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Bioactive peptides from poultry by-product hydrolysate targeting cardiometabolic diseases: production, characterization, and bioavailability.

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Abstract

Food-derived bioactive peptides are promising health-promoting ingredients that can act on a variety of therapeutic targets relevant to the management of cardiometabolic diseases (e.g., type 2 diabetes mellitus (T2DM) and cardiovascular diseases). Food processing side streams/by-products are an underutilized resource that have a great potential as a raw material to produce such bioactive peptides. The main aim of the dissertation was to develop and implement bioanalytical methods for the discovery and characterization of bioactive peptides from the hydrolysate of poultry by-product (i.e., mechanically deboned chicken residue (MDCR)). The selected bioactivities were antihypertensive (angiotensin-1-converting enzyme (ACE-1) inhibition), antioxidant (1,1-diphenyl-2-picrylhydrazil (DPPH) radical scavenging), and antidiabetic (dipeptidyl peptidase 4 (DPP4) inhibition).

The first objective was to produce a library of hydrolysates from MDCR using different processing conditions and screen bioactivities (**paper I**). Sixty hydrolysates were produced using ten enzymes and six hydrolysis times. ACE-1 inhibitory and DPPH radical scavenging properties of the hydrolysates were evaluated using *in vitro* assays. Enzyme choice was shown to have a major influence on both DPPH radical scavenging activity and ACE-1 inhibitory activity. The DPPH radical scavenging activity generally decreased with increasing hydrolysis time. On the other hand, hydrolysis time had no systematic effect on the ACE-1 inhibitory activity. Optimization of the processing parameters allowed to tailor composition of the hydrolysates and improve their bioactive properties.

The second objective was to develop predictive models of bioactivities based on Fourier transform infrared (FTIR) spectra and molecular weight distribution (MWD) of the hydrolysates (**paper I**). Partial least squares regression (PLSR) models developed based on IR spectra and MWD showed adequate prediction performances for both ACE-1 inhibitory and DPPH radical scavenging activities. However, PLSR models based on FTIR spectra performed better than the MWD based models. In addition, FTIR is a rapid analytical method; thus, it has a high potential as a rapid screening and analytical tool for quality control in the industrial production of bioactive peptides.

The third objective was to identify peptides with promising ACE-1 and DPP4 inhibitory properties (**paper II** and **paper III**). Bioactivity-guided fractionation in combination with liquid chromatography with tandem mass spectrometry (LC-MS/MS) led to identification of potent bioactive peptides. Two peptides, VL and IY, were identified from the most potent

fractions for DPP4 and ACE-1 inhibition, respectively (**paper II**). The bioactivity-guided fractionation revealed that low molecular weight peptide fraction (LMWPF) exhibited promising dual ACE-1 and DPP4 inhibitory activity. Dipeptide YA identified in LMWPF was one peptide responsible for the dual ACE-1 and DPP4 inhibition (**paper III**).

The fourth objective was to evaluate gastrointestinal (GI) stability and intestinal permeability of identified bioactive peptides (**paper III**). Three identified peptides and LMWPF were demonstrated to resist the GI conditions in a simulated *in vitro* digestion (i.e., INFOGEST static model). Membrane permeability experiments across the epithelial Caco-2 cell monolayer showed that although the peptides were absent from the apical side, none were detected at the basolateral side of the cell layer. This could either be due to peptide degradation by brush border peptidases or that the cells themselves metabolise the peptides. Furthermore, the gene expression of several peptide transporters, transcription factors and tight junction proteins in the Caco-2 cells was changed upon peptide stimulation, indicating that the peptides elicited a response in Caco-2 cells.

This dissertation demonstrated that MDCR is a promising raw material for production of bioactive hydrolysates and peptides for management of cardiometabolic diseases. One of the hydrolysate fractions (LMWPF) demonstrated a dual pharmacological effect. Therefore, the LMWPF is a health-promoting ingredient with high potential, especially for complex diseases requiring multidrug regimens (such as T2DM). The study successfully developed a prediction model for bioactivities of hydrolysates based on FTIR fingerprints, identified peptides from a MDCR hydrolysate responsible for bioactivities using bioactivity-guided fractionation and LC-MS/MS, and characterized the bioactive peptides including their potency, GI stability and intestinal permeability. The results from the study revealed that, particularly, the LMWPF from MDCR hydrolysate can be considered for further *in vivo* evaluation as promising health promoting ingredient in functional foods or nutraceuticals.

Sammendrag

Bioaktive peptider er lovende helsefremmende ingredienser som kan ha effekt på en rekke terapeutiske mål relevante for behandling av kardiometabolske sykdommer (f.eks. type 2 diabetes mellitus (T2DM) og kardiovaskulære sykdommer). Proteinrike sidestrømmer fra matforedling er en underutnyttet ressurs med stort potensial som råstoff for produksjon av slike bioaktive peptider. Hovedmålet med avhandlingen var å utvikle og gjennomføre bioanalytiske metoder for å finne og karakterisere bioaktive peptider fra hydrolysat av sidestrømmer fra fjærfeforedling (dvs. restfraksjon etter mekanisk utbeining av kylling (RMUK)). De utvalgte bioaktivitetene var blodtrykksreduksjon (angiotensin-1-converting enzym (ACE-1) hemming), antioksidant (1,1-diphenyl-2-picrylhydrazil (DPPH) radikalfjernende), og blodsukkerregulering (dipeptidyl peptidase 4 (DPP4) hemming).

Det første delmålet var å produsere et bibliotek av hydrolysater fra RMUK ved bruk av forskjellige prosessbetingelser og screening av bioaktiviteter (**artikkel I**). Seksti hydrolysater ble produsert ved bruk av ti enzymer og seks hydrolysetider. ACE-1-hemmende og DPPH-radikalfjernende egenskaper hos hydrolysatene ble evaluert ved bruk av *in vitro* analyser. Enzymvalg viste seg å ha stor innflytelse på både DPPH-radikalfjernende aktivitet og ACE-1-hemmende aktivitet. Den DPPH-radikalfjernende aktiviteten minket generelt med økende hydrolysetid, mens ingen systematisk effekt av hydrolysetid ble sett for ACE-1-hemmende aktivitet. Optimalisering av prosessparameterne gjorde det mulig å tilpasse sammensetningen av hydrolysatene og forbedre deres bioaktive egenskaper.

Det andre delmålet var å utvikle prediktive modeller for bioaktiviteter basert på Fourier transform infrarød (FTIR) spektra og molekylvektfordeling (MWD) av hydrolysatene (**artikkel I**). Partial least squares regression (PLSR) modeller utviklet basert på IR-spektra og MWD viste tilstrekkelige gode predikasjonsmuligheter for både ACE-1-hemmende og DPPH-radikalfjernende aktiviteter. Imidlertid presterte PLSR-modeller basert på FTIR-spektra bedre enn de MWD-baserte modellene. I tillegg er FTIR en rask analysemetode; dermed har den et høyt potensial som et raskt screenings- og analytisk verktøy for kvalitetskontroll ved industriell produksjon av bioaktive peptider.

Det tredje delmålet var å identifisere peptider med lovende ACE-1- og DPP4-hemmende egenskaper (**artikkel II** og **artikkel III**). Bioaktivitetsveiledet fraksjonering i kombinasjon med væskekromatografi-tandem massespektrometri (LC-MS/MS) førte til identifisering av potente bioaktive peptider. To peptider, VL og IY, ble identifisert fra de mest potente

fraksjonene for henholdsvis DPP4- og ACE-1-hemming (**artikkel II**). Den bioaktivitetsstyrte fraksjoneringen avslørte at lavmolekylær peptidfraksjon (LMWPF) viste lovende dobbel ACE-1- og DPP4-hemmende aktivitet. Dipeptid YA identifisert i LMWPF var ett peptid ansvarlig for den doble ACE-1- og DPP4-hemmingen (**artikkel III**).

Det fjerde delmålet var å evaluere gastrointestinal (GI) stabilitet og intestinal permeabilitet for de identifiserte bioaktive peptidene (**artikkel III**). Tre identifiserte peptider samt LMWPF ble vist å være resistente mot GI-betingelsene i en simulert *in vitro* fordøyelse (dvs. INFOGEST statisk modell). Membranpermeabilitets studier gjort i humane epitelcelle, Caco-2, viste at selv om peptidene ble borte fra øverste del av cellelaget var det ikke mulig å detektere de samme peptidene på andre siden. Dette indikerer at peptidene ikke krysser transepitelbarriæren, men at de enten brytes ned lokalt av peptidaser, eller at cellene metaboliserer peptidene. I tillegg viste ekspresjonen av flere gener relatert til peptidtransportører, transkripsjonsfaktorer og tight junction-proteiner hos Caco-2-celler betydelig endring når cellene ble stimulert av de bioaktive peptidene; som indikerer at peptidene fremkalte en lokal respons i Caco-2-celler.

Avhandlingen viste at RMUK er et lovende råmateriale for produksjon av bioaktive hydrolysater og peptider for regulering av kardiometabolske sykdommer. En av hydrolysatfraksjonene (LMWPF) viste en dobbel farmakologisk effekt. Derfor er LMWPF en helsefremmende ingrediens med høyt potensial, spesielt for komplekse sykdommer som krever multidrug-regimer (som T2DM). Studien lyktes i å utvikle en prediksjonsmodell for bioaktiviteter i hydrolysater basert på FTIR-fingeravtrykk, å identifisere peptider fra et RMUK-hydrolysat ansvarlig for bioaktiviteter ved bruk av bioaktivitetsveiledet fraksjonering og LC-MS/MS, og å karakterisere de bioaktive peptidene inkludert deres styrke, GI-stabilitet og intestinal permeabilitet. Resultatene fra studien viste at spesielt LMWPF fra RMUK-hydrolysat kan vurderes for videre *in vivo* evaluering som en lovende helsefremmende ingrediens i funksjonelle matvarer eller nutraceuticals.

Preface

The present dissertation was part of the research project Peptek, and financed by Nofima, the Norwegian Institute of Food, Fisheries and Aquaculture Research. The main aim of Peptek was to develop 'a comprehensive approach and procedures for the treatment of unused or remaining biomass in the form of residual biomass after food production'.

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Liudmila Sorokina Ås, January 2024

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Paper I

Liudmila Sorokina, Anne Rieder, Shiori Koga, Nils Kristian Afseth, Rita De Cássia Lemos Lima, Steven Ray Wilson and Sileshi Gizachew Wubshet. Multivariate correlation of infrared fingerprints and molecular weight distributions with bioactivity of poultry by-product protein hydrolysates. *Journal of Functional Foods* **95**, 105170 (2022).

Paper II

Liudmila Sorokina, Josipa Matic, Anne Rieder, Shiori Koga, Nils Kristian Afseth, Steven Ray Wilson and Sileshi Gizachew Wubshet. Low Molecular Weight Peptide Fraction from Poultry Byproduct Hydrolysate Features Dual ACE-1 and DPP4 Inhibition. *ACS Food Science & Technology* **3**, 2219-2228 (2023).

Paper III

Liudmila Sorokina, Anne Rieder, Nina Therese Solberg, Shiori Koga, Sissel Beate Rønning, Nils Kristian Afseth, Steven Ray Wilson and Sileshi Gizachew Wubshet. *In vitro* gastrointestinal stability and intestinal absorption of ACE-1 and DPP4 inhibitory peptides from poultry by-product hydrolysate. *Prepared for submission to Food & Function*.

List of abbreviations

ACE-1	angiotensin-1-converting enzyme
AP	apical
BL	basolateral
Caco-2	Cancer coli-2
CMDs	cardiometabolic diseases
CVDs	cardiovascular diseases
DPP4	dipeptidyl peptidase 4
DPPH	1,1-diphenyl-2-picrylhydrazil
DH	degree of hydrolysis
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EC number	Enzyme Commission number
EFSA	European Food Safety Authority
EIC	extracted ion chromatogram
FAPGG	N-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly
FTIR	Fourier transform infrared
GI	gastrointestinal
GIP	glucose-dependent insulinotropic peptide
GLP-1	glucagon-like peptide 1
HAT	hydrogen atom transfer
HBSS	Hanks' balanced salt solution
HHL	N-Hippuryl-His-Leu
HPLC	high performance liquid chromatography
IC_{50}	half-maximal inhibitory concentration
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LMWPF	low molecular weight peptide fraction (SEC Fr II)
MDCR	mechanically deboned chicken residue
M_w	weight average molecular weight
MWD	molecular weight distribution
m/z	mass-to-charge ratio
NDA	Panel on Dietetic Products Nutrition and Allergies
NIR	near-infrared
OPA	*o*-phthaldialdehyde
PCA	principal component analysis
PepT1	peptide transporter 1
PLSR	partial least squares regression
QSAR	qualitative structure-activity relationship
Q-ToF	quadrupole Time-of-Flight
RAAS	renin-angiotensin-aldosterone system
RNS	reactive nitrogen species
ROS	reactive oxygen species
SEC	size exclusion chromatography
SET	single electron transfer
T2DM	type 2 diabetes mellitus

TEER	transepithelial electrical resistance
TFA	trifluoracetic acid
TNBS	trinitrobenzensulfonic acid
WHO	World Health Organization

List of amino acids: 3-letter and 1-letter abbreviation

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1 Background

Cardiometabolic diseases (CMDs) are the leading cause of death worldwide [1, 2]. With the growing world population and increasing life expectancy [3], the number of people suffering from lifestyle-related diseases, including CMDs, is likely to increase [4, 5]. Dietary protein-derived bioactive peptides may contribute to the management of these diseases in the form of nutraceuticals or functional food. Such bioactive peptides can be produced using biotechnological methods (e.g., enzymatic hydrolysis) from food processing side streams/by-products, for example, from the meat and fish industry [6-8].

Biotechnological transformation of underutilized resources, such as food processing by-products, into value-added products contributes to a more sustainable economy. Currently, during processing, 25% to 60% of an animal (depending on the species) becomes a side stream [6, 8]. Reducing by-products by converting them into a product for human consumption is a vital step towards the sustainable use of resources. Such raw material is an excellent resource for producing bioactive peptides. Extensive research on food-derived bioactive peptides over the last decades has shown that these peptides have a broad spectrum of pharmacological activity [7-14]. The discovery and development of bioactive peptides allow the food processing industry to generate higher revenues by transforming low-value by-products into high-value ingredients.

This dissertation aimed to develop and implement bioanalytical methods for the discovery and characterization of bioactive peptides from a poultry by-product, with a focus on peptides acting on therapeutic targets related to CMDs. In the following chapters, aspects related to the aim, such as protein hydrolysates, bioactive peptides, discovery platforms, methods used for the characterization of hydrolysates and peptides, as well as identification of bioactive peptides, are covered.

Chapter 2 Protein hydrolysates and bioactive peptides

2.1. Enzymatic protein hydrolysates

A *protein hydrolysate* is a complex mixture of protein fragments, peptides of varied length and amino acid composition, and free amino acids with different functional, biological, and nutritional properties [9]. Protein hydrolysates can be produced using chemical hydrolysis (i.e., acidic or alkaline conditions), subcritical water processing, and enzymatic hydrolysis. Chemical hydrolysis requires extreme pH and temperature conditions, which make it challenging to control the process and properties of the product. In addition, the nutritional parameters of hydrolysates are reduced due to destruction of certain amino acids, contamination by residual organic solvents and/or some toxic chemicals, and high salt concentration [15]. Subcritical water processing is a new method for protein hydrolysis, which degrade proteins by applying high temperatures (150-300 ℃) and pressure (4-10 MPa) [16]. In contrast, enzymatic protein hydrolysis is a mild biotechnological process that allows to control and modify the properties of the product (e.g., bioactivity, rheological properties, and sensory attributes) without losing nutritional parameters [11]. Therefore, enzymatic protein hydrolysate is currently the most studied method to produce bioactive peptides [17].

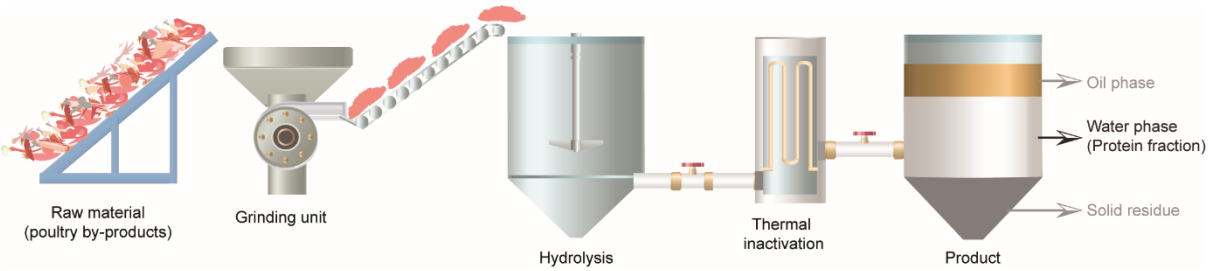

Figure 1 – Main unit operations of enzymatic protein hydrolysis. Figure modified from Wubshet et al. [18].

Figure 1 demonstrates the main unit operations of the enzymatic protein hydrolysis process. The process typically starts with homogenization of a raw material using a grinder. This is followed by addition of water to achieve good mixing and make a substrate better accessible by an enzyme [11]. The mixture is preheated to a temperature optimal for a given enzyme, and subsequently, the enzyme is added. The hydrolysis reaction is performed for a determined time, followed by thermal enzyme inactivation. After the enzyme inactivation, the mixture is separated into three phases by centrifugation in a lab-scale process or by a three-phase decanter in an industrial scale one [11]. The three phases are the oil phase (top layer), water phase or liquid hydrolysate (middle), and solid phase (bottom). In a lab setting the liquid phase is usually freeze-dried, while in an industrial setting liquid phase can be up-concentrated using

evaporator, which can be followed by spray-drying [11]. In addition, pre-treatment of raw material can be used (e.g., microwave, ultrasound, pulsed electric field) to achieve optimal protein yield [19]. Similarly, downstream processing (e.g., filtration) can be performed to improve the characteristics of the final product, such as bioactivity and sensory attributes [20].

Processing parameters influence the properties (e.g., bioactivity) of hydrolysates. For example, Nongonierma et al. [21] evaluated process conditions (temperatures, hydrolysis time, and enzyme-to-substrate ratios) to optimize the production of hydrolysates containing peptides with dipeptidyl peptidase 4 (DPP4) inhibitory properties from bovine milk protein isolate. They reported that temperature and hydrolysis time had a significant effect on the bioactivity [21]. Slizyte et al. [22] studied the influence of enzymes and hydrolysis time on the bioactive properties of hydrolysates. Their results showed, for example, that angiotensin-1-converting enzyme (ACE-1) inhibitory activity were dependent on enzyme and increased with hydrolysis time, while 1,1-diphenyl-2-picrylhydrazil (DPPH) radical scavenging increased only with hydrolysis time [22]. Therefore, processing parameters, such as pre-treatment of substrate, amount of water, enzyme-to-substrate ratio, enzyme, temperature, and pH, should be studied and adjusted to produce an optimal product in a cost-effective manner.

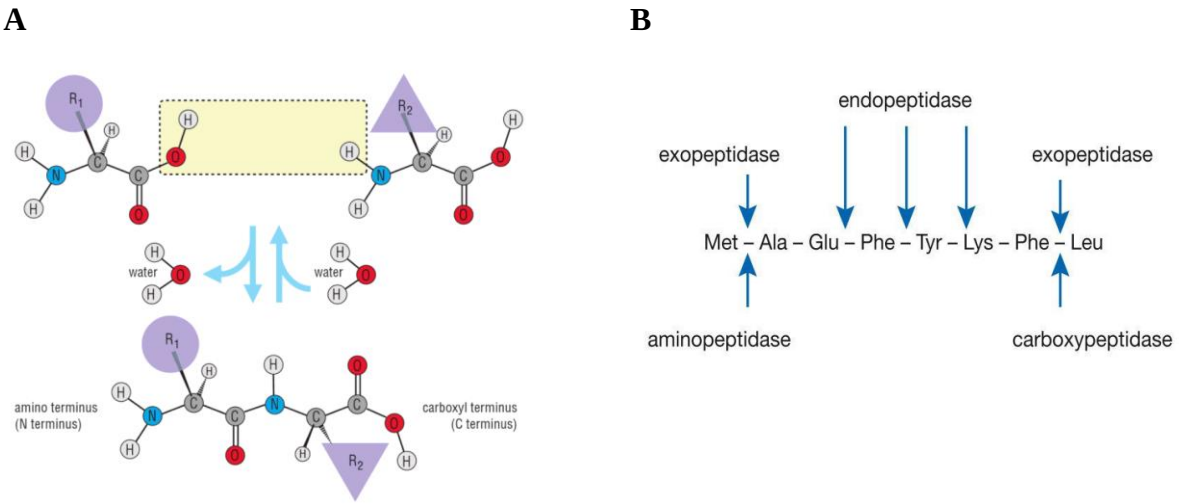

Figure 2 – Formation and hydrolysis of peptide bond with the loss and addition of a water molecule, respectively. R1 and R2 represent amino acids' side chains (A). Specific cleavage sites of different proteases (B). Figures reproduced from Hooper [23], Pestko and Ringe [24].

Enzyme choice is one of the parameters that enables modification of hydrolysate properties. A group of enzymes – proteases – is responsible for cleaving peptide bonds between amino acids in a protein or a peptide. A peptide bond between two amino acids is formed by a condensation reaction, where the amine group of one amino acid reacts with the carboxylic acid of another amino acid with the loss of a water molecule (Figure 2A) [24]. A variety of proteases can

facilitate the hydrolysis of a peptide bond. Proteases are classified in several ways, including cleavage specificity (Figure 2B). Exopeptidases facilitate cleavage at N- (aminopeptidases) or C-termini (carboxypeptidases), while endopeptidases can hydrolyze peptide bonds at non-terminal positions [25]. Endopeptidases are divided further into several classes according to the amino residue or co-factor that is essential in a catalytic site [26]. Examples of those enzyme classes are serine proteases, cysteine proteases, aspartic proteases, threonine proteases and metalloproteases. In addition to the broad selectivity (i.e., endo- vs exo-), endopeptidases and certain exopeptidases have sequence specificity, while other exopeptidases are generally nonspecific [27]. For example, trypsin cleaves after basic residues (lysine or arginine), while chymotrypsin requires aromatic residues at the site of cleavage [28].

Another classification of enzymes is based on their origin, such as mammalian, plant and microbial [17]. Most often commercial enzymes are produced using bacteria, yeast, and fungi [20]. In addition, for production of hydrolysates for human consumption, food grade enzyme preparations must be used according to food safety regulations (e.g., the European Food Safety Authority (EFSA) in Europe) [29].

Enzymatic protein hydrolysis can be performed using endogenous or exogenous enzymes. Hydrolysis with endogenous enzymes is a process that is difficult to control, while use of exogenous enzymes for hydrolysis allows control of the process and produces a product with desired specifications [11]. Therefore, all studies presented in this thesis are based on food-grade exogenous proteases. Enzymatic protein hydrolysis can be performed using pure enzymes with well-characterized activity (e.g., trypsin, and pepsin) or industrial enzyme preparations which are often mixtures of several proteases and where their exact formulations are often either unknown [30] or proprietary. For example, Merz et al. [31] identified seven peptidases in Flavourzyme 1000L (an industrial enzyme preparation from *Aspergillus oryzae*), which consists of three endopeptidases, two aminopeptidases and two dipeptidyl peptidases. Merz et al. [30] also identified peptidases in ten industrial enzyme preparations (including Flavourzyme 1000L). In a preparation of a widely used enzyme Alcalase 2.4 L, they identified three endopeptidases (including subtilisin) and one exopeptidase (aminopeptidase) [30], while the producer specifies only subtilisin in the product data sheet [32]. More precise information about enzyme preparations is required for better control of the hydrolysis process and final product properties.

2.2. Bioactive peptides liberated by enzymatic hydrolysis

Protein hydrolysates have been reported to possess a variety of bioactive peptides [9, 33-35]. *Bioactive peptides* are short chains of amino acids that, in addition to nutritional properties, affect the physiological function of the human body. They usually are 2-20 amino acids in length [19]. Bioactive peptides derived from dietary proteins exhibit a variety of health-promoting properties (i.e., preventing diseases or modulating physiological systems) [36, 37]. Such peptides remain inactive in the parent protein and only exhibit their activity upon liberation. Food-derived bioactive peptides can be liberated by a variety of methods including gastrointestinal digestion, food processing, fermentation, chemical or enzymatic hydrolysis [7, 38].

