in the scientific community to explore new therapeutic approaches. Although targeting the renin–angiotensin system (RAS) has not received much attention, it is a crucial factor in COVID-19 pathophysiology, with implications for effective therapeutics that could ameliorate lung injury and reduce disease progression.

Besides its role as the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) receptor, angiotensin II-converting enzyme (ACE2) is established for its role in hypertension by negatively regulating the RAS pathway through modulating blood pressure and maintaining vascular homeostasis. The unique interaction of SARS-CoV-2 and host cell receptor ACE2 provides a critical link between COVID-19 and, hypertension and CVDs (Hoffmann et al., 2020; Zhou et al., 2020). Despite some controversies (Mehta et al., 2020; South et al., 2020), a large number of studies has reported a significant association between the use of ACE inhibitors, and reduced risk of COVID-19 (Hippisley-Cox et al., 2020). In this context, the molecular interactions between the whey-derived ACE inhibitory peptides IPP, LIVTQ, IIAE, LVYPFP, and human ACE2 were assessed employing the

same molecular docking approach developed in Chapter 2. Peptides IPP, IIAE, LIVTQ, and LVYPFP all formed strong hydrogen bonds and salt bridge interactions with key residues in the active site of human ACE2 (Chapter 3). Among the four peptides examined, IIAE was found to be the most promising candidate to exert an antiviral activity on SARS-CoV-2, through inhibiting ACE2 via specific molecular interactions with all the key residues of ACE2. IIAE also formed strong interactions at the active site of ACE2, in common with known potent pharmaceutical ACE2 inhibitors, such as Carnosine (Saadah et al., 2020) and chloroquine phosphate (Upreti et al., 2021).

These results show that the whey-derived peptides IPP, IIAE, LIVTQ, and LVYPFP may be potential candidates to be used in the treatment of COVID-19 via inhibition of the host cell receptor ACE2. Moreover, in comparison with well-known ACE inhibitory drugs such as Captopril and Lisinopril, the natural peptides produced from whey have a dual inhibitory action against both ACE and ACE2, and may be associated with fewer side effects, which may represent advantages in the treatment of COVID-19. The structural insights provided by this molecular docking study are valuable in understanding the regulation of ACE2 and help to design novel potent therapeutic inhibitors against SARS-CoV-2 based on ACE2 inhibitory activity. However, further *in vitro* and *in vivo* studies are needed to further elucidate the whey-derived peptides' underlying inhibitory mechanisms against ACE2. It also remains unknown whether inhibiting ACE2 would be efficient at attenuating infections by SARS-CoV-2, and further research is needed to understand the underlying molecular mechanisms related to the ACE2 inhibitory capacities.

5.3. General conclusion

In this research, we have managed to answer questions in relation to the application of molecular docking as a pre-screening tool to determine the ACE inhibitory activities of bioactive whey proteins derived peptides. Molecular docking can aid to elucidate structure–activity relationships, making it a reliable and fast approach to predict which peptide sequences are contributing to the *in vitro* ACE inhibitory activities, without having to apply cumbersome purification methods to isolate such BAPs. Therefore, this novel approach offers a very useful tool which could be used in the development of functional foods, with peptides as bioactive compounds, for treatment of hypertension. Our results also indicate that whey proteins derived peptides can inhibit ACE activity, one of the main enzymes involved in blood pressure regulation.

5.4. Further studies

However, there are possibly many more unanswered questions than the ones that were addressed. As peptides are prone to gastrointestinal digestion before reaching the target organ, there is a possibility that peptides with high *in vitro* ACE inhibitory activity may not exhibit an *in vivo* effect. Therefore, to further develop potent ACE inhibitory peptides obtained from whey proteins, it is very important to better characterise the mode of action of such peptides. Further research is thus needed to completely elucidate the mechanisms of actions underlying the whey-derived peptides' ACE inhibitory properties, and the structures associated with these mechanisms.

In vivo studies provide more sufficiency of the efficacy of BAPs under metabolic interactions and bodily transformations (Nong & Hsu, 2022). To this end, further *in vivo* studies need to be carried out to determine the potential of these peptides to act as anti-hypertensive agents. It is also of utmost importance to confirm the *in vitro* and *in silico* effects of these peptides by human trials and determine the clinical efficacy of the peptides identified in this work before considering their exploitation as physiologically functional foods. In this regard, various aspects such as the safety characteristics of these peptides should also be assessed, namely the allergenic potential which is commonly associated with whey protein-based materials. Other aspects to be investigated include peptide dose, potential side effects, maintenance of bioactivity during transit through the gastrointestinal tract, peptide absorption across the intestinal mucosa, and site(s) of action *in vivo.*

Furthermore, the interaction of these peptides with other components in the food matrix should be investigated if these peptides (or hydrolysates) were to be ingested in food formulations. In this sense, the incorporation of peptides into such formulations, as encapsulated or free functional ingredients, could also be an interesting topic for future research. The necessity of large-scale production methodologies to warrant sufficient material for food formulations and *in vivo* studies should also be further explored. Future technological research is thus required to establish experimental conditions leading to higher yields of these bioactive molecules.

In any case, the findings presented here contribute to the advancement of the vast research area of BAP production, particularly in the exploitation of the health benefits of milk proteins-derived BAPs. Therefore, I believe this work could have an impact in the future of human health and food technology.

5.5 References

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