The health-beneficial effects of a bioactive peptide are influenced by amino acid composition, sequence, length, hydrophobicity and structure of a molecule [14, 19, 35, 39]. Currently, more than 4700 bioactive peptides are listed in the BIOPEP-UWM database [40] (accessed October 2023). The reported bioactivities include immunomodulating, antidiabetic, antibacterial, antioxidant, opioid receptor binding, anti-inflammatory, antihypertensive, etc. [40]. These bioactivities are important for treatment and prevention of several diseases, such as cardiovascular diseases (CVDs), type 2 diabetes mellitus (T2DM), metabolic syndrome, immune disorders, and cancer [12-14, 35, 41-43]. There are several products containing bioactive peptides already available on the market [43, 44]. Many bioactive peptides are derived from milk or fish proteins, with a variety of health-beneficial activities including, among others, blood pressure lowering activity, blood sugar regulation, modulation of inflammation, and stress relieving (Table 1).

Table 1 – Examples of commercial products with health benefits based on bioactive peptides or protein hydrolysates. Table reproduced from Chalamaiah et al. [44].

Brand name	Manufacturer (country)	Source	Health benefits
Lactoprodan® Hydro 365	Arla Food Ingredients (Denmark)	Whey, casein	Regulating blood sugar and promoting the synthesis of muscle glycogen, helping for placement glycogen stores in muscle tissue and the liver, leading to enhanced recovery after training
Lacprodan® ALPHA	Arla Food Ingredients (Denmark)	Whey	Preventing of sarcopenia during ageing
Lacprodan® Whey Protein	Arla Food Ingredients (Denmark)	Whey	Regulating blood sugar level
SureStart™ 917; SureStart™ 948; SureProtein™ 911; SureProtein™ 817	NZMP (Fonterra) (New Zealand)	Whey, milk	Promoting digestive comfort and prevention of allergy
Beautycoll® (Peptan)	Beautycoll (UK)	Fish collagen	Promoting healthy ageing, joint and bone health for an active lifestyle, preventing skin aging, supporting connective tissues
ProMod Liquid Protein Fruit Punch (Pro-Stat)	Abbott (USA)	Collagen	Helping improvement of pressure ulcer treatment
Capolac®	Arla Food Ingredients (Denmark)	Milk	Helping calcium absorption
Prodiet F200/Lactium	Ingredia (France)	Milk	Stress-relieving effects
Bonito peptide	Nippon Supplement Inc. (Japan)	Bonito fish	Helping to regulate the ACE-1
Vasotensin®	Metagenics (USA)	Bonito fish	Helping to regulate the ACE-1
Seacure®	Proper Nutrition Inc. (USA)	Pacific whiting fish	Helping to wound healing and supporting to the immune system
Fortide	Chengdu Mytech Biotech Co. Ltd (China)	Soybean	Improving feed efficiency, nutrient digestion, and intestinal histology
Calpis	Calpis Co. (Japan)	Sour milk	Antihypertensive effect (ACE-1)
Valtyron	Senmi Ekisu Co. (Japan)	Sardine muscle	ACE-1 inhibitory effect
PEPTIBAL®	Virage Santé INC., (Canada)	Shark fish	Maintains healthy immune system at gut and reduces the inflammation
Verisol	Gelita (Canada)	Porcine and bovine	Improving skin physiology
PeptAIde™	BASF (Germany)	Brown rice	Help modulate inflammation
Replexium™	BASF (Germany)	Patented peptides	Reducing the appearance of wrinkles and provides skin firming benefits

2.3. Food processing by-products as promising substrates for bioactive peptides

Enzymatic protein hydrolysis produced from a wide range of raw materials has been characterized with the focus on bioactive properties [42, 43, 45, 46]. Bovine milk proteins are one of the most explored sources of bioactive peptides [17]. This could mainly be due to the availability of relatively homogeneous protein fractions (e.g., whey) as a by-product from dairy processing. In recent years, however, a wide range of by-products from the fish and meat processing industry have also been indicated as valuable sources of bioactive peptides [7, 9, 10, 47, 48]. Aspevik et al. [11] suggested that enzymatic protein hydrolysis is a promising method for the valorization of food industries' by-products or residual raw materials.

In chicken meat production approximately 25-30% of live animal weight constitutes by-product (mechanically deboned chicken residue, blood, feathers, viscera, etc.) [49]. In 2020, 133 million tonnes of poultry meat (90% chicken meat) were produced, which accounts for almost 40% of global meat production [50]. Norway produced nearly 102 thousand tonnes of chicken meat in 2020 [51].

Mechanically deboned chicken residue (MDCR) is one of the major low-end by-product of poultry processing. Mechanical deboning is a downstream processing technology where carcasses (typically after stand filleting) are further used to optimally recover a protein rich meat mince [52, 53]. The process constitutes grinding of the carcasses, followed by separation of the meat-rich fraction from the bone-rich fraction using a fine screen or slotted surface [52]. The bone-rich fraction (i.e., used as a raw material in the studies covered in this thesis) is the low-value by-product referred to as MDCR. This by-product constitutes muscle and connective tissue proteins mixed with bones and fat. The chemical composition of MDCR was evaluated to contain 19.0% protein, 8.4% fat, and 6.8% ash [54], with water being the remaining constituent. It has to be noted that this ratio is an estimation because batch-to-batch variation is expected due to deboning settings, intra-species variations, etc. [18, 54]. Muscle tissue proteins are myofibrillar proteins, with actin and myosin being the main ones, and connective tissue proteins are collagen proteins [55]. In MDCR, collagen was estimated to be 28% of the total protein content [54].

One of the main challenges for the production of protein hydrolysate with defined characteristics from by-products is heterogeneity in the raw materials. Thus, commercial production of such hydrolysates requires a robust process that takes into account variations in raw material composition and produces a product with consistent characteristics [11]. Wubshet

et al. [18] proposed a solution where statistical models were used to predict end-product characteristics (yield and average molecular weight) using raw material quality and hydrolysis time as an input [18]. The authors evaluated four spectroscopic techniques (fluorescence, Raman, near-infrared (NIR) imaging scanner, and miniature NIR) and their combinations to characterize raw material composition [18]. The most accurate prediction was done using a combination of miniature NIR and fluorescence spectroscopies [18].

It is important to note that in this work, the term 'by-product' is used in a broad sense, referring to parts of raw materials that are not currently utilized for human consumption but are deemed safe for human consumption. In accordance with the EU regulations, the term 'by-product' specifically applies to raw materials lacking food-grade quality [11]. Following the EU regulations, the accurate term for residual raw materials with food-grade quality that can be utilized in food production would be 'co-product' [11].

2.4. The effect of bioactive peptides on cardiometabolic diseases

CMDs are a group of diseases which include CVDs, diabetes, and non-alcoholic fatty acid disease [1, 56]. Lifestyle, genetic and environmental factors are associated with CMDs [57]. According to the World Health Organization (WHO) [58], CVDs are the leading cause of death, with 17.9 million people annually, while diabetes accounts for 2 million deaths (Figure 3).

In the last decades, the scientific community has been showing that bioactive peptides can act on targets which are relevant for management of CMDs [6, 59]. However, the contribution of bioactive peptides to the management of CMDs is still not fully explored and therefore requires further investigation in both *in vitro* and *in vivo* as well as human intervention studies [8].

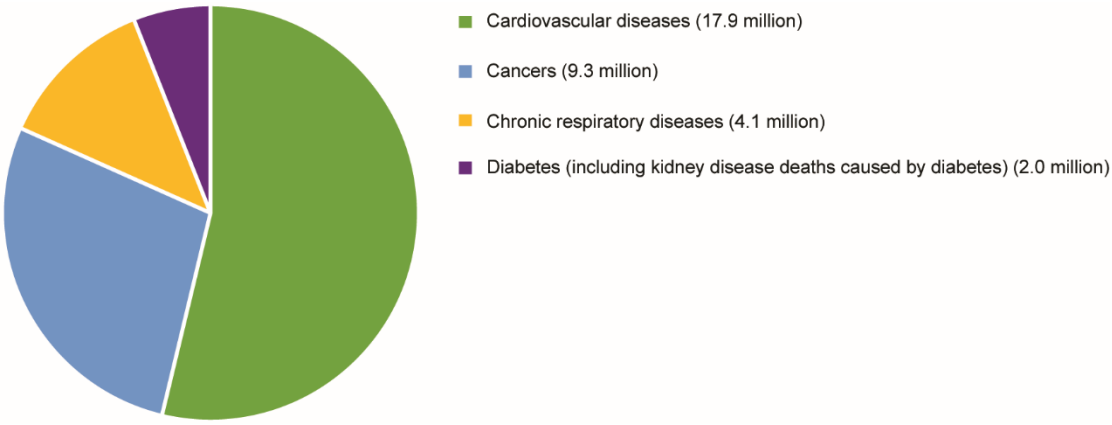

Figure 3 – Annual number of deaths due to chronic diseases according to causes. Figure made based on the report from WHO [58].

2.4.1. Diabetes type 2 and hypertension: the bad companions

Diabetes affects 537 million people worldwide, with T2DM accounting for more than 90% of these cases [60]. T2DM is characterized by dysregulation of glucose metabolism due to reduced insulin secretion, insulin resistance or a combination of both [61]. More than 85% of T2DM patients suffer from hypertension [62]. It is therefore recognized that diabetes and hypertension are 'the bad companions' [63] as hypertension is one of the main causes of CVDs [64].

Management of T2DM often requires multi-drug regiments, which is problematic due to possible drug to drug interactions, toxicity, side effects and poor patient compliance [65, 66]. A multi-target drug discovery approach offers a promising solution. This solution employs the discovery and development of polypharmacological drugs, composed of one or several molecules, which can act on multiple targets relevant to the same disease [67, 68]. In this context, peptides from food-derived protein hydrolysates are of interest because they exhibit various bioactivities, including the potential to modulate targets currently employed in the management of T2DM, such as DPP4, ACE-1, or antioxidant properties [6, 14, 59].

2.4.2. DPP4 inhibition as a mechanism of antidiabetic peptides

Antidiabetic peptides can control T2DM through several mechanisms, including regulation of incretin hormones and insulinemia levels, reduction of the activity of carbohydrate digestive enzymes and satiety response [69]. Inhibition of DPP4 (EC 3.4.14.5) is one of the mechanisms to regulate blood glucose level in diabetic patients [70]. DPP4 is an enzyme that is broadly distributed in the human body and has several roles in various physiological processes, including the regulation of incretin hormones [69]. The role of DPP4 is to inactivate

glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) through cleavage of N-termini dipeptides [69] (Figure 4).

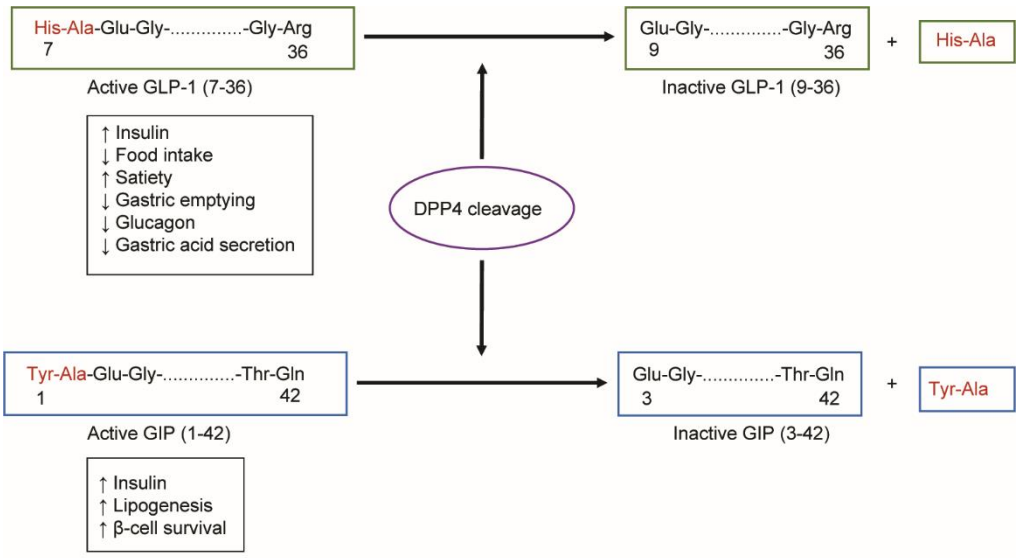

Figure 4 – Regulation of incretin hormones (GLP-1 and GIP) by DPP4. Figure modified from Patil et al. [69].

Incretin hormones GLP-1 and GIP are gut hormones that are released after a meal and are responsible for glucose homeostasis in the body [71]. These hormones promote insulin secretion in response to food intake in a glucose-dependent manner [69]. GLP-1 is also involved in inhibition of glucagon release, delay of gastric emptying and reduction of appetite [72]. Incretin hormones have short half-lives (3-5 minutes) due to the activity of DPP4 [70]. T2DM patients show reduced incretin response, which leads to higher glucagon levels after food intake, and elevated glucose levels [73]. Thus, one of the T2DM treatment strategies is the inhibition of DPP4 in order to extend half-lives and increase concentrations of GIP and GLP-1 in the circulation [70]. Table 2 shows the example of DPP4 inhibitory peptides produced from a variety of food proteins, including fish, milk, egg, beans, and wheat gluten [74]. These peptides differ in sequence, length, and inhibitory potential (half-maximal inhibitory concentration (IC_{50})).

Table 2 – Examples of DPP4 inhibitory peptides identified in hydrolysates. Table modified from Nongonierma and FitzGerald [75].

Peptide sequence	IC_{50} (μM)	Sample	References
IPI	3.2	Bovine whey protein hydrolysate	[76, 77]
VPL	15.8	Wheat gluten hydrolysates; bovine milk protein isolate hydrolysates	[21, 76, 78, 79]
INNQFLPYPY	40.1	Bighead carp muscle hydrolysate; bovine milk protein isolate hydrolysate	[21, 79-81]
GPGA	41.9	Atlantic salmon skin gelatin hydrolysate	[82]
ILAP	43.4	*Palmaria palmata* hydrolysate	[83]
WP	45	Wheat gluten hydrolysates	[78, 84]
LKPTPEGDL	45	Whey protein hydrolysate; bovine milk protein isolate hydrolysate	[21, 79, 85]
IPGDPGPPGPPGP	65.4	Tilapia skin gelatin hydrolysate	[86]
TQMVDEEIMEKFR	69.8	Mare whey protein hydrolysates	[87]
VL	74	Bovine whey protein hydrolysate; bovine milk protein isolate hydrolysates	[21, 77, 79, 88]
LPGERGRPGAPGP	76.8	Tilapia skin gelatin hydrolysate	[89]
SPQ	78.9	Wheat gluten protein hydrolysate	[90]

2.4.3. ACE-1 inhibition as a mechanism of antihypertensive peptides

Food-derived bioactive peptides can exhibit antihypertensive effect by inhibition of ACE-1 or renin [91]. Both are key enzymes in the renin-angiotensin-aldosterone system (RAAS), which regulates blood pressure. The role of RAAS is to control fluid and electrolyte balance in the body, which is done through tightly regulated effects on blood vessels, heart, and kidneys [92]. ACE-1 (EC 3.4.15.1) contributes to increased blood pressure by inducing blood vessels' constriction [93]. The main location of ACE-1 is the vascular endothelial lining of the lungs, while also present in several other body tissues [94].

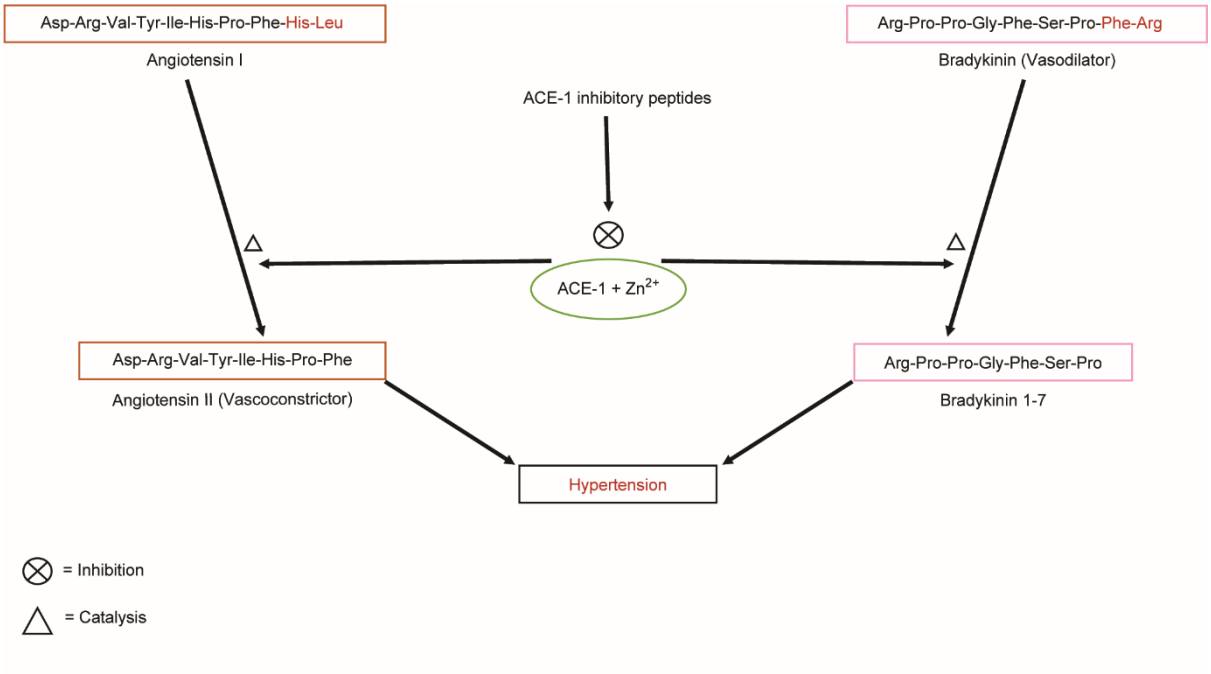

Figure 5 – Schematic representation of antihypertensive mechanism by ACE-1 inhibition. Figure modified from Mada et al. [12].

ACE-1 is a zinc protease, which converts inactive angiotensin I into a potent vasoconstrictor, angiotensin II (Figure 5). The activation is performed by cleavage of dipeptide from the C-terminus of angiotensin I. ACE-1 is also responsible for degradation of bradykinin, a known vasodilator, by removing a dipeptide from the C-terminus [12, 94]. Some food-derived bioactive peptides are reported to inhibit ACE-1, thus preventing the degradation of angiotensin I and bradykinin [95]. Xue et al. [95] summarised ACE-1 inhibitory peptides derived from a variety of foods including chicken meat, dairy, fish, beef, shrimp, soybean, and egg (Table 3).

Table 3 – Examples of ACE-1 inhibitory peptides derived from food-hydrolysates. Table modified from Xue et al. [95].

Peptide sequence	Origin	IC_{50} (μM)	Reference
GPL; GPV	Bovine Skin gelatin	2.6; 4.7	[96]
VGPV; GPRGF	Bovine collagen	405.1; 200.9	[97]
IKW	Chicken	0.2	[98]
IY; IKP	Bonito	2.1; 1.6	[98]
LKP	Chicken/fish muscle	0.3	[98-100]
VAP	Grass carp	16.6	[101]
QIGLF	Egg white protein	75.0	[102]
MKP	Casein	0.4	[103]
LSW	Soybean	2.7	[104, 105]
VNP; VWP	Rice	6.4; 4.5	[106]
FQLPKF; GFPTLKIF	Barley	28.2; 41.2	[107]
LY	Rapeseed	110.0	[108]

2.4.4. Mechanisms of action of antioxidant peptides

An imbalance between the oxidants and the antioxidant system's capacity in an organism results in oxidative stress [109]. Certain oxidants, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), play important roles in the maintenance of the host defense system, maturation processes of cellular structures, and DNA repair mechanisms [110]. Thus, the presence of ROS and RNS in the body is vital, but both excess and very low levels are detrimental [109]. Oxidative stress results in alteration of the cell membranes and damage of essential biomolecules (i.e., proteins, lipids, and DNA) [110]. Such an oxidative damage can lead to the initiation of many chronic diseases including diabetes, CVDs, neurodegeneration, or tumorigenesis [111, 112].

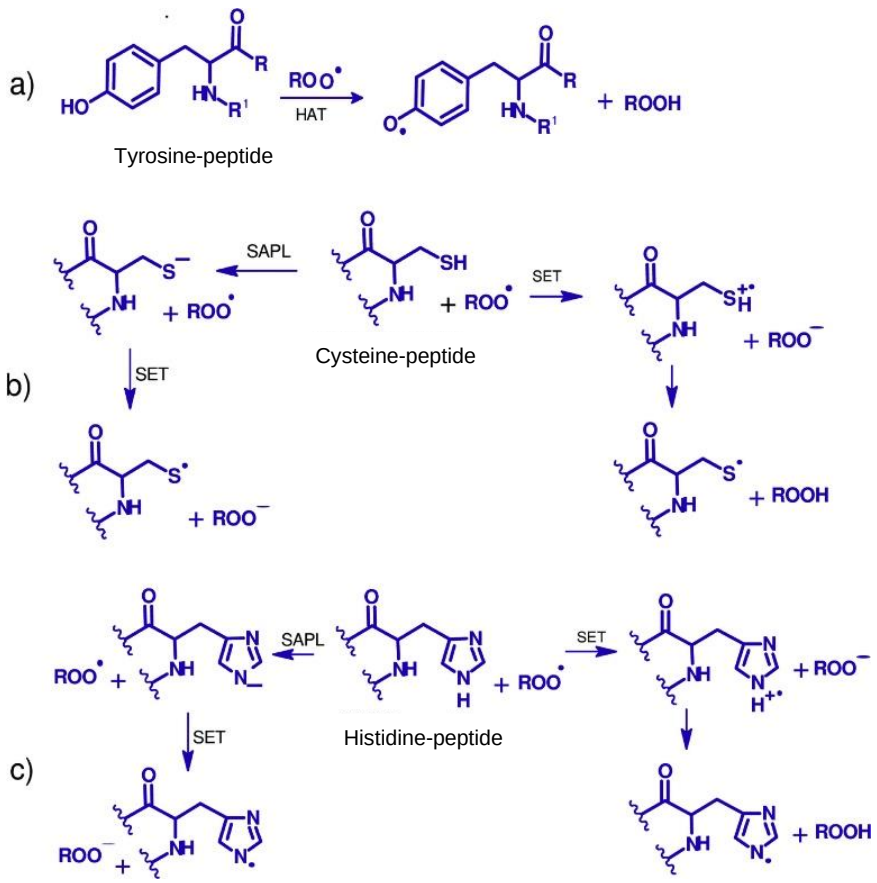

Figure 6 – Radical scavenging mechanisms of peptides: hydrogen atom transfer (HAT) mechanism by a tyrosine containing peptide (A), single electron transfer (SET) mechanism with (left) and without (right) solvent-assisted proton loss (SAPL) by a cysteine containing peptide (b) and a histidine containing peptide (c). Figure reproduced from Esfandi et al. [109].

As illustrated in Figure 6, bioactive peptides can neutralize free radicals by two main mechanisms: hydrogen atom transfer (HAT) and single electron transfer (SET) [109]. Depending on the environmental conditions (pH and acid-base properties), the proton is either directly transferred between the reacting moieties or is involved in solvent-assisted proton loss [109]. The mechanism of action of HAT is characteristic of peptides containing tyrosine, while SET is characteristic of peptides with histidine, cysteine, and tryptophan [109]. In addition to radical species, lipid oxidation in biological systems or food can be initiated by transition metals, such as copper and ferrous ions [109]. Esfandi et al. [109] showed that bioactive peptides can inhibit lipid oxidation by metal chelation. Wei et al. [113] also demonstrated that tetrapeptide DHHQ has a protective action against the oxidation of low-density lipoprotein induced by Cu^{2+} using a chelating mechanism. Antioxidant peptides have been reported from a variety of food proteins including fish, milk, shrimp, soybean, and sweet potato [114] (Table 4).

Table 4 – Examples of antioxidant peptides derived from food-hydrolysis.

Peptide sequence	**Sample**	**References**
LLSGTQNQPSFLSGF, NSLTLPILRYL, TLEPNSVFLPVLLH	Lentil protein	[115]
YSK	Rice bran protein	[116]
VLYSTPVKMWEPGR, VITVVATAGSETMR, HIGININSR	*Tinospora cordifolia* stem proteins	[117]
PGPIPN, PFPGPIPN, YPFPGPIP, VYPFPGPIPN, MPFPKYPVEP, EPVLGPVRGPFP, QEPVLGPVRGPFP, TPVVVPPFLQPE, TQTPVVVPPFLQPE	Casein from bovine milk	[118]
AEERYP, DEDTQAMP	Chicken egg white	[119]
VLPVPQK	Buffalo milk casein	[120]
RPNYTDA, TSQLLSDQ, TRTGDPFF, NFHPQ	Rice	[121]
VCSV, CAAP	Flounder fish (*Paralichthys olivaceus*)	[122]
LANAK, PLSVGRPPVGKLTL, VKVLLEHPVL	Oyster	[123]
CSQAPLA, YPKLAPNE, YPQLLPNE	Corn gluten	[124]
CQV, QCV, QVC, QCA	Rye secalin	[125]

Chapter 3 Bioactive peptide discovery platform

Health claims for food or food constituents are regulated and controlled by countries' regulatory authorities. For example, in the European Union, the Panel on Dietetic Products Nutrition and Allergies (NDA) of EFSA is responsible for regulating health claims in food products [126]. Under the regulation by EFSA, health claims are divided into two categories: nutritional and health claims [44]. Some of the examples of nutritional claims are 'source of protein', 'high protein', or 'low sugars' [126]. Health claims are subdivided into three categories: general function claims, disease risk reduction claims, and claims relating to children's development [127]. Examples of NDA-authorized health claims include 'docosahexaenoic acid contributes to the maintenance of normal vision' and 'beta-glucans contribute to the maintenance of normal blood cholesterol levels' [128]. Bioactive peptides lie under the category of food constituents and, therefore, a product containing bioactive peptides can be submitted for a health claim evaluation. To claim a health effect on a product, a dossier with supporting information needs to be assembled. While human studies play a key role in the scientific evidence of health claims, the characterization of an active constituent is also evaluated [126]. Upon submission of a health claim application, an NDA's expert panel carries out a thorough scientific assessment [126].

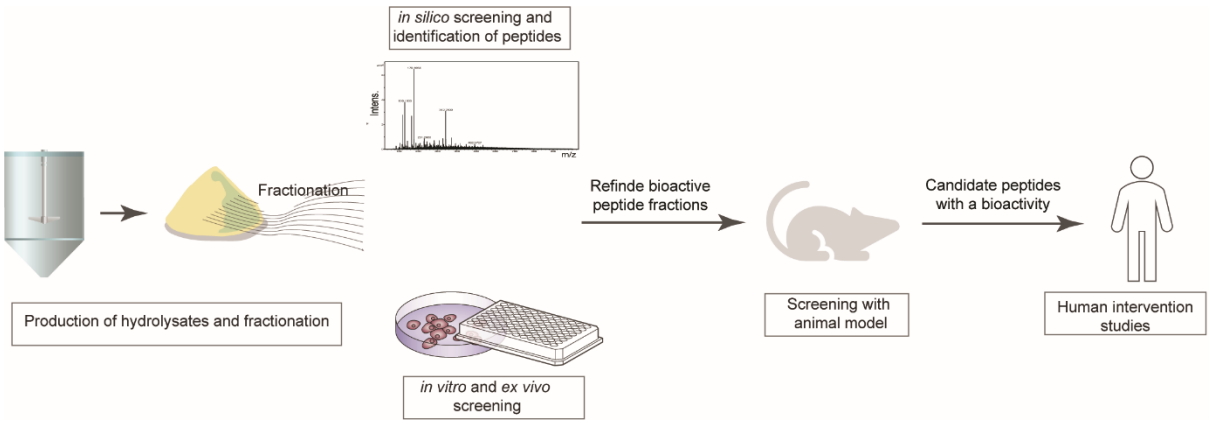

Figure 7 – Pipeline for the preparation of a health claim application for bioactive peptides based on EFSA recommendations. Figure based on Chalamaiah et al. [44].

A promising application of food-derived bioactive peptides lies in functional foods and nutraceuticals [44]. The process from identification of a bioactive peptide to a product with health effect in humans can be divided into several steps (Figure 7). The first step in the search for bioactive peptides is the identification of a potential food protein source. The second step entails the generation of peptides using, for example, enzymatic hydrolysis. The third step focuses on the characterization of these peptides. In the next step, bioactivities are evaluated

using *in vitro* models, followed by animal models. Finally, the clinical efficacy of the candidate ingredient must be studied in a human intervention study. Following these studies, the types of health claims are formulated, and a comprehensive review of the available evidence is conducted, including the identification of any gaps in the data. When all necessary information is obtained, the health claim application can be submitted to the regulatory authority. This chapter discusses the state-of-the-art discovery platforms relevant to the scope of the dissertation.

3.1. Screening for bioactive peptides: conventional vs *in silico* approaches

Discovery of new bioactive peptides from food-derived or novel proteins is often divided into two approaches: conventional and *in silico* (Figure 8).

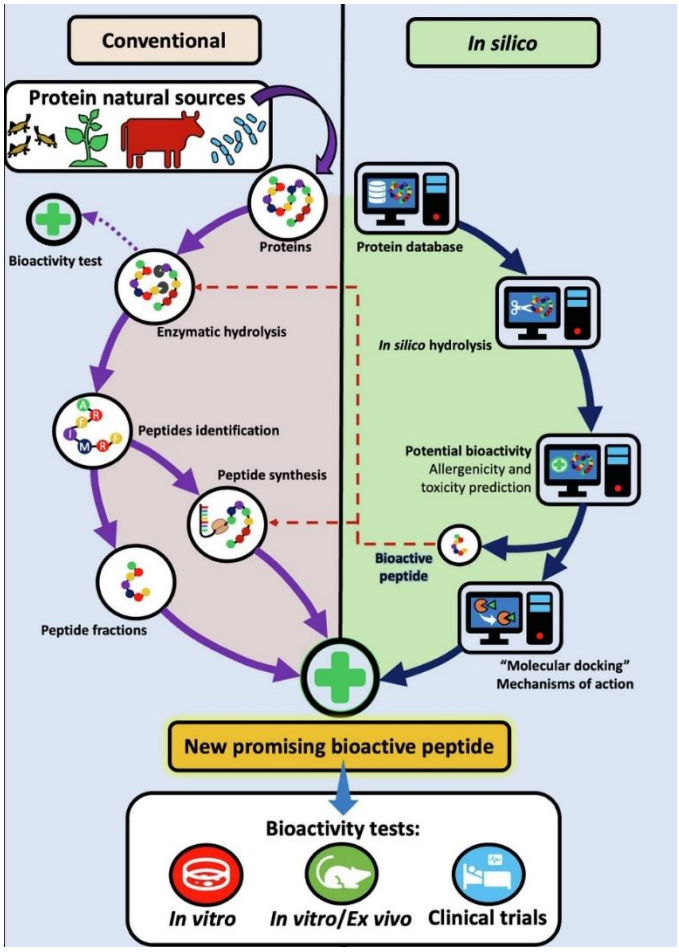

Figure 8 – Schematic presentation of a conventional approach (to the left), an in silico *approach (to the right), and a hybrid approach (red dashed line) for discovery of new bioactive peptides from food-derived proteins. Figure reproduced from Peredo-Lovillo et al. [41].*

The *conventional approach* is an empirical approach which consists of several steps (Figure 8). The first step is to choose a protein source and an enzyme followed by the liberation of encrypted bioactive peptides. Afterward, the evaluation of bioactivities of interest is performed using a variety of *in vitro* assays. The next step is the identification of bioactive peptides using bioactivity-guided fractionation. The fractionation is typically performed using different preparative chromatographic techniques. Size exclusion, reversed-phase, and ion exchange are the most common chromatographic techniques used for the fractionation and isolation of bioactive peptides [12]. Due to the high degree of complexity, purification of specific peptides from a crude hydrolysate often requires several cycles of fractionation steps using more than one separation technique. After each fractionation step, bioactivity is evaluated and the fraction with the highest potency is taken to the next fractionation step. When there is a pure peptide or a few peptides in a fraction, the identification is typically performed using mass spectrometry [12]. The potency of purified inhibitory peptides is then determined using *in vitro* bioassays. If there is more than one peptide in the isolated fraction, a verification of structure and bioactivity is performed using synthetic peptides.

This conventional approach has been successfully used for the discovery of bioactive peptides from different protein sources. For example, the identification of ACE-1 inhibitory and anticoagulant peptides from protein hydrolysate of poultry slaughter waste (combs and wattles) [129]. Another example is the discovery of two peptides (Pro-Ala-Leu and Lys-Val-Glu-Pro-Leu-Pro) with dual ACE-1 and DPP4 inhibitory properties in a hydrolysate of Antarctic krill protein [130].

The *in silico approach* represents a series of bioinformatics tools that can provide accelerated discovery of bioactive peptides (Figure 8) [41]. The first step in this approach is a selection of the source proteins and accessing the protein sequence from databases, such as UniProtKB [131] and the National Center for Biotechnology Information [132]. Afterward, the simulation of enzymatic hydrolysis is performed using computational tools, such as *'enzyme(s) action'* in the BIOPEP-UWM database [40] to predict the protein breakdown and generate a list of peptides. When all possible peptides are identified, their selected bioactivities are predicted using computational tools, such as PeptideRanker [133] or qualitative structure-activity relationship (QSAR) [41]. The QSAR method builds on a recognized connection between the structural characteristics of a peptide and its chemical or biological properties [134]. The results obtained using *in silico* methods need to be verified using *in vitro* and *in vivo* studies [41]. The potential mechanism of interaction of bioactive peptides with their targets can be elucidated

using a molecular docking tool, which allows to predict the binding mode of peptides with enzymes of interest (e.g., ACE-1 or DPP4) and to estimate affinity of a peptide within the enzymes' binding sites [41, 134]. Another *in silico* method focuses on the statistical optimization of bioactive peptides' release performed using the design of experiments together with response surface methodology [17].

One example of an *in silico* approach is the identification of novel DPP4 inhibitory peptides by using the QSAR model to evaluate peptide analogs of Ile-Pro-Ile [135]. Another example is an evaluation of the antioxidant activity of peptides from pea protein hydrolysate with PeptideRanker [136].

Both conventional and *in silico* approaches have limitations. The conventional approach is a time-consuming, expensive, laborious process, which also lacks the possibility to control all variables and includes guessing factors [17, 41]. In turn, the application of the *in silico* approach is restricted to the number of proteins and bioactive peptides available in databases and the number of proteolytic enzymes present in online cutter tools [17]. Further, enzyme cutter tools do not take into account processing parameters (pH, enzyme-to-substrate ratio, temperature, hydrolysis time, etc.) which contribute to the variety of peptides potentially released [17]. In fact, several studies using QSAR models have reported large differences between predicted and experimental IC_{50} values for DPP4 and ACE-1 inhibitions [135, 137-139]. Therefore, Nongonierma et al. [135], emphasized that it is important to include physiochemical parameters when building the model. In addition, the mode of action of the inhibitory peptides has to be considered when selecting peptides for docking studies, peptides with non-competitive mode of action should not be used for docking analysis [17].

Advantages of the conventional approach relative to the *in silico* include the possibility to identify bioactive peptides from proteins not listed in the databases, also using enzyme preparations that contain several proteases or proteases not available in enzyme cutter online tools. Additionally, hydrolysis conditions (temperature, enzyme-to-substrate ratio, pH, etc.), and substrate composition (pure proteins vs complex raw materials) are taken into account under *in vitro* hydrolysis. The main advantages of the *in silico* approach include much faster results acquisition and lower usage of chemicals and reagents [17].

A hybrid approach also exists (red dashed line in Figure 8), where *in silico* and conventional methods are combined [41]. Peredo-Lovillo et al. [41] are convinced that this combination approach can improve the identification and prediction of new promising bioactive peptides.

An example of a study that combines methods from both approaches is identification and purification of antioxidant and antidiabetic peptides from protein hydrolysates of chickpea [140]. Gui et al. [141] also identified and purified antioxidant, DPP4, and ACE-1 inhibitory peptides from sturgeon skin hydrolysate with combined methods. In both studies, production, purification, and bioactivity evaluation of bioactive peptides were performed using methods from the conventional approach, and molecular docking analysis was performed with *in silico* approach studying the interaction between enzymes and inhibitory peptides interactions [140, 141].

Selection of approach or methods should be based on the scientific question and starting conditions. In the present work, the conventional approach was used. The choice was made based on the complexity of raw material and enzyme preparation with unknown specificity of proteases.

3.2. Characterization of hydrolysates with bioactive peptides

The analysis of bioactivity and chemical characteristics of protein hydrolysates provides detailed information for understanding the potential health benefits of protein hydrolysates. This information is crucial for both the optimization of the hydrolysis process and the development of functional food ingredients or nutraceuticals.

3.2.1. Bioactivity

In vitro assays are used as a first screening tool to select candidate molecules/leads based on their bioactivities. *In vitro* methods represent simplified models of intricate biological systems that enable higher throughput and facilitate the identification of mechanisms of action [142]. However, understanding the limitations of such models is vital for interpretation of results. Certain aspects of bioactivity assays, which should be considered when interpreting and comparing results to others, are discussed below using examples from ACE-1 inhibition, DPP4 inhibition, and DPPH radical scavenging assays.

Both ACE-1 and DPP4 inhibition are biochemical assays based on enzymes as therapeutic targets. In such assays, the IC_{50} value can be determined to compare the efficacy of several candidate molecules. Assay parameters, such as enzyme-to-substrate ratio, substrate type, buffer, detection method, influence the IC_{50} value [75, 143, 144]. Therefore, it is essential that the assays were performed under the same conditions when comparing peptides from different studies. Henda et al. [144] evaluated the influence of three substrates for ACE-1 inhibitory

assay: two synthetic – N-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly (FAPGG) and N-Hippuryl-His-Leu hydrate (HHL) – and one natural – angiotensin I. Their study (Table 5) demonstrated that IC_{50} values for eleven peptides and captopril (a therapeutic drug), were not consistent when different substrates were employed [144]. Interestingly, some of the evaluated peptides showed IC_{50} values that were either lower or equal to that of captopril when angiotensin I was used as a substrate. Henda et al. [144] also highlighted the importance of reporting the substrate and the results for reference molecules (positive and negative controls) to facilitate interstudy comparison.

Table 5 – Examples of half-maximal inhibitory concentrations (μM) of captopril, peptides, and losartan (used as a negative control). Table adapted from Henda et al. [144].

ACE-1 substrate drug/peptide sequence	**FAPGG**	**HHL**	**angiotensin-I**
captopril	0.00179 ± 0.0003	0.0151 ± 0.005	16.71 ± 1.9
Losartan (negative control)	17.13 ± 1.4	49.61 ± 3.7	146 ± 11
VY	1.64 ± 0.2	0.067 ± 0.009	0.22 ± 0.01
IY	140 ± 8	0.88 ± 0.08	1.03 ± 0.06
GPL	3.31 ± 0.4	1020 ± 9	1074 ± 7

Another important aspect of enzyme assays that can affect the determination of an IC_{50} value of a peptide, comes from different modes that a molecule can inhibit an enzyme. Enzymes can be inhibited either reversibly or irreversibly with several modes [145]. The reversible inhibition is the main focus for food-derived bioactive peptides, where competitive, non-competitive, uncompetitive, and mixed modes of inhibition have been explored [143]. Different modes of inhibition have distinct effects on the kinetics of the enzyme-substrate interaction [143], and they may influence the interpretation of IC_{50} values. The outlined types of reversible inhibition modes can be identified by studying changes in kinetic parameters (K_m and V_{max}) [145].

A *competitive inhibitor* acts by competing with the substrate for the active site of an enzyme. When the inhibitor occupies the active site, the substrate is prevented from binding to the enzyme [145] (Figure 9A). A competitive inhibitor is often structurally similar to the substrate, the inhibitor forms an enzyme-inhibitor complex but without catalytic reaction [145]. This mode of inhibition is dependent on the substrate concentration since an increase in the amount of substrate will outcompete the inhibitor [145]. Therefore, the amount of substrate present in the assay influences the IC_{50} value – an increase in the substrate concentration causes an increase in the IC_{50} value [146]. An example of a competitive inhibitor for DPP4 is a tripeptide IPI [147].

An *uncompetitive inhibitor* binds to a site distinct from the active site of an enzyme and specifically interacts with the enzyme-substrate complex, thereby preventing the enzyme from reacting with its substrate [145] (Figure 9B). Lan et al. [148] tested 19 tripeptides, Trp-Arg-Xaa, where Xaa represents 19 different amino acids. They reported that all 19 tripeptides were uncompetitive inhibitors for DPP4, with Trp-Arg-Glu identified as the most potent one [148]. A *mixed inhibitor* binds to a site distinct from the active site and can bind to both the enzyme-substrate complex and the enzyme while exhibiting different affinities for one or the other [149] (Figure 9C). A peptide, YYGYTGAFR, from salmon skin showed a mixed mode of inhibition of DPP4 [150]. A *non-competitive inhibitor* is a special case of a mixed inhibitor when an inhibitor binds equally well to the enzyme-substrate complex or the enzyme [149]. Two ACE-1 inhibitory peptides, VGPV and GPRGF from bovine collagen, were reported to act in non-competitive mode [97].

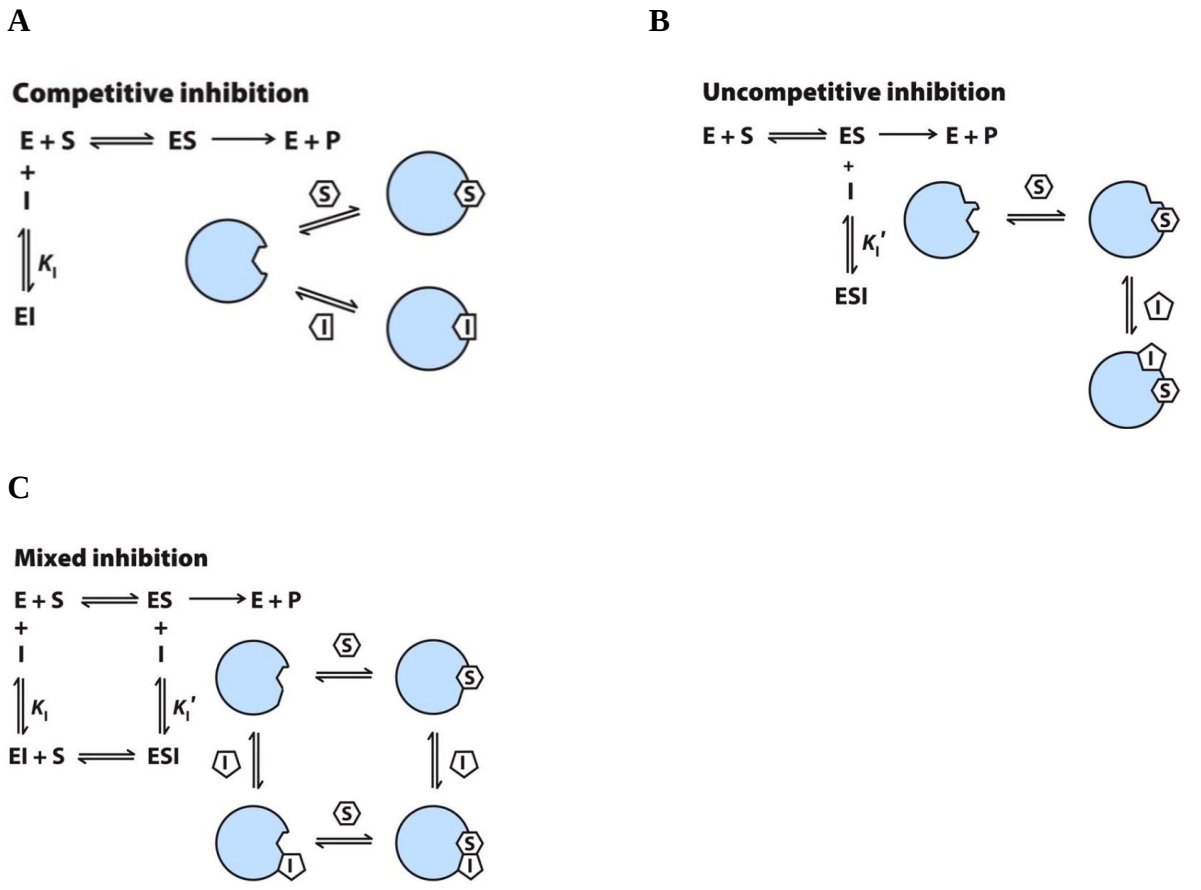

Figure 9 – Types of reversible inhibitors: competitive (A), uncompetitive (B), and mixed (C) inhibition. K – the equilibrium constant, E – enzyme, S – substrate, P – product, I – inhibitor, ES – enzyme-substrate complex, EI – enzyme-inhibitor complex, ESI – enzyme-substrate-inhibitor complex. Figure reproduced from Nelson and Cox [145].

DPPH radical scavenging assay is a chemical assay where antioxidant activity is attributed mainly to the SET mechanism [151]. The DPPH radical is a stable, nitrogen-centered, and sterically hindered radical [152]. These characteristics differ DPPH radicals from *in vivo* targets which are short-lived, small, and readily accessible radicals such as superoxide ($O_2^{-\bullet}$), hydroxyl radicals ($HO^{\bullet}$), or lipid oxyl radicals. [153]. Therefore, this assay provides information on the intrinsic antioxidant capacity of candidate molecules in a system with minimal environmental interference [151]. Results are often reported as the effective concentration required to decrease the initial DPPH radical concentration by 50% [151]. Correct assessment of antioxidant activity requires understanding of the influence of assay conditions on the results, such conditions include pH, solvent, reagent concentrations, light, temperature, oxygen [151, 152]. The electron transfer mechanism is pH-dependent, the rate increases with increasing pH and degree of ionization [153]. The DPPH radical is a hydrophobic molecule and requires an organic solvent such as methanol [152]. When the assay is run in methanol, the HAT mechanism is inhibited because hydrogen atoms are strongly bound. However, when the assay is performed with 50% methanol, water disrupts the binding and facilitates hydrogen atom transfer making the HAT reactions possible [152].

The examples above demonstrate how assay parameters can influence the results. This highlights the need for a detailed description of assay parameters and the establishment of standardized protocols to facilitate feasible interstudy comparisons. A meaningful comparison of results from different laboratories is essential for improving the translatability of *in vitro* studies to *in vivo*.

3.2.2. Chemical characterization

Chemical characteristics – such as peptide chain length, amino acid composition and sequence – are factors of the utmost importance for the determination of peptide bioactivity. Therefore, several chemical parameters of a protein hydrolysate are usually evaluated to get an insight into the bioactive potential including degree of hydrolysis (DH), molecular weight distribution (MWD), protein content, amino acid composition, and sequences of peptides. The DH is used to measure the extent of protein degradation and is defined as "the proportion of the total number of peptide bonds that are cleaved during hydrolysis" [154]. The most common methods to determine the DH are *o*-phthaldialdehyde (OPA), trinitrobenzensulfonic acid (TNBS), and pH stat [155]. Both the OPA and the TNBS methods are based on derivatization of primary amino groups, thus quantifying the amount of free amino groups released as a result of

hydrolysis [155]. The pH stat method is a titration method in which the release of protons is detected by a change in pH [156]. The amount of broken peptide bonds is estimated from the quantity of base used to maintain a constant pH during the hydrolysis [155]. The method to determine DH must be selected based on the advantages and disadvantages of each method, including considerations of the enzyme type and protein substrate. For example, the TNBS method has shown optimal results when estimating the DH value of whey protein hydrolysate [155]. Since a DH value is a relative measurement, which depends on the number of peptide bonds in the starting material, the total number of peptide bonds present in the substrate protein has to be estimated and this estimation may cause inaccuracy [154].

An alternative approach to characterize the degree of protein breakdown in a given hydrolysate is a MWD of peptides [157]. Typically, size exclusion chromatography (SEC) is used to obtain the MWD of a hydrolysate. A comparison of DH with MWD for monitoring of hydrolysis shows that the MWD parameters (specifically average molecular weight) provide more comprehensive information about the process [158]. Notably, a connection between bioactive properties and the average molecular weight of hydrolysates has been demonstrated in several studies [159-162].

It is important to determine the amount of protein in a sample. A variety of methods exist to estimate *protein content* in a hydrolysate including Kjeldahl nitrogen and Dumas combustion methods. Both methods report a total nitrogen content by using a nitrogen-to-protein conversion factor [163]. A source-specific conversion factor ensures a more accurate estimation of protein content because the relative nitrogen content in a protein depends on the amino acid composition [164]. Secondly, the presence of non-protein nitrogen-containing compounds (such as amino acids, ammonia) can lead to overestimation of the protein content [163].

The *amino acid composition* of a hydrolysate is another parameter that is often evaluated due to its importance for the nutritional value and bioactive potential of a hydrolysate. Various methods are employed to determine amino acid composition, given the necessity to adapt to the different stability of individual amino acids and the resistance of certain peptide bonds to the hydrolysis procedure [165]. Total hydrolysis with strong acid or base followed by liquid chromatography is currently the most commonly used method for separating and quantifying amino acids [165].

The *amino acid sequence* of a peptide is a crucial factor for its bioactivity. Tandem mass spectrometry (MS/MS) is widely used for identification of amino acid sequences [143]. The sequence of a peptide can be identified in two ways: database-assisted and *de novo* sequencing [143]. In the case of database-assisted sequencing, it is limited to the proteins in the databases, while *de novo* sequencing allows for the identification of peptides from unknown proteins and short-chain peptides (<5 amino acids) [143]. Short bioactive peptides are particularly interesting because they have generally improved bioavailability compared to larger peptides [166]. However, the identification of peptide sequences in the range of 2 to 4 amino acids remains a challenging task [166].

Another tool that has been proven successful in characterization of crude protein hydrolysates is Fourier transform infrared spectroscopy (FTIR). Infrared (IR) absorption spectra of proteins and peptides contain information about the amino acids side chains, peptide backbone, and their secondary structure [167-170]. FTIR has therefore been an excellent tool for monitoring hydrolysis processes and obtaining information on the structural characteristics of the resulting peptides [171-174]. Multivariate statistical models based on IR spectra have been utilized to predict various characteristics of protein hydrolysates, such as average molecular weight, DH, solubility, foaming properties, emulsification, bitterness, and also end product quality [157, 158, 175-177].

3.3. Bioavailability of bioactive peptides

When bioactive peptides are identified, the next step is to evaluate their bioavailability. It is noteworthy that the preferred way of administration for bioactive protein hydrolysates is the oral route [178]. Oral bioavailability is defined as the proportion of the orally administered dose that reaches systemic circulation in an unchanged form, which becomes available at its target site of action to produce the desired therapeutic effect [179]. The oral administration route poses multiple barriers for bioactive peptides to reach their targets. A bioactive peptide must escape metabolism in the gastrointestinal (GI) tract and in the liver to be able to interact with its target. Thus, GI stability, intestinal absorption and liver metabolism need to be evaluated.

3.3.1. Gastrointestinal stability

The environment in the GI tract is harsh due to the digestive enzymes and the low pH in the gastric compartment [180]. Stability challenges for peptides arise from a number of enzymes that exist in the human digestive system to degrade proteins and peptides (Figure 10). In the gastric compartment of the GI tract, the primary protein-degrading enzyme is pepsin [180]. In the intestinal compartment, the trypsin, chymotrypsin, and carboxypeptidases are the main enzymes in the pancreatic juice, while brush border peptidases are located on the epithelial cells [180]. It is generally recognized that low molecular weight peptides are more resistant to the GI conditions compared to larger peptides and proteins [59, 181]. Wang et al. [182] reported that relatively small peptides, an ACE-1 inhibitory peptide (PTGNPLSP) and an antioxidant peptide (VTAGLVGGGAGK) identified in the hydrolysate of blue-green microalgae, have strong stability in the GI tract. Fan et al. [183] also studied ACE-1 inhibitory peptides derived from hydrolysis of egg white proteins and reported that six ACE-1 inhibitory peptides with short amino acid sequences (LAPYK, LKISQ, LKYAT, INKVVR, LFLIKH, and LGHWVY) had good GI stability. Liao et al. [184] identified an ACE-2 upregulating peptide, AKSLSDRFSY from a pea protein hydrolysate, that was resistant to pepsin degradation but susceptible to proteases in pancreatin.

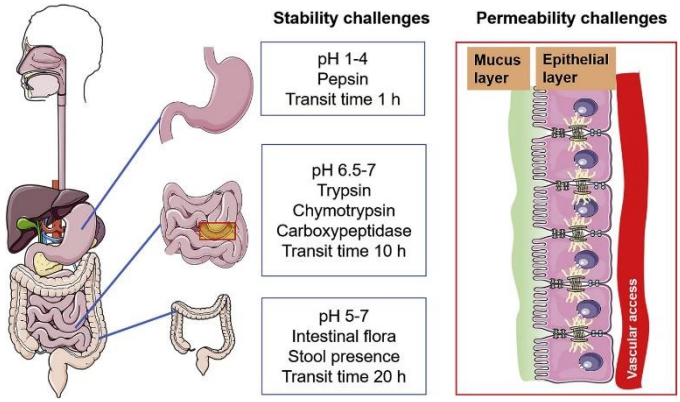

Figure 10 – Physiological barriers in the GI tract for bioavailability of peptides. Stability challenges associated with pH gradients and digestive enzymes. Permeability challenges posed by mucus layers and epithelial layers. Figure reproduced from Zhu et al. [180].

Gastrointestinal stability of bioactive peptides can be evaluated using *in vitro* digestion methods. There are three types of such methods: static, semi-dynamic and dynamic methods [185]. *In vitro* methods simulate physiological conditions *in vivo* (such as digestive enzymes and their concetrations, pH, digestion time, salt concetrations) typically of oral, gastric, and intestinal phases [186]. Simulated digestion is used to evaluate the digestability of foods and the stability of certain compounds (such as bioactive peptides or pharmaceuticals) [186]. The

static method is the simplest one, allowing a relatively large number of samples to be run in parallel. Therefore, it fits well for a screening purpose of a bioactivity discovery platform. Several models employing different parameters such as enzyme sources (human, porcine, and rabbit origin), activities of enzymes, digestion times, pH, and composition of digestive fluids for static *in vitro* digestion have been developed [186]. Because of the complexity however, it is difficult to compare interstudy results. Therefore, the international network INFOGEST, which focuses on food digestion research, has come to an international consensus about a standardized static model. This model standardization allows for the comparison of data between laboratories [186, 187]. The INFOGEST static method proposes a set of key parameters based on available physiological data for the relevant conditions of the upper GI tract (oral, gastric, intestinal phases) [187]. This method provides assessment of endpoints of each digestion phase, while the more complex semi-dynamic and dynamic models are better suited to assess digestion kinetics [185]. There are, however, limitations of the INFOGEST static method and these should be considered when interpreting results. In the case of the evaluation of bioactive peptides' stability, the biggest challenge is the lack of brush border peptidases, which are necessary for completing the digestion process [186].

3.3.2. Intestinal permeability of bioactive peptides

The next physiological barrier encountered by bioactive peptides on their route to target sites is the intestinal epithelial monolayer (Figure 10). The intestinal epithelial monolayer has three main functions: absorption of nutrients and food components (such as amino acids, peptides, glucose, vitamins), barrier function with detoxification systems and efflux transporters, and signal recognition and transduction [188]. The epithelial monolayer consists of several cell types, each with specific functions. Enterocytes, constituting approximately 90% of the cells in the intestinal monolayer, serve as the primary absorptive cells [180]. Goblet cells, the second most abundant type, are responsible for mucus secretion [189]. The challenges for the transport of intact bioactive peptides arise from the presence of a mucus layer, which impedes the diffusion of peptides towards the cell monolayer; brush border peptidases located on the microvilli of the epithelial cells; and cytosol peptidases if a bioactive peptide enters the cell [180].

While the intestinal permeability of bioactive peptides is commonly recognized as low with estimates generally indicating less than 1% [8, 190], the availability of bioactive peptides upon oral administration has been demonstrated in human trials and animal models, although in the

nanomolar and picomolar range [59, 191]. Bioactive peptides can be transported across intestinal monolayer via several routes (Figure 11): carrier-mediated transport, paracellular diffusion, transcytosis, and passive transcellular diffusion [190].

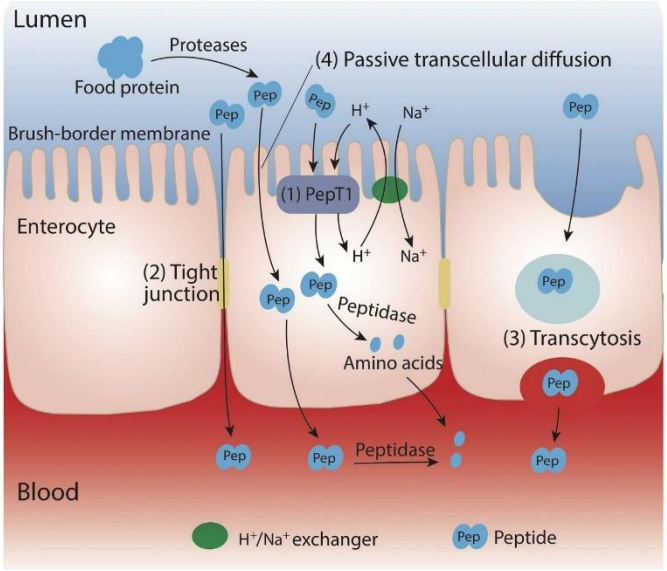

Figure 11 – Four possible routes for the transport of bioactive peptides across an intestinal epithelial cell monolayer include: the carrier-mediated route (1), the paracellular route via tight junctions (2), transcytosis via vesicles (3), and passive transcellular diffusion (4). Brush border peptidases, peptidases present in the cytosol and the bloodstream can hydrolyze peptides. Figure reproduced from Xu et al. [190].

Carrier-mediated transport is performed by specific cell membrane proteins that transport peptides against concentration gradients [192]. One of such carrier is transporter 1 (PepT1), a high capacity and low-affinity proton-coupled peptide transporter [190]. Thousands of di- and tripeptides can be recognized and taken up into the cells by PepT1 [181, 192]. In addition to the peptide length, amino acid composition, charge and characteristics of side chains influence the affinity between a peptide and the PepT1 [181]. For example, it has been demonstrated that neutral peptides have the highest affinity, while negatively charged peptides have the weakest affinity for the PepT1 transporter [181]. Not all di- and tripeptides can be transported by PepT1. For example, it was reported that bovine PepT1 was not able to transport peptides KK and KWK [193]. Studies show that certain peptides (FY, YY, and MPP) serve as high-affinity inhibitors of human PepT1 [194, 195]. Peptides transported by PepT1 into the cells have several fates. In the cytosol, some peptides will be degraded by peptidases. Peptides that are resistant towards this hydrolysis can be transported to the basolateral (BL) side either by H^+/Na^+ exchanger, or it is hypothesized that peptides can also be transported by BL carriers [190].

Paracellular diffusion is an energy-independent route through water-filled pores/channels between cells, mediated by tight junctions [181]. The diameter of pores in tight junctions ranges from 0.4 – 0.9 nm in the villi to 5 – 6 nm in the crypts of the intestinal membrane [196]. The estimated pore diameter of the Caco-2 cell monolayer is within the range of 1.2 – 2.1 nm [190, 197]. The paracellular permeability of a peptide is influenced by its structural characteristics, such as molecular dimensions, overall charge, hydrophilicity, and conformational flexibility [181]. It has been reported that low molecular weight, hydrophilic, and negatively charged peptides can utilize paracellular diffusion route [192].

Transcytosis is an energy-dependent transport mechanism that involves endocytotic uptake on the apical (AP) side, transcytotic transport via internalized vesicles, and secretion on the BL side [192]. It is considered that transcytosis favours the transport of long-chained (>4 amino acids) and hydrophobic peptides [192].

Passive transcellular diffusion is an energy-independent and concentration-based transport route via AP and BL membranes [192]. It is widely recognized that lipophilicity is a key factor for this transport route [192]. Characteristics of peptides such as hydrophobicity, charge, and size are crucial for the passive diffusion [192].

The human colon carcinoma cell line, Cancer coli-2 (Caco-2), is widely utilized to study *in vitro* intestinal permeability and transport mechanisms of drugs and other molecules, including bioactive peptides [198, 199]. Cultured on permeable membrane inserts, Caco-2 cells spontaneously differentiate into a monolayer with a phenotype similar to that of small intestinal enterocytes [198, 200]. The differentiated Caco-2 cells form microvilli structures on the AP side and tight junctions between the cells. They also produce many of the brush border enzymes and transport proteins that are responsible for the active transport and efflux of drugs in the intestine [188, 198]. A variety of bioactive peptides have been reported to cross Caco-2 cell monolayers using different routes [190]. F. Xu et al. [201] reported, for example, that an antioxidant peptide (WDHHAPQLR) from rapeseed protein was transported through the paracellular pathway with an estimated absolute bioavailability of 3.56%. Similarly, Miguel et al. [262] demonstrated that an antihypertensive tripeptide (YPI) from egg protein crossed the Caco-2 cell monolayer (approximately 0.38%) using the PepT1 transporter.

The main advantage of employing the Caco-2 cell monolayer model is its relative simplicity which allows for screening and mechanistic studies [200]. However, there are several differences between the Caco-2 cell monolayer model and *in vivo* which should be considered

when interpreting the results. The first difference is the number of cell types. Physiological intestinal epithelium consists of several different cell types [200], while the Caco-2 cell monolayer models consist of a single cell type. Other differences include the lower permeability of the Caco-2 cell monolayers, the lack of a mucus layer and certain transporter functions in comparison to the human intestinal epithelium [188, 190, 200]. Nevertheless, because of the advantages, the Caco-2 cell monolayers have been a popular tool to study the permeability of bioactive peptides with various methods. Some studies employ a range of experimental conditions, including different transport media, transport times, and concentrations of peptides [202-207]. In addition, variations in growth conditions influence the expression of transporters, which play a key role in intestinal transport [199, 208]. This makes it difficult to compare the results obtained, and therefore, method standardization is crucial for the reproducibility and interstudy comparison of results.

Chapter 4 Aim of the study

The main aim of the dissertation was to develop and implement bioanalytical methods for discovery and characterization of bioactive peptides from a poultry by-product protein hydrolysate. Special focus was placed on the bioactive effects towards therapeutic targets related to CMDs. To achieve the aim, hydrolysates were produced, screened, and characterized with following objectives:

(1) Produce a library of hydrolysates from MDCR using different processing conditions (enzyme choices, time of hydrolysis) and evaluate bioactivities of the hydrolysates (**paper I**).
(2) Characterize crude hydrolysates and develop predictive models of bioactivities based on FTIR and SEC (**paper I**).
(3) Isolate and characterize peptides with promising ACE-1 and DPP4 inhibitory effect (**paper II**).
(4) Evaluate gastrointestinal stability and intestinal permeability of identified bioactive peptides using *in vitro* models (**paper III**).

Chapter 5 Methods

The main principles of the methods applied in the study are described in this chapter.

5.1. Laboratory scale hydrolysis of MDCR

The laboratory scale hydrolysis was performed in a 2 L reactor with ca. 1.5 L reaction volume (Figure 12B). As described in section 2.1., raw material is homogenized prior to enzymatic protein hydrolysis. However, because MDCR used in this study has already been through grinding process during the mechanical deboning (Figure 12A), homogenization process was omitted. The first step was to mix the MDCR with water in 1:2 ratio, followed by a pre-heating of the mixture to a desired temperature for a specific enzyme. At the optimum temperature, a selected enzyme was added. In this study (**paper I**), six enzyme preparations were used, and a percentage of enzyme relative to the weight of raw material varied from 1 to 5%. The hydrolysis was performed for six different durations (i.e., 10, 30, 45, 60, 90, and 120 minutes). At the end of hydrolysis, samples were taken from the reactor and the reaction was terminated by heat-inactivation. Firstly, temperature was increased rapidly (several seconds) in a microwave followed by incubation of hydrolysates in a water bath at 90℃ for 15 minutes. Samples were thereafter centrifuged to separate into oil, liquid (the protein hydrolysate) and sediment (minerals and insolubilized proteins) phases. Then, the liquid phase was collected, filtered through a Seitz T 2600 depth filter sheet and freeze-dried. An image of a MDCR hydrolysate after freeze-drying is shown in Figure 12C.

The aim was to create a library of 60 hydrolysates under various conditions, enabling the production of hydrolysates with diverse compositions of peptides and distinct chemical characteristics. The hydrolysates were examined for different chemical characteristics and bioactivities, and the relationship between these was explored (**paper I**).

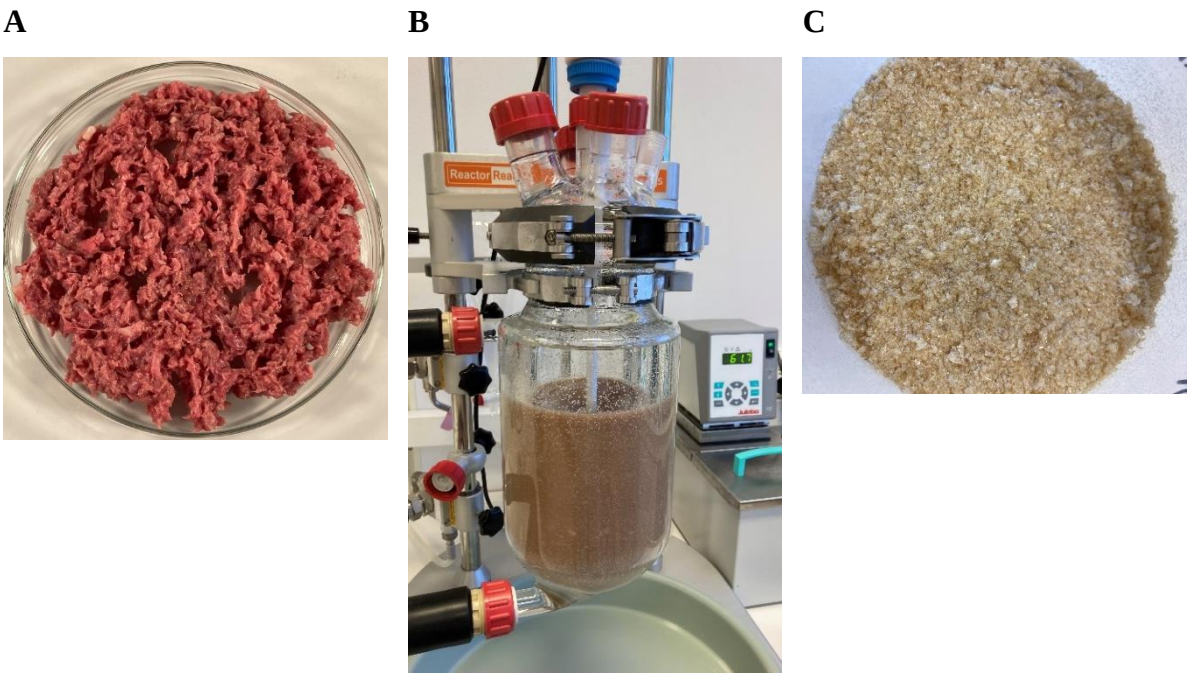

Figure 12 – Raw material (MDCR) for enzymatic hydrolysis in this study (A). Enzymatic protein hydrolysis of MDCR using a laboratory scale setup with 2 L reactor (B) and freeze-dried hydrolysate from MDCR (C).

5.2. Chemical characterization of protein hydrolysates

This section introduces the analytical methods used for characterization of hydrolysate throughout the study. These includes FTIR, high performance liquid chromatography (HPLC), and liquid chromatography-mass spectrometry (LC-MS).

5.2.1. Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy is a widely used technique for studying secondary structure of various molecules, including proteins, and their structural changes [157, 167, 169, 171]. FTIR is a vibrational spectroscopic technique, where an IR spectrum is generated based on interaction between the sample (matter) and the IR radiation [209]. Thus, each compound has a complex and unique IR spectrum called a chemical fingerprint [209]. When exposed to IR radiation, the bonds within a molecule (e.g., in a protein or a peptide) vibrate at specific frequencies [167, 210]. These frequencies depend on the bond type and the surrounding environment, leading to absorption at particular wavelengths [167, 210]. Information about the protein structure can be deduced from spectral parameters, such as band position, width, and absorption coefficient [167]. Second derivatives are commonly employed to improve resolution of the individual band components and distinguish the distinct components of individual bands [211]. Advantages of FTIR include rapid, non-destructive analysis, and relatively low cost of analysis [209, 210, 212]. However, bands often overlap, especially for

larger molecules [210] or complex food matrices. This makes interpretation of FTIR spectra for large molecules or complex samples difficult.

FTIR has been used for monitoring of enzymatic hydrolysis both of single proteins [174, 213] and complex mixtures of proteins [171]. Table 6 and Figure 13 show an example of IR band annotation for hydrolysates from MDCR, prepared using Alcalase [171]. Böcker et al. [171] demonstrated that the FTIR signature of MDCR hydrolysis changes systematically as a function of hydrolysis time. Several spectral regions systematically change with increasing hydrolysis time [171] (Figure 13). For example, the band *iv* at approximately 1400 cm^{-1}, assigned to free carboxylate groups, increased with hydrolysis time [171]. This trend is consistent with the increasing number of C-terminus groups [171].

Table 6 – Second derivative bands between 1700 and 800 cm^{-1} for MDCR. Modified from Böcker et al. [171].

Annotation	Region	Band positions [cm^{-1}]
C=O amide I: turns	*i*	1675–1664
C=O amide I: α-helix		1645
COO^- (asym stretch)	*ii*	1583
Amide II: α-helix		1547
$–NH_3^+$ (scissor)	*iii*	1516
CH_2 (scissor)		1454
COO^- (sym stretch)	*iv*	1405
Amide III, CH2 (def, rock), OH (def, bend)		1313
Amide III, C–O (stretch)		1242
CNH_3 (rock), CH2 (wag)	*v*	1118
CO, CC, CN (stretch)	*vi*	1045
CCOO (wagging)	*vii*	997
CH_2 (twist)		928
Not assigned		851

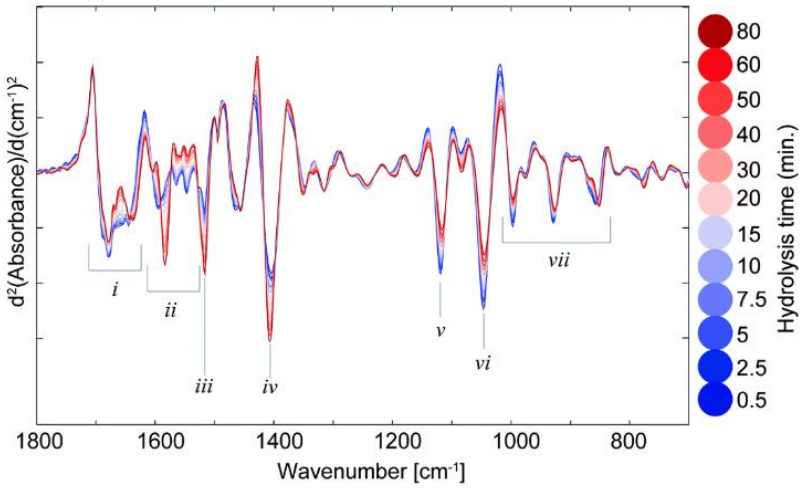

Figure 13 – The second derivative spectra (1800–1200 cm^{-1}) from Alcalase catalysed hydrolysis of MDCR. Each spectrum represents the sampling time given in the legend to the right. Figure reproduced from Böcker et al. [171].

In this study, FTIR spectroscopy was used in **paper I** to obtain the IR spectra of 60 hydrolysates produced from MDCR. The hypothesis was that IR spectra can predict bioactivities of hydrolysates (e.g., antioxidant and antihypertensive properties) as it contains complex information of a hydrolysate. Multivariate analyses (e.g., principal component analysis (PCA) and partial least squares regression (PLSR)) were performed to evaluate correlation between the IR spectra of the hydrolysates and their DPPH radical scavenging and ACE-1 inhibitory properties. Since an FTIR spectrum is a complex data, multivariate statistics (i.e., chemometric methods) are often applied to extract important features related to sample composition and quality. PCA is a method that reorganizes information in a dataset and simplifies it by producing new variables called principal components (PCs) [214, 215]. These PCs "account for the majority of the variability in the data" [214]. Similarly, PLSR allows processing of predictor (e.g., IR spectra) and predicted variables (e.g., bioactivity) to identify factors that can explain the maximum variance and correlation [215].

5.2.2. Chromatography

HPLC is a well-established technique for characterization and purification of various molecules including proteins and peptides [216]. In liquid chromatography, different components in a mixture are separated based on their relative affinities towards mobile and stationary phases [217]. The stationary phase is packed into a column and the sample is pumped with the mobile phase through the column [217]. SEC and reversed-phase chromatography (RPC) are two widely used techniques for characterization of proteins and peptides [212, 216]. In SEC, molecules are separated based on their hydrodynamic volume (a property largely correlated to

weight) as they flow through a porous stationary phase with varying pore sizes [212]. Molecular weight of peptides is calculated based on retention time of known molecules (calibration standards) [218, 219]. On the other hand, the molecules are separated based on their hydrophobicity with RPC. In a typical RPC, the proportion of the apolar solvent (e.g., acetonitrile or methanol) to water is gradually increased during the elution period. The gradual increase in polarity allows good resolution and a timely elution of constituents with a wide range of polarities. In RPC, polar analytes elute early while apolar analytes elute late due to their interaction with the stationary phase [217]. Both SEC and RPC can be used for analytical and preparative applications [216, 217].

In **paper I**, SEC was used to determine weight average molecular weights (M_w) of 60 hydrolysates. When calculating M_w, both the number of molecules and the mass of individual molecules are taken into account. Thus, due to higher mass, larger molecules contribute more to M_w than smaller molecules [220, 221]. MWD of a hydrolysate contains more information than the M_w of a hydrolysate [219]. Figure 14 illustrates that two hydrolysates with similar M_w can differ markedly in the MWD profiles. Both in **Paper I** and **II,** the SEC chromatograms were used to explore the MWD of hydrolysates. The results were used to develop prediction model of bioactivity in **paper I**. Additionally, SEC was used in a semi-preparative mode for bioactivity-guided fractionation in **paper II**. In **paper III**, *in vitro* digests of a bioactive peptide fraction were separated on a SEC column and *in vitro* GI digestibility was evaluated according to the MWD of peptides.

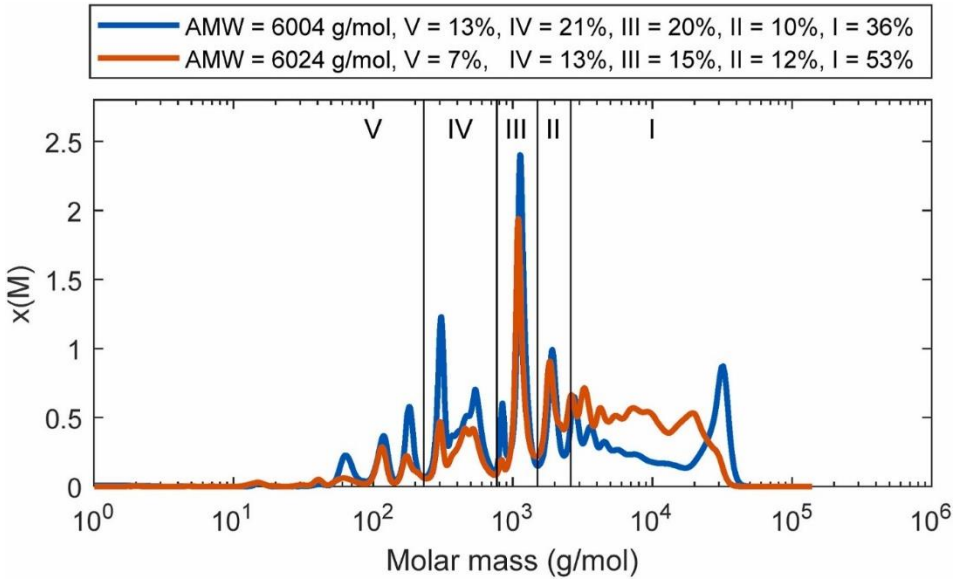

Figure 14 – Molecular weight distribution of two MDCR hydrolysates with approximately equal average molecular weight (AMW). The distributions are divided into five molecular weight regions (I-V), and the relative weight proportion of each fragment is calculated from the cumulative weight distribution. Differential log molecular weight distribution, x(M), uses the logarithm of molecular weight as a basis. Figure reproduced from Måge et al. [219].

RPC was used in **paper I** to detect the substrate HHL vs its cleaved version, hippuric acid, and His-Leu (more details in section 5.3.1.) for the assay evaluating the inhibition of ACE-1 enzyme by the hydrolysate. RPC was also employed in preparative mode for bioactivity-guided fractionation as well as in analytical mode to separate peptides as part of LC-MS analysis (**paper II** and **paper III**).

5.2.3. Mass spectrometry

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) has been widely used for identification of bioactive peptides from complex matrices of protein hydrolysates [77, 166, 222, 223]. A quadrupole Time-of-Flight (Q-ToF) mass spectrometer coupled to HPLC was used in this study. Eluents from the HPLC flows into the Q-ToF system, where the molecules are ionized by the electrospray ionization technique (Figure 15). The generated ions are then directed into a quadrupole mass filter (Q1), where ions with specific mass-to-charge ratios (*m/z*) can be selected. Next in the MS/MS mode, the selected ions move into the collision cell (Q2), where they undergo fragmentation due to collisions with neutral gas molecules [224]. This fragmentation process is known as collision-induced dissociation. When operating the instrument in the MS mode, since fragmentation is not necessary, Q2 operates in mass filter mode, similar to Q1. After leaving Q2, ions enter the ToF analyser. Here, an ion pulser accelerates ions with an electric field. Subsequently, ions move into the flight tube, where they

are separated according to *m/z* [224]. Ions with lower *m/z* have shorter times of flight compared to ions with higher *m/z*. A reflection device plays a crucial role in correcting for the spatial spread and the kinetic energy dispersion of ions with the same *m/z* [224]. This correction ensures that ions with the same *m/z* reach the detector at the same time [224].

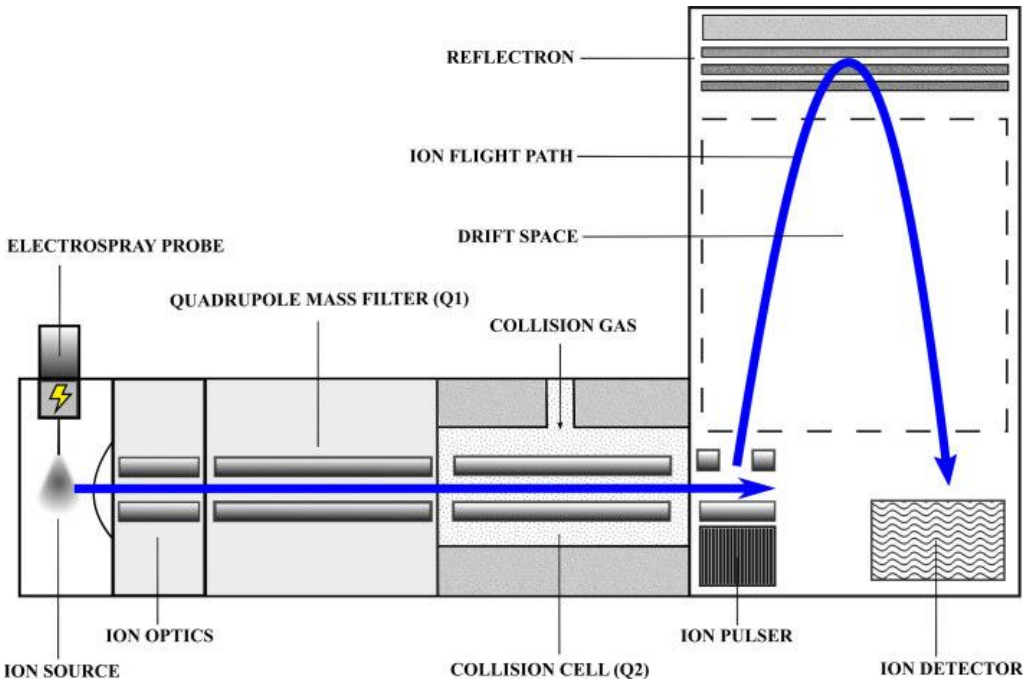

Figure 15 – Schematic diagram of a quadrupole time-of-flight mass spectrometer. Figure reproduced from Allen and McWhinney [224].

A peptide sequence can be deduced from its *m/z* and fragmentation pattern. The nomenclature for fragment annotation was suggested by Roepstorff and Fohlman [225] and modified by Johnson et al. [226] and Biemann [227]. The nomenclature of fragments is based on the specific cleavages that occur in a peptide. When one bond in the main chain is cleaved, one of the six types of fragments occurs [228] (Figure 16). When the charge is on the C-terminal side, the fragment is labelled as either *a*, *b*, or *c*. On the other hand, when the charge is on the N-terminal side, the fragment is labelled as either *x*, *y*, or *z* [228]. Another type of fragment results from the cleavage of multiple bonds in a peptide are immonium ions of amino acids [228]. Peptidomics strategies can be employed [222, 229, 230] for automated identification of large peptides. While short peptides (≤5 amino acids) typically require manual identification due to high noise ratio and small number of fragment ions [231].

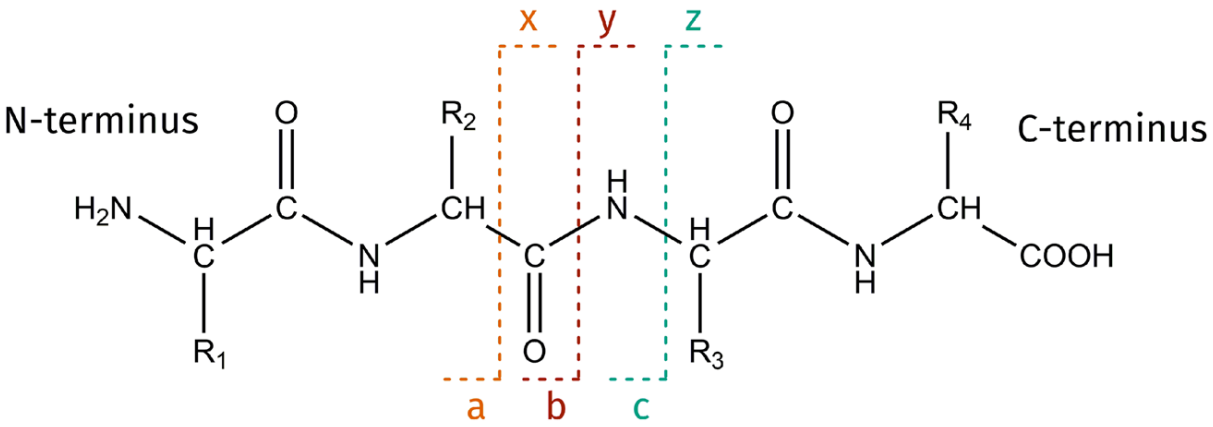

Figure 16 – Annotation of peptide fragment ions: a, b, c ions are N-terminal fragments, while x, y, z fragments are C-terminal. Figure reproduced from Hale and Cooper [232].

The Q-ToF was used for identification of peptides in one of the hydrolysate fractions with the highest bioactivity (**paper II**) and our target peptides in *in vitro* digests, and cell media (**paper III**).

5.3. Bioactivity evaluation

Three *in vitro* bioactivity assays are used in this study.

5.3.1. ACE-1 inhibitory assay

One common method for evaluation of ACE-1 inhibition uses the synthetic substrate HHL and detect the reaction products with RPC [144, 233, 234]. The enzyme ACE-1 cleaves the substrate HHL into hippuric acid and dipeptide His-Leu. When an inhibitor is present, the amount of substrate remains the same or decreases slowly according to the strength and concentration of the inhibitor. The ACE-1 inhibition is calculated based on the differences in the RPC chromatogram peak areas (Figure 17) of hippuric acid between samples with and without an inhibitor. This is an end-point assay which measures enzymatic activity after inactivation of the reaction mixture. Sixty hydrolysates prepared under the different conditions were evaluated with this assay in **paper I**.

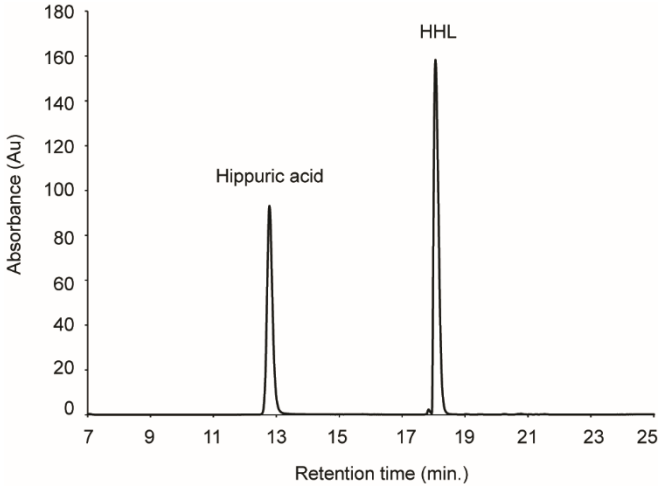

Figure 17 – RPC separation of HHL (substrate) and hippuric acid (product) upon hydrolysis by ACE-1.

Another widely used method is a fluorogenic assay [235-237]. In such assay, a fluorescent product is generated when ACE-1 cleaves a synthetic *o*-aminobenzoyl peptide (Figure 18) [238]. This product is measured in relative fluorescence units using a fluorescence microplate reader with an excitation wavelength of 330 nm and an emission wavelength of 430 nm [238]. The advantage of this assay is that inhibition measurement can be performed in a kinetic mode. Captopril, a known inhibitor for ACE-1, is a commonly used as a positive control for the assay. This assay was used in **paper II** and **paper III** to evaluate potency of the hydrolysate fractions and synthetic dipeptides. The main advantage of this method over the method used in **paper I** is the rapid analysis time.

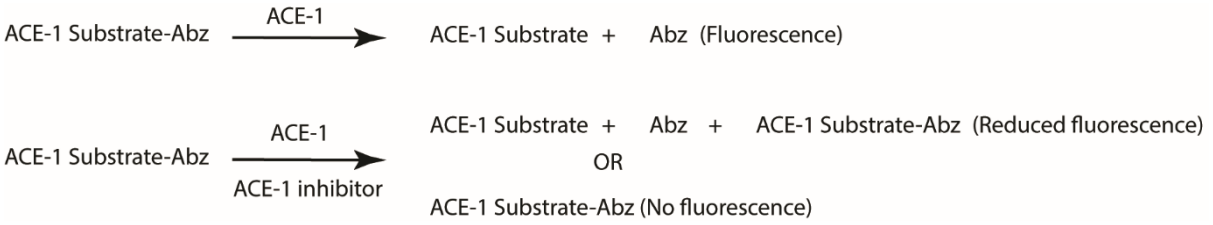

Figure 18 – Principle of ACE-1 inhibitory assay with aminobenzoyl (Abz)-based peptide substrate, which upon enzymatic cleavage release a free fluorophore. Figure adapted from BioVision [238].

5.3.2. DPP4 inhibitory assay

A fluorescence-based assay is commonly used for evaluation of DPP4 inhibitory properties [75, 88, 239, 240]. DPP4 cleaves a fluorogenic substrate, Gly-Pro-Aminomethyl coumarin (AMC), and the free AMC fluorophore is released. The fluorescence is measured with an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm. This assay is performed as end-point measurement of activity and sitagliptin, a known inhibitor for DPP4, is commonly used as a positive control. This assay was used in **paper II** and **paper III** to evaluate potency of the hydrolysate fractions and synthetic dipeptides.

5.3.3. DPPH radical scavenging assay

One of the widely used assays to evaluate antioxidant properties is a DPPH radical scavenging assay [41, 59, 241]. This assay employs colorimetric detection of reduction-induced bleaching of the deep purple DPPH radical, which is measured at 517 nm wavelength. The stable free radical DPPH has a deep purple colour, and colourless or pale yellow products 2,2-diphenyl-1-picrylhydrazine or substituted analogous hydrazine are formed when the radical reacts with a scavenger (Figure 19) [242]. Radical scavenging (i.e., antioxidant) capacity is calculated as a percentage based on the measured absorbance of samples and blanks. This assay was used in **paper I** to evaluated DPPH radical scavenging properties of the sixty hydrolysates.

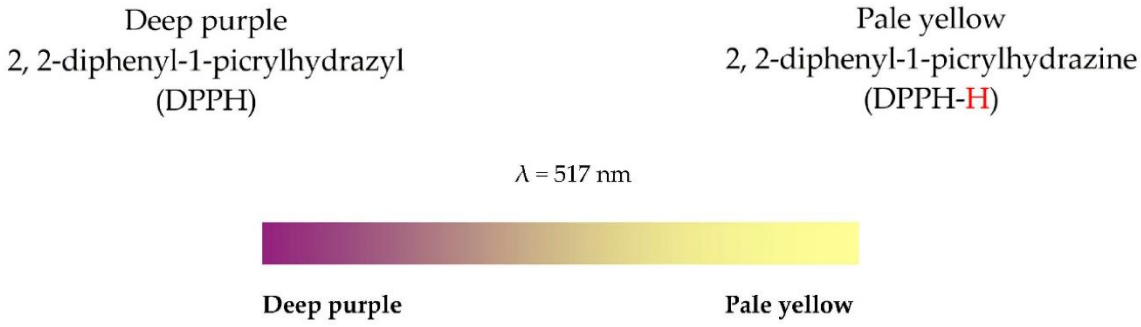

Figure 19 – DPPH radical changes colour upon scavenging by an antioxidant. Figure modified from Bibi Sadeer et al. [243].

5.4. Bioavailability evaluation

In the present study *in vitro* methods were used to evaluate the GI stability and intestinal permeability of bioactive peptides (**paper III**). This section presents the main principles of the *in vitro* methods used with special focus on peptides.

5.4.1. Gastrointestinal stability: INFOGEST static in vitro digestion model

INFOGEST static *in vitro* digestion model is a simulated GI digestion method which recommends standardized parameters for conditions in oral, gastric, and intestinal compartments [186, 187]. Enzyme activities (i.e., pepsin, trypsin and chymotrypsin) were determined prior to the digestion experiment and simulated fluids for each phase were prepared according to the enzyme activities. Figure 20 illustrates the procedure of *in vitro* digestion used in this study. The protocol was slightly modified due to a low amount of hydrolysate fraction. The adjustments included the downscaling from 50 mL tube to the 1.5 mL Eppendorf tube and the addition of three proteases relevant for protein digestion. The first step was the oral phase (pH 7), where the hydrolysate fraction was dissolved in simulated salivary fluid (SSF) without amylase because the samples did not contain carbohydrates. Following a brief incubation for 2 minutes at 37℃, the oral bolus was mixed with simulated gastric fluid (SGF) containing pepsin at pH 3. After a 2-hour gastric incubation at 37℃, the gastric chyme was mixed with simulated intestinal fluid (SIF) containing trypsin and chymotrypsin without bile at pH 7 and incubated for two hours at 37℃. During the intestinal phase, samples were taken at three time points: 10, 30, and 120 minutes. Additionally, two types of control samples were prepared. One was the hydrolysate fraction in the simulated fluids without enzymes, and the other was the enzymes in the simulated fluids without the hydrolysate fraction. The GI stability of the hydrolysate fraction was analysed using SEC profiles of digests in comparison to control samples. Q-ToF was used with targeted mode to evaluate the stability of the three bioactive peptides through GI digestion.

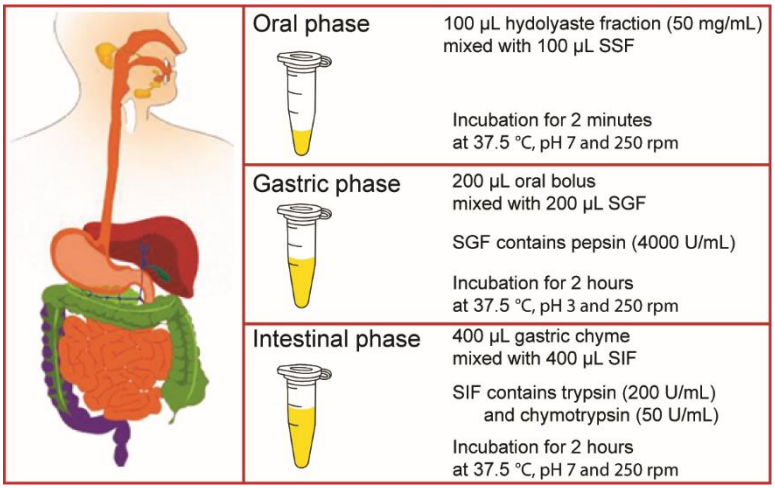

Figure 20 – Phases of the INFOGEST standardized international consensus static model. The phases were modified for the digestion of a hydrolysate fraction. Figure modified from Brodkorb et al. [187].

5.4.2. Intestinal permeability using Caco-2 cell monolayers

Intestinal permeability of the peptides, another important aspect of bioavailability, was evaluated using Caco-2 cell monolayers. The protocol was adapted based on the method described in Hubatsch et al. [198]. Prior to the transport experiment, the cells were cultivated on permeable membranes for a minimum of 21 days with regular change of culture medium (Dulbecco's modified Eagle's medium (DMEM) with supplementation) in order to obtain a tight monolayer of differentiated Caco-2 cells (Figure 21A). Upon reaching confluence, cells start to differentiate spontaneously. After a total culture period of approximately 21 days, dense microvilli characteristic of small intestinal enterocytes appear on the AP side [200]. It is reported that the cells maintain their morphofunctional properties from 21 to 30 days after seeding [244]. During the cultivation, the development of the monolayer was evaluated based on transepithelial electrical resistance (TEER) values measured using a voltohmmeter with a chopstick electrode (Figure 21B). Before and after the transport experiment, the integrity of the cell monolayers was measured for TEER on a separate plate. Additionally, the integrity of cell monolayers was analyzed after exposing them to the conditions of the transport experiment by using a fluorescent paracellular marker (fluorescein isothiocyanate-dextran, average molecular weight 10 kDa).

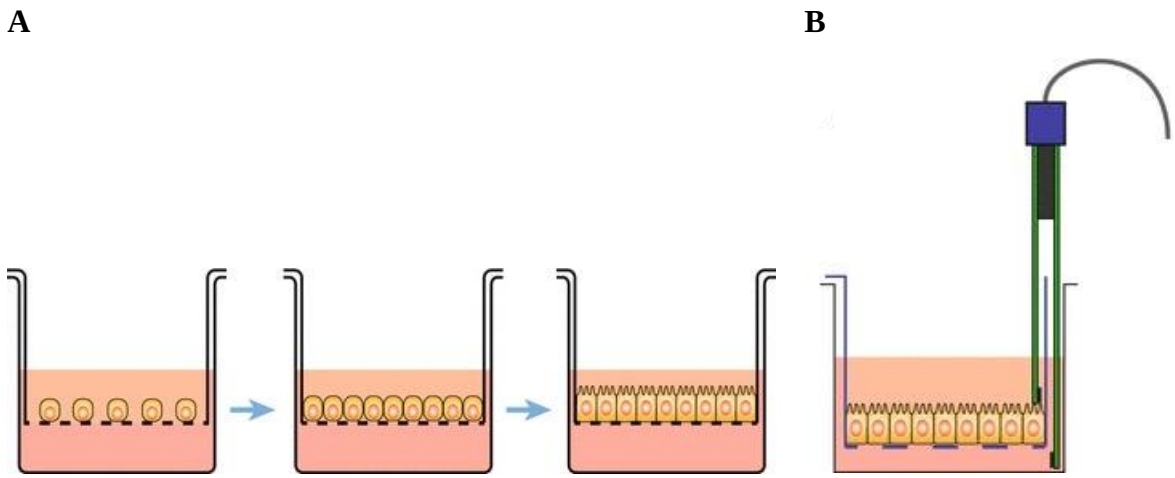

Figure 21 – Caco-2 cells cultured on permeable membrane inserts (A). Measurement of TEER value by a chopstick electrode (B). Figures reproduced from Lea [200], Lea [245].

One day before the experiment, the culture medium was replaced with the fresh one. On the day of the experiment, the culture medium was removed, and cells were washed with phosphate-buffered saline followed by addition of the transport medium (i.e., DMEM). The plates were incubated for 30 minutes in the transport medium to let the cells to adjust to the new conditions. Afterwards, the transport medium in the AP side was replaced with pre-warmed hydrolysate fraction or synthetic peptides (also prepared in pre-warmed to 37℃ transport medium) (Figure 22). After a 4-hour incubation, the media from AP and BL compartments were collected separately. Peptide permeability was analyzed using Q-ToF with targeted mode.

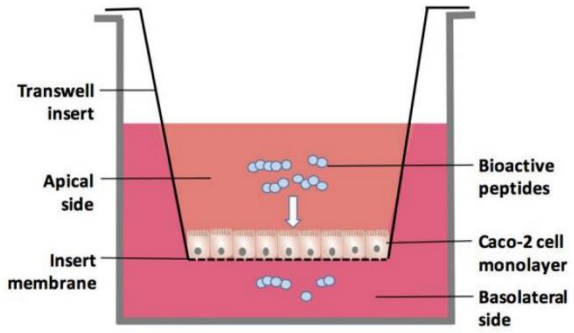

Figure 22 – Transport model of bioactive peptides across Caco-2 monolayer cultured on permeable membrane inserts. The peptides are added to the apical compartment, and those transported across the monolayer can be detected in the basolateral compartment. Figure reproduced from Xu et al. [190].

Chapter 6 Results and discussions

6.1. Paper I

Multivariate correlation of infrared fingerprints and molecular weight distributions with bioactivity of poultry by-product protein hydrolysates

The aim of the **paper I** was (1) to understand the effect of processing parameters (e.g., enzyme and time of hydrolysis) on hydrolysate characteristics (e.g., composition and bioactivity) and (2) to develop predictive models for estimation of bioactivity based on chemical characteristics/fingerprints. The study was based on a library of 60 MDCR hydrolysates produced using ten industrial enzyme preparations and six hydrolysis times (ranging from 10 to 120 minutes).

6.1.1. Effect of processing parameters on bioactive characteristics

Our results demonstrated that both enzyme choice and hydrolysis time influenced DPPH radical scavenging and ACE-1 inhibitory activity of the hydrolysates (Figure 23). In the case of DPPH radical scavenging activity, the general trend for the hydrolysates was a decrease in the activity with increasing hydrolysis time. Additionally, enzyme choice was also an important factor for radical scavenging activity (Figure 23A). In the case of ACE-1 inhibitory activity, enzyme choice had a stronger influence, and no unifying trend was observed in connection with hydrolysis time (Figure 23B). Some enzyme preparations demonstrated no dependency on hydrolysis time (e.g., PNL), while others showed two opposite trends: a decrease (e.g., NPU) or an increase (e.g., E03) in inhibitory activity.

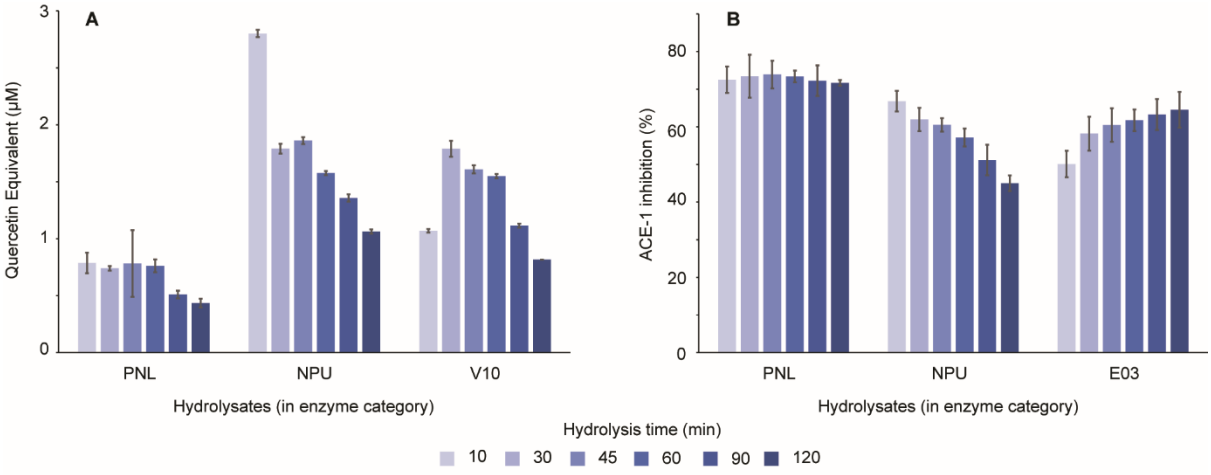

Figure 23 – Bioactive properties of the hydrolysates: DPPH radical scavenging activity. The hydrolysates' concentration was 0.47 mg/mL. Results were expressed in quercetin equivalents (Q Eq) in µM (A). ACE-1 inhibition (%). The hydrolysates' concentration was 0.25 mg/mL (B). Error bars show standard deviations based on three replicates. Bioactivities of hydrolysates produced with three enzymes representing different trends are presented.

Our results demonstrated that bioactivity depends on the processing parameters and different trends were observed for DPPH radical scavenging and ACE-1 inhibition (Figure 23). Thus, investigation of the impact of the processing parameters is required to determine optimal conditions for production of a hydrolysate with a particular bioactivity. For the industrial production, optimal enzyme preparations are those that give high bioactive properties after short hydrolysis time. For example, PNL in this experimental setup is a promising candidate for ACE-1 inhibition (Figure 23B). It can be advantageous to use design of experiment together with response surface methodology for process optimization to obtain a product with certain characteristics. For example, this methodology has been successfully used for optimization for production of hydrolysates with ACE-1 inhibitory activity from whey [246] and stone fish [247], and with antioxidant and DPP4 inhibitory properties from casein [248].

6.1.2. Predictive models

Firstly, univariate correlations of chemical characteristics, such as M_w, of the hydrolysates with bioactivity (i.e., DPPH radical scavenging and ACE-1 inhibition) were explored (Figure 24). However, no strong correlations were observed between any of the parameters (M_w, % protein, and % moisture) and the bioactivities. Therefore, multivariate models (i.e., PLSR) based on FTIR spectra or SEC chromatograms were developed for prediction of DPPH radical scavenging and ACE-1 inhibition (Figure 25).

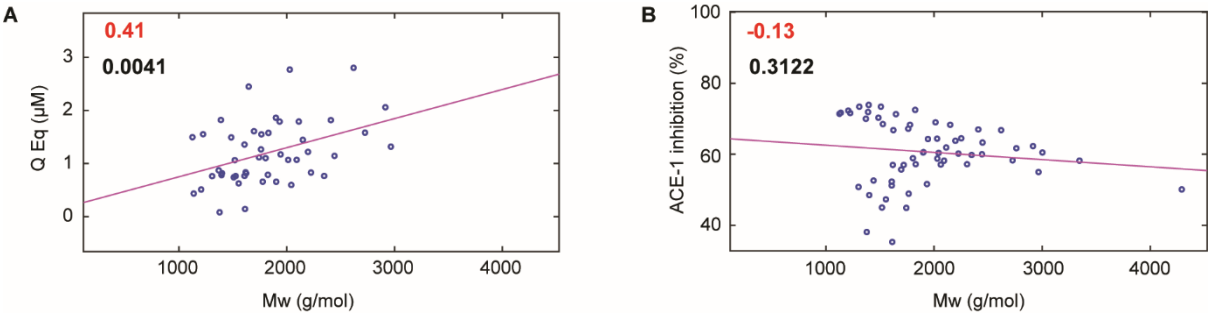

Figure 24 – Correlation plots between M_w and antioxidant activity (Q Eq) (A), M_w and ACE-1 inhibition measured for chicken protein hydrolysates (B). Pearson's correlation coefficients (red font) and p-values (black font) are inserted to the top-left corner of the plots.

The PLSR model based on FTIR spectra predicted ACE-1 inhibition better than the PLSR model based on the SEC chromatograms. Both models equally predicted DPPH radical scavenging, while FTIR based models demonstrated better robustness than SEC based models upon validation. One reason for these results could be that FTIR spectra contain more information about a hydrolysate, such as secondary structure, peptide backbone and side chains of amino acids, than MWD. Another contributing factor could be the fewer samples for DPPH radical scavenging in comparison to ACE-1 inhibition.

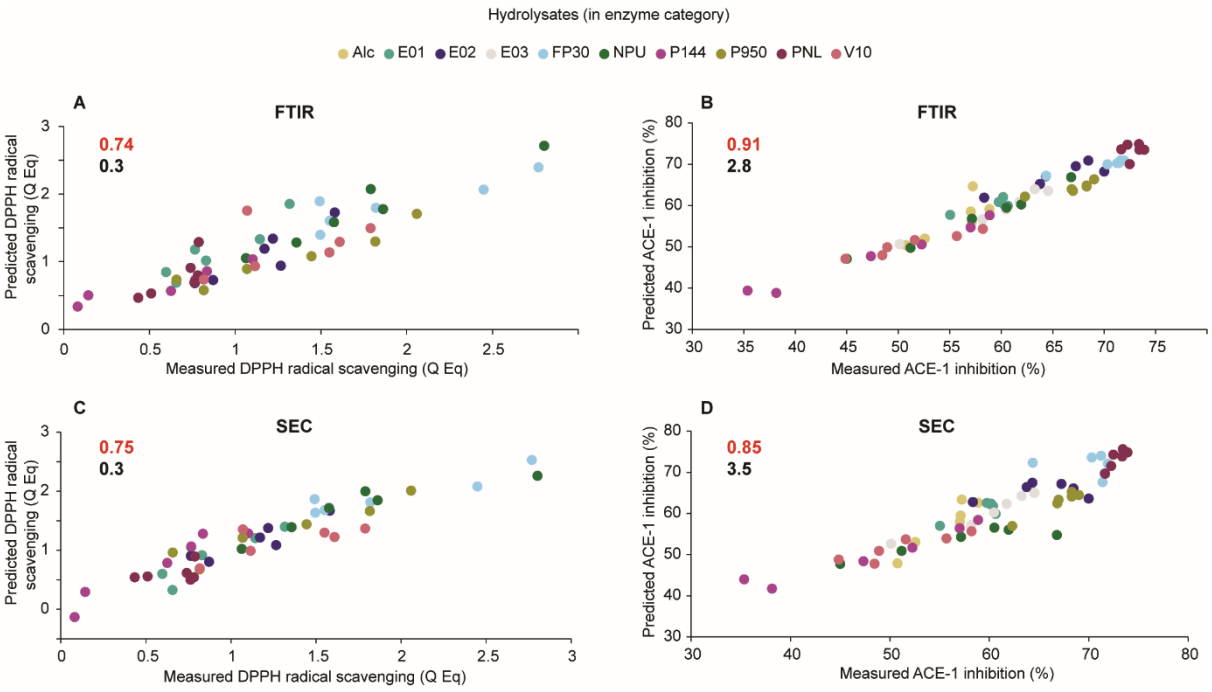

Figure 25 –PLSR models for prediction of DPPH radical scavenging (A) and ACE-1 inhibition (B) based on FTIR fingerprints. PLSR models for prediction of DPPH radical scavenging (C), and ACE-1 inhibition (D) based on SEC fingerprints. Inserted values to the top-left corner of each figure are R-square (red font) and RMSECV (black font) for each model with leave-one-out cross-validation.

Our results demonstrated that the PLSR models based on FTIR spectra can adequately predict the bioactivity of the hydrolysates. In an industrial setting, rapid analysis is crucial when the quality of raw material varies (e.g., by-products), and timely adaptation of processing parameters is essential to maintain the product quality. Since FTIR is a rapid analytical method, it has the potential to become an industrially relevant solution for monitoring the quality of a product with a specific bioactivity. PLSR models can also be a useful tool for bioactivity prediction of hydrolysates and selecting hydrolysates with bioactive potential. This will reduce the time and cost of searching for new bioactive hydrolysates and peptides by testing the bioactivity only of those with high potential. As a successful proof of concept, this study opens the possibility to explore PLSR models based on FTIR and SEC in combination with other bioactivities. The methods presented in the study can be applied to hydrolysates produced from different raw materials with various enzymes. PLSR models require adequate calibration and validation using larger datasets, however when this is established, they are considered to be robust predictive models.

Protein hydrolysate is a complex mixture of various peptides and amino acids, some of which are responsible for a certain bioactivity. Additionally, crude hydrolysates of MDCR contain bone minerals and fats, that can influence the bioactivity measurements and cause, for example, false positive results. Therefore, **paper II** focused on isolation and identification of peptides responsible for bioactivity in a hydrolysate.

*Main results of **paper I**:*

(1) processing conditions influence bioactive properties of hydrolysates:

- ✓ *DPPH radical scavenging decreases with increasing time of hydrolysis.*
- ✓ *enzyme specific trends were observed for ACE-1 inhibition.*

(2) PLSR models based on FTIR spectra and SEC of hydrolysate can adequately predict ACE-1 and DPPH radical scavenging properties:

- ✓ *FTIR based models performed more robustly than SEC based models.*
- ✓ *ACE-1 inhibition was more accurately predicted than DPPH radical scavenging.*

6.2. Paper II

Low Molecular Weight Peptide Fraction from Poultry Byproduct Hydrolysate Features Dual ACE-1 and DPP4 Inhibition

In **paper II**, bioactive peptides with ACE-1 and DPP4 inhibitory properties were isolated from the hydrolysate, which exhibited the highest ACE-1 inhibitory activity in **paper I**. The isolation was performed using bioactivity-guided fractionation, and the peptides were identified using LC-MS/MS. Hydrolysates of MDCR consist of a mixture of various peptides due to the complex nature of the raw material and the use of a non-specific enzyme preparation. Therefore, a conventional bioactivity-guided fractionation involving a series of filtration and fractionation steps was used for discovery of bioactive peptides.

6.2.1. Bioactivity-guided isolation of a potent fraction

The first step of fractionation indicated that bioactive peptides for both ACE-1 and DPP4 inhibition have molecular weight below 3 kDa (Figure 26A). The following fractionation step using SEC demonstrated that Fr II (M_w 514 Da) had the highest inhibition for both therapeutic targets (Figure 26B and C).

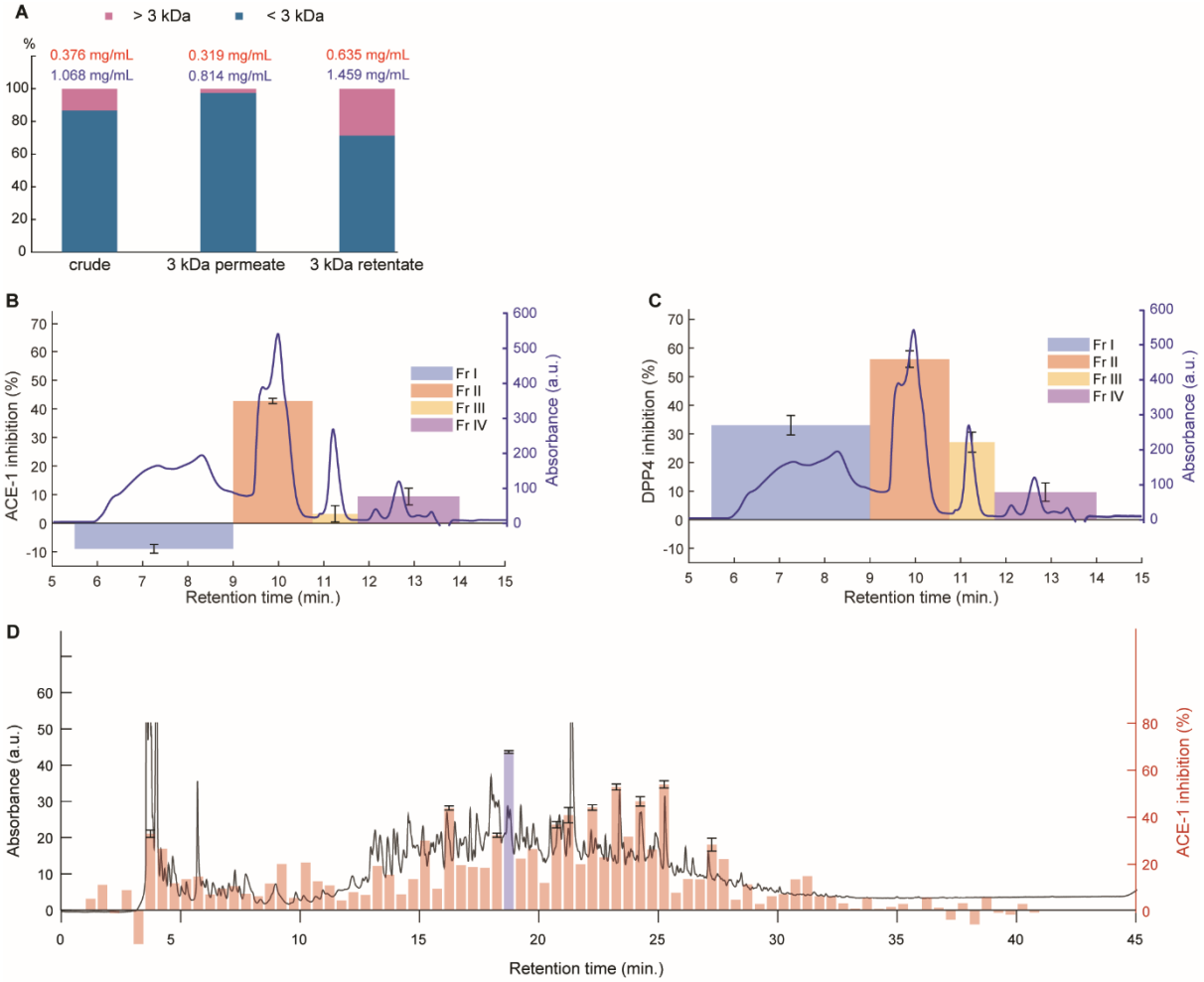

Figure 26 – ACE-1 and DPP4 inhibitory properties of fractions. The proportion (%) of peptides with M_w > 3kDa (pink) and < 3 kDa (blue) in the crude hydrolysate, permeate and retentate. The values over the bar chart shows IC_{50} values for ACE-1 (red) and DPP4 (blue) inhibition (A). SEC chromatogram (blue line) of the 3 kDa permeate and percentage (bar plot) of ACE-1 inhibition (B) and DPP4 inhibition (C) of the four SEC fractions at final assay concentration of 0.1 mg/mL. The colour-highlighted region (the bar width) indicating the retention time range corresponding to the collection period for each fraction. ACE-1 inhibition (%) of 80 fractions (30 seconds per fraction) collected from RPC fractionation of LMWPF (D). Eleven fractions with the highest activities were measured in triplicate (shown with standard deviations as error bar). The fraction with the highest inhibition% is highlighted in blue. RPC chromatogram of LMWPF (black) is overlayed.

Further fractionation of Fr II using an RP column followed by bioactivity screening showed that several fractions had inhibitory potential for both targets (Figure 26D and Figure 5B in **paper II**). While the further identification process was focused on a fraction with the highest activity (the fraction with blue color in Figure 26D), it is important to note that several other peptides consituting Fr II were bioactive (Figure 26D) and may have additive or synergistic effect. For example, chemical diversity of ACE-1 and DPP4 inhibitory peptides was emphasized by van der Ven et al. [246] and Nongonierma and FitzGerald [75], respectively. The study suggested that Fr II has potential as a commercial product, since our results

demonstrated that several RP fractions consist of bioacitve peptides with diverse chemical characteristics.

6.2.2. Identification of bioactive peptides

The sequence of bioactive peptides from RPC fractions with the highest ACE-1 and DPP4 inhibitory activities was identified based on *m/z* and fragmentation patterns obtained by LC-MS/MS (Figure 27). Manual peptide identification represents a bottleneck in the screening process. To simplify the workflow, libraries of known peptides can be analyzed using LC-MS/MS as a preparative step for identifying unknown peptides. Analyzing unknown peptides using the same LC-MS/MS method will enable sorting based on retention time and intensities of fragments, in addition to *m/z*. This approach can facilitate the identification process, leading to faster and more accurate identification.

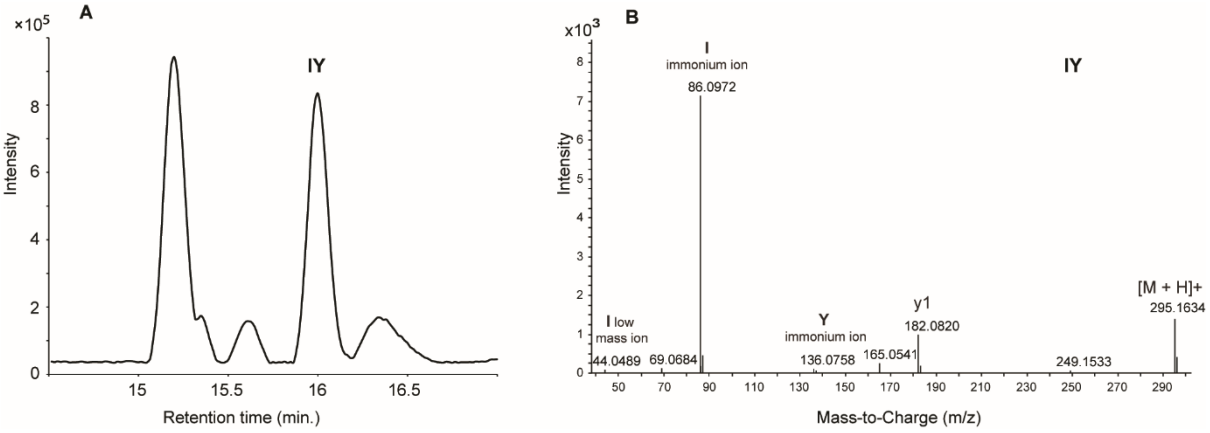

Figure 27 – Base peak chromatogram of the most active fraction from RPC fractionation – ACE-F36 marked blue in Figure 26D (A) and MS/MS spectra of the dipeptide, IY, eluted at retantion time 16 min (B). The peak of dipeptide, IY, was identified based on the MS/MS spectra for m/z 295.1634.

The fractionation process reduces the complexity of the hydrolysate fractions but exposes peptide to chromatographic conditions (e.g., mobile phase and modifiers) in every step. It is well-known that trifluoracetic acid (TFA) counter-ion can bind to positively charged peptides residues and remain as part of the peptide after lyophilization [249]. The resulting residual TFA can interfere with bioactivity measurements. Therefore, TFA, which is commonly used as mobile phase modifier for analytical SEC, was substituted by formic acid for SEC fractionation in this study.

In addition to providing a documentation of active constituents of hydrolysates, isolation and identification of a bioactive peptide is an important step for further targeted studies of important properties, such as bioavailability. When a given bioactive peptide is identified, its

bioavailability can be studied *in vivo* by measuring plasma concentration or using *in vitro* models.

Paper II demonstrated that low molecular weight peptide fraction Fr II (LMWPF) possessed ACE-1 and DPP4 inhibitory properties. Two bioactive peptides were identified from the most potent RPC fractions. Identification of bioactive peptides and determination of IC_{50} values are important steps for documentation of bioactive constituents of the hydrolysate. LMWPF is considered to be a feasible (i.e., scalable) product that can be up-concentrated from industrial scale hydrolysate using downstream processing (e.g., nanofiltration) and holds potential as a health-promoting functional ingredient or nutraceutical with dual effect. The bioavailability of the LMWPF, with a focus on the identified dipeptides in **paper II**, was investigated in **paper III**.

*Main results of **paper II**:*

(1) A LMWPF with M_w of 514 Da possesses poly-pharmacological effect (i.e., dual ACE-1 and DPP4 inhibition).

(2) Two dipeptides IY and VL, which were responsible for high bioactivities (IC_{50} values of 7.00 ± 0.43 µM for ACE-1 and 1.22 ± 0.25 mM for DPP4 inhibition activities, respectively), were identified from a MDCR hydrolysate.

6.3. Paper III

In vitro gastrointestinal stability and intestinal absorption of ACE-1 and DPP4 inhibitory peptides from poultry by-product hydrolysate

Paper III aimed to evaluate GI stability and intestinal permeability of LMWPF with special focus on the dipeptides: VL, IY (identified in **paper II**), and YA. The dipeptide YA was identified from the LMWPF and evaluated to have dual activity with the IC_{50} values for ACE-1 and DPP4 inhibition of 0.42 ± 0.06 mM and 1.13 ± 0.11 mM, respectively (**paper III**). INFOGEST static *in vitro* model was used to determine GI stability, and Caco-2 cell monolayers were used to evaluate intestinal permeability of the peptides.

6.3.1. Gastrointestinal stability

No significant changes were observed in the MWD of the peptides after simulated digestion (Figure 28A) indicating a good stability of the peptides in the upper GI tract. Furthermore, the individual bioactive dipeptides (VL, IY, and YA) were evaluated based on peak areas derived from the ion counts of extracted ion chromatograms (EIC) corresponding to their respective molecular ion peaks ($[M + H]^+$). The results showed that both LMWPF and the dipeptides were stable during *in vitro* GI digestion (example for IY in Figure 28B). In particular, the peptides were resistant to hydrolysis by pepsin, trypsin, and chymotrypsin because the used variant of the INFOGEST static *in vitro* model lacked several other digestive proteases (e.g., carboxypeptidases, pancreatic elastase, brush border peptidases). In this study, individual enzymes (pepsin, trypsin, and chymotrypsin) were used instead of pancreatin because complex nature of pancreatin could interfere with the identification and quantification of the bioactive peptides. Pancreatin is a mixture of enzymes and contains other peptidases (e.g., carboxypeptidases and elastase) in addition to trypsin and chymotrypsin [250]. Therefore, it is worth testing the alternative variant of the INFOGEST static *in vitro* model, which uses pancreatin instead of individual enzymes.

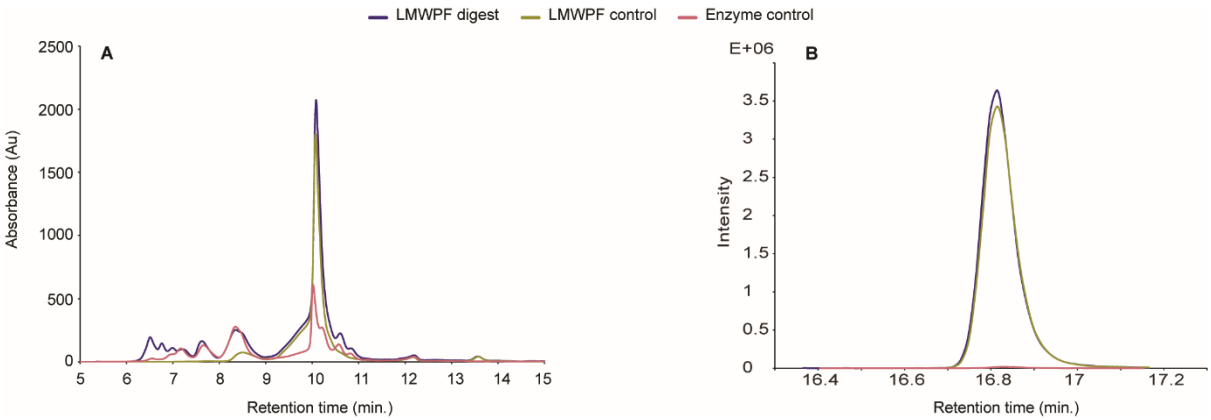

Figure 28 – Size exclusion chromatograms of the samples at the end of intestinal phase (120 min). LMWPF digest (purple), LMWPF control (green), and enzyme control (pink) at the end of intestinal phase (A). Ion count of the $[M + H]^+$ corresponding to the dipeptide IY (B).

The GI stability of VL, IY, and YA demonstrates that, in addition to their bioaccessibility, these dipeptides can reach and act locally on targets (i.e., DPP4 and ACE-1) in the GI lumen. DPP4 is abundantly expressed by enterocytes and endothelial cells of the small intestine [251]. Thus, DPP4 inhibition in the GI lumen can increase the half-lives of GLP and GIP hormones, which is one of the strategies for treatment of T2DM [70]. On the other hand, ACE-1 expressed in endothelium is targeted for blood pressure regulations [252-254], therefore, inhibitory peptides need to reach blood circulation to interact with the target. Several studies have reported that bioactive peptides regulate nutrient absorption, gut motility, secretion and activities of GI enzymes, contribute to intestinal damage repair, and exhibit anti-inflammatory effect [190, 255-258]. As LMWPF showed overall stability to GI digestion, this fraction could be further investigated for health-promoting effects on therapeutic targets in GI tract.

6.3.2. Intestinal permeability

For evaluation of peptides transport across Caco-2 cell monolayers, it is crucial to optimize the conditions that hold the integrity of monolayers. Therefore, Caco-2 cell monolayer integrity was measured using TEER values under various incubation conditions prior to the evaluation of intestinal permeability. We have tested Hanks' balanced salt solution (HBSS), commonly used as a transport medium [204, 206, 207], DMEM, and culture medium which consisted of DMEM with supplementation (10% fetal bovine serum, 1% non-essential amino acids solution and 1% penicillin-streptomycin). Duration of transport experiment was tested at 0, 1 and 5 hours, as peptides' transport is often tested after two hours or longer [202, 203, 205], while transport of drugs takes less than one hour [198]. We observed that, during incubation in HBSS, the breaking point for monolayer integrity occurred earlier than one hour. TEER values

dropped below 100 ohm/cm^2 for six out of nine wells after one hour incubation (Figure 29A). After an additional 4-hour incubation in HBSS, all wells exhibited TEER values below 30 ohm/cm^2 indicating lack of monolayer integrity. In comparison, the monolayer integrity in wells incubated with DMEM remained stable, and TEER values were similar to the values of wells with culture medium (Figure 29A). Furthermore, the fluorescent images of cell nuclei revealed differences in the nuclei shapes in cells incubated in HBSS compared to those in culture medium and in DMEM (Figure 29B-D). Our results clearly showed that HBSS compromised monolayer integrity already after 1-hour incubation and altered the cell status. Thus, in this study, transport experiments were conducted in DMEM.

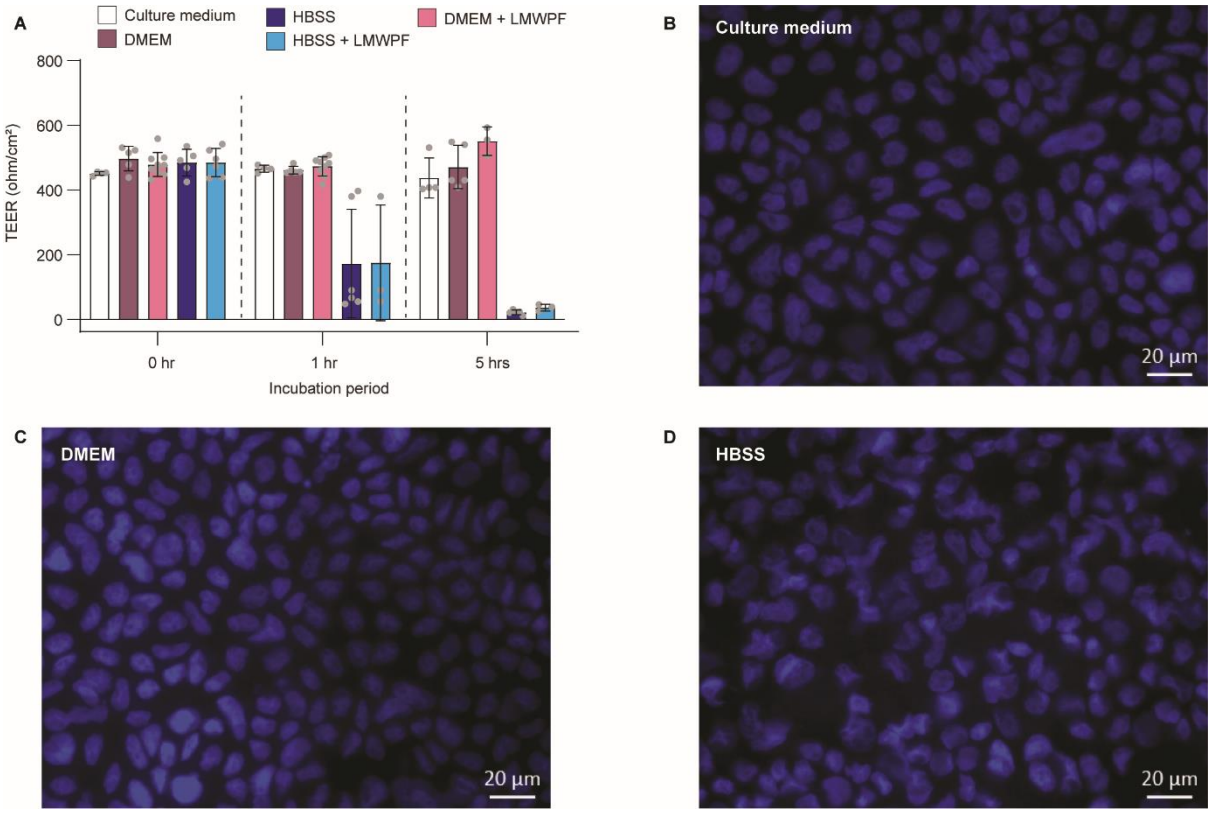

Figure 29 – Influence of transport solutions on TEER of Caco-2 cells: HBSS with/without LMWPF, DMEM with/without LMWPF and culture media. Measurements are taken at three time points: a day before exposure (0 hr), after one and five hours of exposure. All TEER values at 0 hr were measurement of monolayer in the culture medium. For treatments with LMWPF: 1-hour pre-incubation in the corresponding transport medium (DMEM or HBSS), followed by 4-hour incubation in transport medium with LMWPF. Average values of replicate samples were plotted as bar with standard deviation and individual replicate values are inserted as grey circles. Different number of wells were used per treatment (A). Fluorescence microscopy images of cells' nuclei after a 5-hour incubation with culture medium (B), DMEM (C) and HBSS (D).

The peptides were not detected on the BL side using targeted MS/MS, which indicated no transepithelial transport of the dipeptides. However, the peptides' concentration was considerably decreased on the AP side after 4-hours incubation. Thus, the amount of the peptide on the AP side was compared to that at the starting point for both LMWPF and synthetic

peptides, based on the peak areas. For the synthetic peptides, the peak areas decreased by more than 95% for IY and more than 88% for YA, after 4-hour incubation. Figure 30A illustrates the EIC of IY in the AP side after 4-hour incubation compared to the corresponding stock solution applied in the transport experiment. Similarly, in the transport experiment with LMWPF, the decrease in concentration of YA, IY and VL in the AP was observed after 4-hour incubation, while none of the peptides were detected in the BL side. Such decrease in the concentration of peptides in the AP side and no detection on the BL side, indicates that peptides are either degraded by brush border peptidases or taken up by Caco-2 cells (e.g., by PepT1). In the cells, the peptides are exposed to cytosolic peptidases and eventually can be degraded [190].

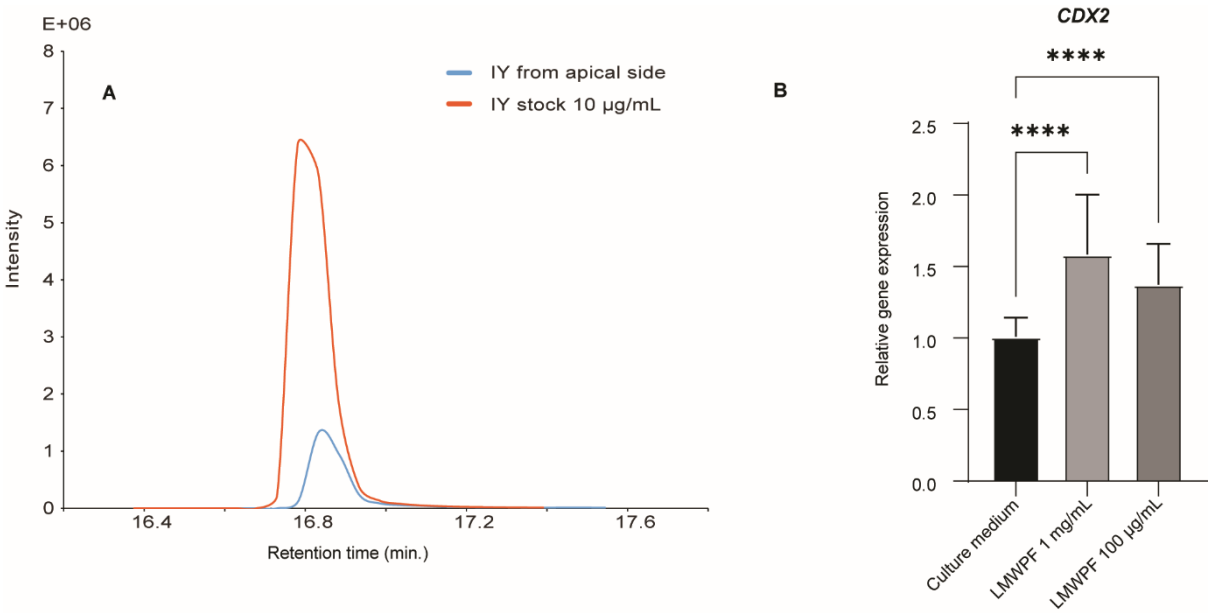

*Figure 30 – Extracted ion chromatogram of the molecular ion peak $[M+H]^+$ corresponding to the dipeptide IY from the apical side (blue) of the Caco-2 cell monolayer after 4-hour incubation. The starting concentration of IY was 100 µg/mL. The IY stock solution (orange) was diluted to 10% for comparison (A). Relative expression of CDX2 gene in Caco-2 cells incubated with LMWPF (1 mg/mL and 100 µg/mL) in comparison to the control treatment with culture medium. Differences between treatments are determined by one-way ANOVA. Asterisks denote significant differences between treatments ****: p value < 0.0001 (B).*

There is well-established knowledge that peptides can adsorb to plastic surfaces [259]. Therefore, an interaction between the plastics of the transwell plates and peptides was investigated. A control experiment in transwell plates without Caco-2 cells was performed with application of 1 mg/mL LMWPF in DMEM to AP side. The results demonstrated a peak area reduction of 30% for VL and 41% for YA and IY on both AP and BL sides after 4-hour incubation. The results indicated that the peptides of interest can bind to the plastics of the transwell plates. It should be noted that preparing Caco-2 monolayer for transport experiments requires 21-day cultivation of the cells in the transwell plate before adding peptides. During cell cultivation, amino acids and peptides from the culture medium (e.g., from fetal bovine

serum) can act as prime solutions and lead to minimal adsorption of added peptides. Therefore, reduction of YA, VL and IY concentrations due to interaction with plastic was presumed to be overestimated in the control experiment.

Currently, a variety of protocols have been used to study the transport of bioactive peptides. For example, different concentrations of peptides (from 0.1 to 25 mg/mL) and several transport media have been used for transport experiments [192, 202]. HBSS with or without supplementation is widely used as a transport medium [203, 205, 206]. However, our evaluation of transport medium showed that HBSS compromises the integrity of Caco-2 cell monolayer when incubation lasts longer than one hour. As transport experiments with peptide are usually longer than one hour [205-207], it is crucial to use transport media which can maintain the integrity of cell monolayers. This makes it difficult to compare our results with some of the results reported in literature. Development of an optimized and standardized protocol for evaluating the transport of bioactive peptides through the cell membranes using Caco-2 monolayers is necessary (e.g., considering transport medium, duration of transport experiment and peptide concentration range). Such protocol would enable a better understanding of the transport mechanisms of peptides under more optimal conditions for the cells and could provide comparable results across different studies.

The response of Caco-2 cells stimulated with LMWPF or synthetic peptides was studied by evaluation of relative expression of nine selected genes related to peptide transporters, transcription factors, and tight junction proteins in comparison to control (culture medium). Addition of LMWPF (1 mg/mL) demonstrated statistically significant differences in expression levels for six out of nine genes. Our results suggested that peptides elicited a response in Caco-2 cells on transcription level. For example, the expression of the homeobox *CDX2* gene was increased in response to stimulation with both concentrations of LMWPF compared to the control treatment (Figure 30B). *CDX2* is involved in regulation of PepT1 expression [260]. However, the cells stimulated with LMWPF did not show difference in *SLC15A1* (gene encoding PepT1 [261]) expression levels compared to the control. Additional studies, including multiple time points, various peptide concentrations, different peptides (varying in length and amino acid sequence), as well as transcriptomic and proteomic analyses of Caco-2 cells, will contribute to a more comprehensive biological interpretation of the results in relation to peptide transport.

*Main results of **paper III**:*

(1) A bioactive peptide, YA, which has a potent DPP4 inhibitory and moderate ACE-1 inhibitory effect was identified from LMWPF.

(2) LMWPF and bioactive dipeptides (VL, IY and YA) were resistant to the simulated conditions of in vitro GI digestion indicating adequate bioaccessibility.

(3) In vitro intestinal permeability with Caco-2 monolayers could not be demonstrated for the bioactive dipeptides despite a decrease in their concentration on the AP side.

(4) The HBSS as a transport medium demonstrated to compromise the Caco-2 monolayer integrity after less than one hour of incubation.

(5) The expression levels of several genes related to peptide transporters, transcription factors, and tight junction proteins were significantly changed in the Caco-2 cells stimulated with LMWPF or synthetic peptides.

Chapter 7 Conclusion and further perspectives

The main conclusions of the dissertation address two aspects (1) the perspectives of MDCR as a raw material for bioactive peptides targeting CMDs and (2) the adequacy of bioanalytical methods adapted and employed for discovery and characterization of food-derived bioactive peptides. Regarding the first aspect, the study demonstrated that MDCR is a promising raw material for production of bioactive hydrolysates and peptides, specifically for management of CMDs. One of the hydrolysate fractions (i.e., LMWPF) demonstrated a dual pharmacological effect, which is beneficial as a health-promoting ingredient especially for the complex disease (such as T2DM), that often require multi-drug regimen. The *in vitro* digestion of peptides demonstrated that the bioactive dipeptides were bioaccessible in the GI tract, while their intestinal permeability remained uncertain. Three dipeptides responsible for the bioactivities were identified from LMWPF, while the study also indicated that the fraction consists of more peptides with bioactivities. Therefore, the fraction of hydrolysates consisting of several bioactive peptides (i.e., LMWPF) is hypothesized to be a promising health-promoting ingredient for contribution to managing CMDs.

The selected combination of bioanalytical methods used in the study successfully resolved most tasks, including production of bioactive peptides using enzymatic hydrolysis, their identification employing bioactivity-guided fractionation and LC-MS, and characterization of bioactive peptides using SEC, FTIR, and *in vitro* bioactivity assays, static digestion, and intestinal permeability models. The challenging aspects included laborious process of manual peptide identification as well as evaluation of transepithelial transport of peptides using Caco-2 cell monolayers. The study demonstrated that FTIR is a promising analytical method for predicting bioactivity. In addition to serving as a rapid screening tool, FTIR can aid monitoring and optimization of product quality in future industrial production of bioactive peptides.

Future work can be directed towards the optimization of the methods employed in this study. For example, manual peptide identification requires increased throughput (e.g., using database assisted dereplication of known peptides). The method for testing Caco-2 monolayer permeability also requires further optimization to address the specificities of peptide transport which include focus on different transport routes, transport medium, time of transport, concentration of peptides. Comparison of our results with other studies was often challenging due to difference in the experimental conditions (e.g., enzyme-to-substrate ratio and hydrolysate concentration for enzyme-based bioactivity assays, or transport medium and time

for Caco-2 monolayer transport experiments). Therefore, the dissertation highlighted the importance of method standardization, which is vital for enhancing interstudy comparisons.

Our results demonstrated that several peptides in a hydrolysate possess bioactive characteristics. Therefore, a potential product does not necessarily have to be a single bioactive peptide, but a fraction of hydrolysate consisting of several bioactive peptides, such as LMWPF, as described in **paper II**. For the commercialization of such product on an industrial scale, upscaling of the fractionation method using, for example, nanofiltration should be explored. Further efforts including *in vivo* studies with the focus on safety and efficacy need to be performed with the ultimate aim of estimation of the health effect in humans. For example, the dosage of a bioactive fraction which would elicit a health benefit has to be in a realistic range. Duffuler et al. [8] summarized human intervention studies with a dosage for hydrolysate ranging from 200 mg to 150 g per day with most often dosage of < 35 g per day.

The dissertation successfully developed and implemented the bioanalytical methods for discovery and characterization of bioactive peptides from a MDCR hydrolysate. Moreover, three specific peptides from MDCR hydrolysates were identified and characterized for bioactivities. The study highlighted the potential of bioactive peptides from MDCR hydrolysate to act on relevant therapeutic targets (i.e., ACE-1 and DPP4). This indicates that peptides derived from poultry hydrolysates can serve as health-promoting ingredients, in particular, for management of CMDs.

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Appendix

Paper I

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Multivariate correlation of infrared fingerprints and molecular weight distributions with bioactivity of poultry by-product protein hydrolysates

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ABSTRACT

Characterization of protein hydrolysates is a vital step in developing peptide-based bioactive ingredients. Multivariate correlation of chemical fingerprints and bioactivity of poultry by-product protein hydrolysates is explored as a potential analytical strategy for characterization and quality control. Chemical fingerprints of sixty hydrolysates were acquired using Fourier-transform infrared spectroscopy (FTIR) and size exclusion chromatography (SEC). Bioactivities (2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and angiotensin-1-converting enzyme (ACE-1) inhibition) were measured *in vitro*. Partial least squares regression models based on FTIR fingerprints or SEC chromatograms showed a better prediction performance for ACE-1 inhibition (coefficients of determination (R^2) = 0.91, root mean square error of prediction (RMSECV) = 2.8; R^2 = 0.85, RMSECV = 3.5, respectively) than for DPPH radical scavenging (R^2 = 0.74, RMSECV = 0.3; R^2 = 0.75, RMSECV = 0.3, respectively). Such models are promising tools for rapid prediction of bioactivities and as a quality control technology in production of bioactive peptides.

1. Introduction

Enzymatic protein hydrolysis (EPH) is a versatile processing technology where proteases are used to cleave proteins into peptides of various lengths under moderate conditions of pH and temperature. EPH does not deteriorate the nutritional quality of the proteins and allows to control relevant properties of the product, such as sensory attributes, functional property and bioactivity (Aspevik et al., 2017). A variety of bioactive properties has been reported for EPH-derived peptides from foods or food processing by-products, such as antihypertensive, antioxidant, antidiabetic, antithrombotic, antimicrobial, opioid, and satiety regulating activities (Lafarga & Hayes, 2014; Romero-Garay et al., 2022; Xing et al., 2019; Zamora-Sillero et al., 2018).

In this study, antihypertensive and antioxidant properties of poultry by-product protein hydrolysates were studied. One of the important therapeutic targets for dietary protein-derived bioactive peptides is angiotensin-1-converting enzyme (ACE-1). ACE-1 is a crucial component of the renin-angiotensin-aldosterone system, which is involved in the pathogenesis of cardiovascular disease (Putnam et al., 2012). There are several clinically approved prescription drugs for inhibition of ACE-1, however, they have adverse side effects (Israili & Hall, 1992; Lahogue et al., 2010; Sánchez-Borges & González-Aveledo, 2010). Therefore, the search for alternative sources of ACE-1 inhibitors in the form of nutraceuticals has become a major area of research in recent years. Several studies have shown the potential of protein hydrolysates as promising sources of ACE-1 inhibitors (Lee & Hur, 2017; Mas-Capdevila et al., 2019; Onuh et al., 2013). Another example of bioactivity attributed to food-derived peptides is antioxidant activity (Di Bernardini et al., 2011; Lorenzo et al., 2018; Samaranayaka & Li-Chan, 2011). Oxidative stress causes damage of essential biomolecules (i.e., proteins, lipids, DNA) and this damage can initiate for example inflammation, cardiovascular disease, diabetes, neurodegeneration, or tumorigenesis (Lorenzo et al., 2018; Pisoschi et al., 2021). Studies show that bioactive peptides from hydrolysates can neutralize radicals by hydrogen transfer, electron transfer (Romero-Garay et al., 2022) and metal chelating (Chakka et al., 2015).

Despite several evidence of *in vivo* and *in vitro* ACE-1 inhibitory and antioxidant effects of protein hydrolysates, development of nutraceuticals for such applications remains a challenging task. This is partly due to the chemical complexity of crude hydrolysates and the resulting

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challenges associated with characterization and discovery of the bioactive peptides. Identification and characterization of the bioactive peptides in a given hydrolysate is a vital step in process- and product development and documentation (Chalamaiah et al., 2019; Li-Chan, 2015). Processing parameters, such as choice of enzyme and hydrolysis time, can affect the hydrolysate's chemical composition and hence it's bioactivity. *In vitro* bioactivity screening of crude hydrolysates in arbitrary doses can lead to false positives due to, for example, bone mineral content. One of the solutions for screening for potent hydrolysates is to use analytical strategies to correlate chemical fingerprints with biological effects (i.e., bioactivity). Correlations of chemical fingerprints with bioactivity can also serve as a platform to ensure reproducible production of bioactive peptides. Process control is particularly important in EPH of by-products (e.g., poultry processing by-products) where raw materials are highly varying in composition, which can result in undesirable quality changes in the final hydrolysates (Wubshet et al., 2018).

Methods for chemical characterization of hydrolysates include size exclusion chromatography (SEC), degree of hydrolysis (DH%) and Fourier-transform infrared spectroscopy (FTIR). Molecular weight distribution (MWD), derived from SEC, has been used for comparing hydrolysates produced under different processing conditions and to monitor the hydrolysis process (Damgaard et al., 2015; Lindberg et al., 2021; Silvestre, 1997). Similarly, FTIR has been demonstrated as an effective tool for monitoring changes in the secondary and primary structure induced by enzymatic cleavage of single proteins (Güler et al., 2011; Ruckebusch et al., 1999) and complex biological tissues (Böcker et al., 2017). Wubshet et al. (2017) demonstrated that weight average molecular weight (M_w) of protein hydrolysates is correlated with FTIR fingerprint, so multivariate statistical models based on FTIR fingerprint can be used to predict M_w. In contrast to SEC, FTIR is a rapid technique with great potential for monitoring the hydrolysis process in an industrial setup (Wubshet et al., 2017) and as quality assessment tool for protein hydrolysates (Måge et al., 2021).

Classical bioactivity screening is a laborious and time-consuming process. Therefore, predictive methods are needed to facilitate screening of complex protein hydrolysates. *In silico*-based integrated '-omics' approaches are alternatives, which allow high throughput screening and enable narrowing down potential bioactive peptides for subsequent *in vitro* screening (Agyei et al., 2016). However, *in silico*-based techniques require knowledge of protease specificity and the raw material protein composition. This limits its use, as industrial enzyme preparations are often a mixture of both predominant enzymes and minor enzymes which give side-activities (FitzGerald et al., 2020) and the protein composition of by-products is varying. The literature shows that bioactivity of peptides is closely related to their chemical structure. Since FTIR fingerprints and SEC chromatograms of protein hydrolysates have been successfully used to predict the chemistry of EPH, these analytical techniques most likely also contain relevant information related to bioactivity. Therefore, the main aim of this study was to develop and evaluate FTIR- and SEC-based models for prediction of antioxidant or hypertensive potential of protein hydrolysates. For this purpose, a library of 60 hydrolysates from mechanically deboned chicken residues (MDCR) was produced using ten industrial protease preparations and six hydrolysis times. Partial least squares regression (PLSR) models based on FTIR- and SEC- data of the crude hydrolysates were developed, and performance of the models in predicting the bioactivates (i.e., ACE-1 inhibition and antioxidant activity) was evaluated. This study represents a first example of direct bioactivity prediction from chemical fingerprints of protein hydrolysates.

2. Materials and methods

2.1. Raw material and chemicals

MDCR were provided by a Norwegian slaughterhouse (Nortura, Hærland, Norway). Protease from *Bacillus licheniformis* (Alcalase, 2.4 U/g) was from Sigma-Aldrich (St. Louis, MO, USA); Endocut 01, Endocut 02 and Endocut 03 from Tailorzyme ApS (Søborg, Denmark); FoodPro PNL and FoodPro 30L from DuPont Danisco (Copenhagen, Denmark); MaxiPro NPU from DSM Food Specialties (Delft, the Netherlands); Promod 950 L and Promod 144P from Biocatalyst Ltd. (Cardiff, UK); and Veron L10 was from AB Enzymes GmbH (Darmstadt, Germany). Analytical grade acetonitrile, trifluoracetic acid (TFA) and monosodium phosphate used for SEC were purchased from Sigma-Aldrich. Sulfanilamide used for Dumas analysis; 2,2-diphenyl-1-picrylhydrazyl (DPPH), quercetin, methanol used for DPPH assay; and ACE from rabbit lung (≥2 U/mg, EC 3.4.15.1), N-Hippuryl-His-Leu hydrate (HHL), hippuric acid (HA), captopril, boric acid, hydrochloric acid, sodium hydroxide, sodium chloride used for ACE-1 assay were analytical grade and purchased from Sigma-Aldrich. Water was prepared by deionization and membrane filtration (0.22 μm) using a Millipore Milli-Q purification system (Merk Millipore, USA).

2.2. Production of hydrolysates

The hydrolysis of MDCR was performed according to the method described by Wubshet et al. (2017). First, MDCR were homogenised using a food processor, vacuum packed into plastic bags and stored at −20 °C until further use. The hydrolysis was performed in a Reactor-Ready™ jacketed reaction vessel (Radleys, Saffron Walden, Essex, United Kingdom) connected to a JULABO circulator pump (Julabo GmbH Seelbach, Germany). Water in the vessel jacket was kept at a selected temperature (±1°C) for individual enzymes (Table 1). The homogenized MDCR (500 g) were suspended in 1 L of purified water and mixed at 300 rpm until the suspension reached the selected temperature for the hydrolysis. At that point, a selected enzyme was added, enzyme loading percent (relative to 500 g of MDCR) is specified in Table 1. The hydrolysis was performed for 120 min, and samples (40 mL) were collected at 10, 30, 45, 60, 90 and 120 min. After the sample collection, the enzyme was thermally inactivated by rapid increase of temperature in a microwave oven (ACP, IA, USA) for several seconds followed by heating in a water bath at 90 °C for 15 min. After the enzyme inactivation, samples were centrifuged for 15 min at 4400 rpm and 25 °C, to separate three phases: fat, water, and sediment. The separated water phase was filtered with a Seitz® T 2600 depth filter sheet (Pall Corporation, Fribourg, Switzerland) and lyophilized using a Gamma 1–16

Table 1
An overview of enzymes and hydrolysis conditions. Individual temperatures and enzyme loadings were selected based on the optimal conditions specified by the manufacturers or previous study. The enzymes in powder form were dissolved in purified water.

Enzyme	Code	Enzyme loading (w/w) %	Temperature (°C)	Production organism or biological source
Alcalase	Alc	1	50	*Bacillus licheniformis*
Endocut 01	E01	1	55	*Bacillus subtilis*
Endocut 02	E02	1	60	*Bacillus licheniformis*
Endocut 03	E03	1	62.5	*Bacillus clausii*
FoodPro 30L	FP30	5	55	*Bacillus subtilis*
FoodPro PNL	PNL	5	60	*Bacillus amyloliquefaciens*
MaxiPro NPU	NPU	3	45	*Bacillus amyloliquefaciens*
Promod 144P	P144	2	50	*Carica papaya*
Promod 950L	P950	1	55	microbial
Veron L10	V10	3	50	*Carica papaya*

LSCplus freeze dryer (Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany). A similar procedure was performed for each of the 10 enzymes (listed in Table 1), resulting in 60 different samples.

2.3. Moisture and protein content

Moisture content of the freeze-dried hydrolysates was determined by overnight weight loss after oven drying at 105 °C. Freeze-dried hydrolysates (ca. 5 mg) were packed into tin foils and combustion was performed using a Vario EL cube (Elementar, Langenselbold, Germany) according to Rieder et al. (2021). The instrument was operated in CNS mode and sulfanilamide was used as a standard for correction. Protein content was calculated from total nitrogen using the protein conversion factor 6.25.

2.4. Size exclusion chromatography

SEC was performed as described by Wubshet et al. (2017). The hydrolysates were prepared in ultrapure water at a concentration of 10 mg/mL and filtered through a Millex-HV PVDF syringe filter with pore size 0.45 mm (Merck Millipore, Billerica, MA). Peptides were separated on a BioSep-SEC-s2000 column (Phenomenex, Værløse, Denmark, 300 × 7.8 mm) coupled with a Dionex UltiMate 3000 HPLC system (Thermo Scientific, Waltham, Massachusetts, U.S.). An injection volume of 10 μL was used for all analyses. The mobile phase was acetonitrile (30% v/v) in ultrapure water (70% v/v) containing 0.05% TFA. The flow rate was 0.9 mL/min, and the UV absorption was monitored at 214 nm. Chromatographic runs were controlled using Chromeleon 6.80 software. MWD and M_w of the hydrolysates were calculated using PSS winGPC UniChrom V 8.00 software (Polymer Standards Service, Mainz, Germany). For calculation of MWD and M_w, similar peptide standards were used as described in Wubshet et al. (2017).

2.5. Dry-film FTIR analysis

Dry-film FTIR analysis was performed according to Wubshet et al. (2017). The freeze-dried hydrolysates were dissolved in ultrapure water to 50 mg/mL, followed by filtration. Each of the filtered samples (5 μL) was deposited on to a 96-slot Si-microtiter plate (Bruker Optik GmbH, Germany) and dried at room temperature to form dry films. Each sample was made in five replicates and measured by a High Throughput Screening eXTension unit coupled to a Tensor 27 spectrometer (both Bruker Optik GmbH, Germany). The spectra were recorded in the region between 4000 and 400 cm^{-1} with a spectral resolution of 4 cm^{-1} and an aperture of 5.0 mm. For each spectrum, 40 interferograms were collected and averaged. Data acquisition was controlled using Opus v 6.5 software (Bruker Optik).

2.6. Radical scavenging (antioxidant) activity

The radical scavenging activity of the hydrolysates were determined using a spectrophotometric method described by López et al. (2007) with some modifications. The freeze-dried hydrolysates were dissolved in 50% methanol to obtain concentration of 0.94 mg/mL. DPPH and quercetin were also dissolved in 50% methanol to obtain 0.2 mM and 80 μM, respectively. An aliquot of a hydrolysate (100 μL) was mixed with 100 μL of 0.2 mM DPPH (sample measurement) or with 100 μL 50% methanol (sample blank measurement). The negative control was 200 μL of 0.1 mM DPPH in 50% methanol. Absorbance (Abs) was measured at 515 nm after incubation at 30 °C for 30 min in a microplate reader Synergy H1 (BioTek, Winooski, VT, USA). The radical scavenging capacity was calculated as given in Eq. (1):

$$\text{Radical scavenging activity}(\%) = \left[\left(Abs_{negative\ control} - \left(Abs_{sample} - Abs_{sample\ blank}\right)\right)/Abs_{negative\ control}\right] \times 100 \quad (1)$$

The antioxidant activity of the hydrolysates was expressed as quercetin equivalents (Q Eq). For calculation of Q Eq, a calibration curve was created based on measured activities of 0.62, 1.25, 2.5, 5, 10, 20, 40, 80 μM quercetin (start concentrations). Activities for all hydrolysates were measured in triplicates and reported as averages with standard deviation. Due to poor solubility of samples in the assay conditions, antioxidant activity of hydrolysates from Alcalase and Endocut 03 could not be acquired.

2.7. ACE-1 inhibition activity

ACE-1 inhibitory activity of the hydrolysates was determined according to a protocol by Lahogue et al. (2010) with some modifications. The hydrolysates, ACE-1, HA, and captopril were dissolved in 0.1 M borate buffer (pH 8.3) containing 0.3 M NaCl. The dissolved hydrolysates (1.75 mg/mL) were filtered through a Millex-HV PVDF syringe filter with pore size of 0.45 mm. The 50 mU/mL ACE-1 solution (50 μL) was mixed with 25 μL of sample, borate buffer (negative control) or 3.5 μM captopril (positive control) and incubated in a 48 well plate at 37 °C for 10 min. After incubation, 100 μL of 2.5 mM substrate HHL was added, and the samples were further incubated at 37 °C for 60 min. The reaction was stopped by addition of 1 M HCl (210 μL). The product HA and the substrate HHL were separated on a Luna C18 column (Phenomenex, 4.6 × 150 mm, 3 μm) coupled with a Dionex UltiMate 3000 HPLC system at room temperature. The mobile phase consisted of 0.05% TFA in ultrapure water (solvent A) and 0.05% TFA in acetonitrile (solvent B). A solvent gradient was applied. The mobile phase composition was 15% B for 15 min, increased to 55% B (from 15 to 21 min), increased to 100% B (21 to 35 min) and returning to 15% B (35 to 45 min). The injection volume was 50 μL. The flow rate was 0.5 mL/min and the UV absorption was measured at 228 nm. The inhibition percentage was calculated as given in Eq. (2):

$$\text{ACE-1 inhibition}\ (\%) = [1 - (\text{Area of HA for sample})/\text{Area of HA for negative control}] \times 100 \quad (2)$$

Activities for all hydrolysates were measured in triplicates and reported as averages with standard deviation.

2.8. Statistics

Correlation between univariate variables (% moisture, % protein, M_w, antioxidant activity (Q Eq), and ACE-1 inhibition) were studied by calculating Pearson's correlation coefficients (r) and p-values. Pearson's covariance matrix was calculated in MATLAB (R2018a, The MathWorks, Inc., Natick, MA, USA). Prior to multivariate analysis, SEC and FTIR raw data were pre-processed. SEC chromatograms were normalized against total area and the chromatographic region 5–15 min was chosen for analysis. The five technical replicates of the FTIR spectra were averaged to create a single spectrum per hydrolysate. The averaged FTIR spectra were transformed into second derivative spectra using the Savitzy-Golay algorithm with a polynomial degree of two and a window size of 13

points. Afterwards, the second derivative spectra were normalized using extended multiplicative signal correction and the spectral region 1800–700 cm^{-1} was chosen for analysis. Principal component analysis (PCA) of the FTIR and SEC data was performed to study the overall variation in the SEC and FTIR datasets. Validation of PCA was performed using leave-one-out cross-validation (LOOCV). Correlation between PC scores and bioactivities were studied by fitting linear regression model. PLSR models based on SEC and FTIR were developed for prediction of % ACE-1 inhibition and DPPH radical scavenging (μM Q Eq) of the protein hydrolysates. Cross validation of the PLSR models was performed using both LOOCV and leave-one-group-out cross-validation (LOGOCV) (Baumann, 2003; Montesinos López et al., 2022). In LOGOCV, a group consisted of six samples produced by the same enzyme was held out at a time. Coefficients of determination (R^2), root mean square error of prediction (RMSECV) and number of factors were used for model evaluation. Multivariate analysis was performed using Unscrambler 11 software (CAMO ASA, Oslo, Norway).

3. Results and discussion

A total of sixty hydrolysates were produced from MDCR using different processing conditions. Subsequently, ACE-1 inhibitory and DPPH radical scavenging activity of the hydrolysates were measured. PLSR models based on FTIR fingerprint and SEC were developed for prediction of ACE-1 inhibitory and DPPH radical scavenging activities of the hydrolysates.

3.1. Effect of processing parameters on antioxidant activity and ACE-1 inhibition

The hydrolysates showed varied DPPH radical scavenging and ACE-1 inhibitory properties (Fig. 1). The observed DPPH radical scavenging activity ranged from 0.08 μM Q Eq (P144 120 min) to 2.8 μM Q Eq (NPU 10 min) measured at hydrolysates' concentration of 0.47 mg/mL. The results show that both enzyme choice and time of hydrolysis influence the DPPH radical scavenging capacity of the hydrolysates (Fig. 1 A). Our observation agrees with previous studies showing that enzyme and hydrolysis time influence antioxidant activity of protein hydrolysates from blue mussel (Wang et al., 2013), barley hordein (Bamdad et al., 2011) and silver carp (Malaypally et al., 2015). The hydrolysates made by FP30 and NPU have overall higher activity than the other hydrolysates, when comparing in accordance with hydrolysis time. The variation in antioxidant activity depends on enzyme since various proteases have different specificities and can result in peptides with different sequences.

Another specific trend was a decrease in radical scavenging capacity of the hydrolysates with increasing hydrolysis time (Fig. 1 A). Hydrolysis time is inversely correlated to M_w of hydrolysates. Our results indicated that samples with lower hydrolysis time (higher M_w) have higher DPPH radical scavenging activity (Fig. 1 A). However, an inconsistent relationship between MW and antioxidant activity has previously been reported. For example, Jamdar et al. (2012) showed that antioxidant activity of poultry viscera protein hydrolysate did not depend on MW of the peptides. In contrast, Li et al. (2013) demonstrated that DPPH radical scavenging activity of fish collagen hydrolysates is negatively correlated with the average MW of the peptides. The raw material used in the current study (i.e., chicken deboning residue) is also rich in collagen (Kristoffersen et al., 2022).

The ACE-1 inhibition of the hydrolysates varied from 35% (P144 90 min) to 74% (PNL 45 min) measured at hydrolysates' concentration of 0.25 mg/mL. The results show that the choice of enzyme has a stronger effect on the inhibitory potential than the hydrolysis time (Fig. 1 B). No consistent trend was observed for changes in activity in the course of the hydrolysis time. The hydrolysates can be roughly divided into three groups (Fig. 1 B). One group contains the hydrolysates with no strong dependency of the hydrolysis time on the activity (FP30, PNL, P950 and E01). Another group includes the hydrolysates that showed decrease in activity with the increasing hydrolysis time (Alc, NPU, P144 and V10). The third group comprises the hydrolysates that demonstrated some increase of ACE-1 inhibition with hydrolysis time (E02 and E03). While it is important to have a relatively short peptide (range 2–12 amino acids) for having an adequate ACE-1 inhibitory activity (Hernández-Ledesma et al., 2011), the peptide chain length alone does not result in increased ACE-1 inhibition. This is reflected in the lack of consistent trend between hydrolysis time and ACE-1 inhibitory activity. A specific inhibitor of a therapeutic target such as ACE-1 requires, in addition to being a small molecule, a specific pharmacophore with a strong binding affinity (i.e., small dissociation constant, Kd). Previous *in vitro* and *in silico* studies have indicated that ACE-1 inhibitory potential of peptides is connected to their specific amino acid sequence (Iwaniak et al., 2014; Wu et al., 2006; Zhang et al., 2020). ACE-1 inhibitory peptides have been reported to have competitive, noncompetitive, or mixed modes of action (Ahn et al., 2012; Lee & Hur, 2017; Udenigwe & Aluko, 2012). Our observation agrees with the previous studies showing that several factors influence the ACE-1 inhibitory properties of the hydrolysates such as specific amino acid sequences and peptide length.

Covariance analysis was performed (Fig. 2) to study the correlation of observed bioactivities with gross composition parameters of the hydrolysates. No strong correlation between the bioactivities (i.e., ACE-1 inhibition and antioxidant activity) and protein content, moisture content or M_w of the hydrolysates was found. However, there was a moderate correlation between M_w and antioxidant activity ($r = 0.41$ and p-value $= 0.0041$). Overall, the absence of strong correlation with single variables indicates that a multivariate correlation based on a detailed fingerprinting of constituting peptides is required to establish a relationship with bioactivities.

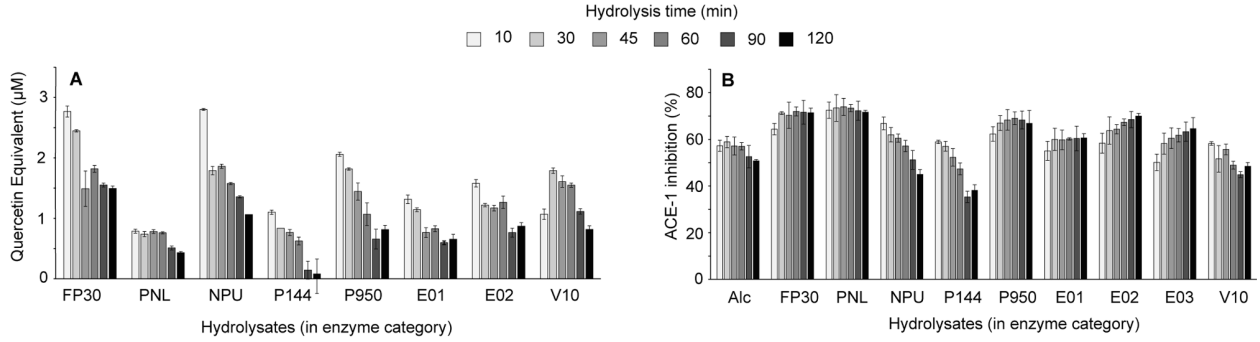

Fig. 1. Bioactive properties of the hydrolysates: (A) DPPH radical scavenging activity (Q Eq in μM). The hydrolysates were tested at a concentration of 0.47 mg/mL. (B) ACE-1 inhibition (%). The hydrolysates were tested at a concentration of 0.25 mg/mL. Standard deviations are shown.

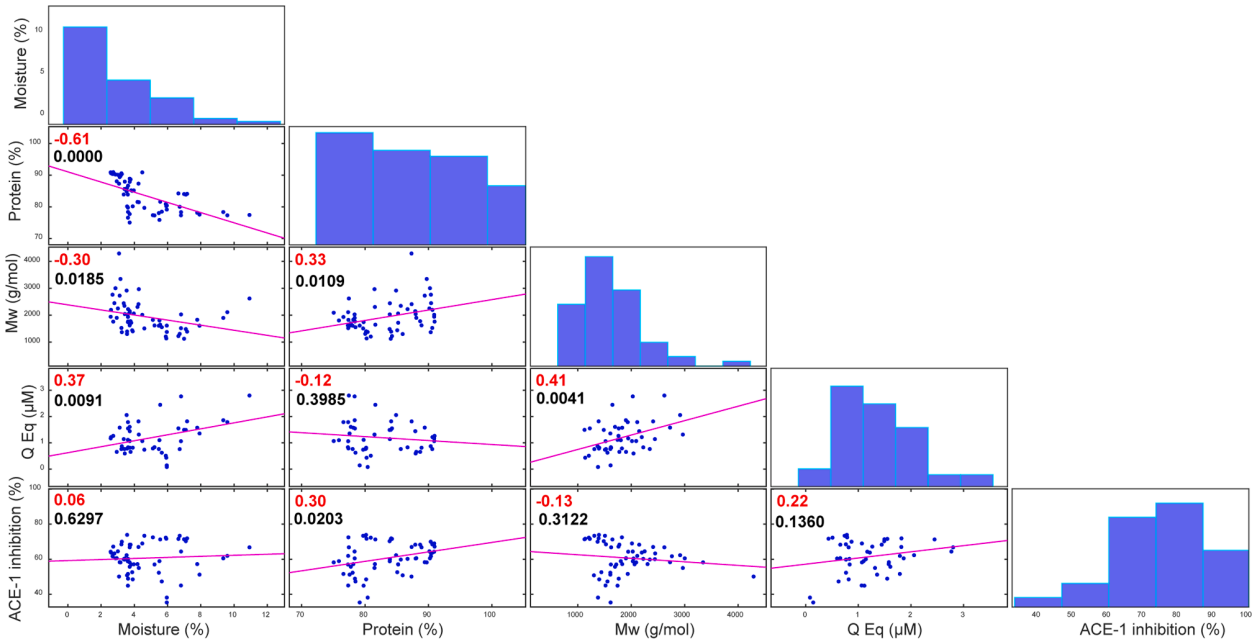

Fig. 2. Pearson's correlation coefficients between all pairs of variables (% moisture, % protein, M_w, antioxidant activity (Q Eq), and ACE-1 inhibition) measured for chicken protein hydrolysates. Histogram showing distribution of the data in each of the variables are presented in the diagonal sub-plot. Inserted to the top-left corner of the off-diagonal subplots are r (red font) and p-values (black font) for each pair of coefficients. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

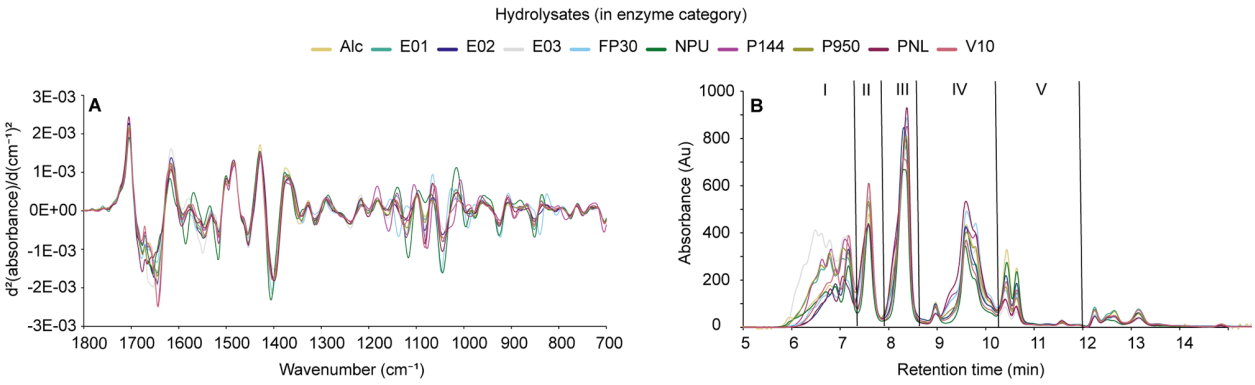

Fig. 3. Chemical characteristics of the hydrolysates produced by ten enzymes after 30 min. (A) Second derivative of FTIR spectra (1800–700 cm^{-1}). (B) SEC chromatograms of the samples measured at 214 nm (from 5 to 15 min).

3.2. *Effect of processing parameters on the chemical fingerprints of hydrolysates*

3.2.1. *FTIR fingerprints of the hydrolysates*

Fig. 3 A shows ten representative FTIR spectra of the hydrolysates prepared with the 10 different proteases (sampled after 30 min of hydrolysis). The spectra of different samples show clear differences in certain areas (e.g., around 1676, 1643, 1585, 1549, 1516, 1454, 1402, 1238, 1118, 1080, 1041 cm^{-1}). These spectral regions have been previously attributed to features of secondary protein structure, peptide backbone, terminal groups of peptides and side chains of amino acids (Barth, 2000; Böcker et al., 2017). Similarly, the influence of hydrolysis time on the hydrolysates prepared by P950 is shown in Fig. S1 A. An increase in the hydrolysis time resulted in a decrease in the absorption regions around 1645 and 1547 cm^{-1}. These absorption areas have been previously assigned to alpha-helical structures (amide I and amide II, respectively) (Böcker et al., 2017). Additionally, an increase in the absorption regions around 1516 and 1402 cm^{-1} was observed. These regions are considered to be characteristic for free amino- and carboxyl-termini (Böcker et al., 2017). Both the decrease in the absorption areas characteristic for secondary structure and the increase in the absorption regions assigned to terminal groups of peptides are consistent with changes during hydrolysis process, such as loss of secondary structure and increasing number of peptides.

PCA of the FTIR spectra was carried out to evaluate the variation between the 60 hydrolysates and study the influence of processing conditions on hydrolysates composition. The first principal component (PC-1) (Fig. 4 A) explained 44% of the variance and the grouping of the samples indicates that time of hydrolysis (within the time series produced by each enzyme) is the main factor. The loadings for PC-1 (Fig. S2 A) show that the sample variance is related to changes around 1645, 1583, 1548, 1518 and 1410 cm^{-1}. These absorption regions were previously assigned to alpha-helices in amide I region (1645 cm^{-1}) and amide II region (1548 cm^{-1}), free carboxyl- (1583 and 1410 cm^{-1}) and amino-groups (1518 cm^{-1}) (Böcker et al., 2017). PC-2 explained 20% of the sample variance and seemed to group samples according to enzyme

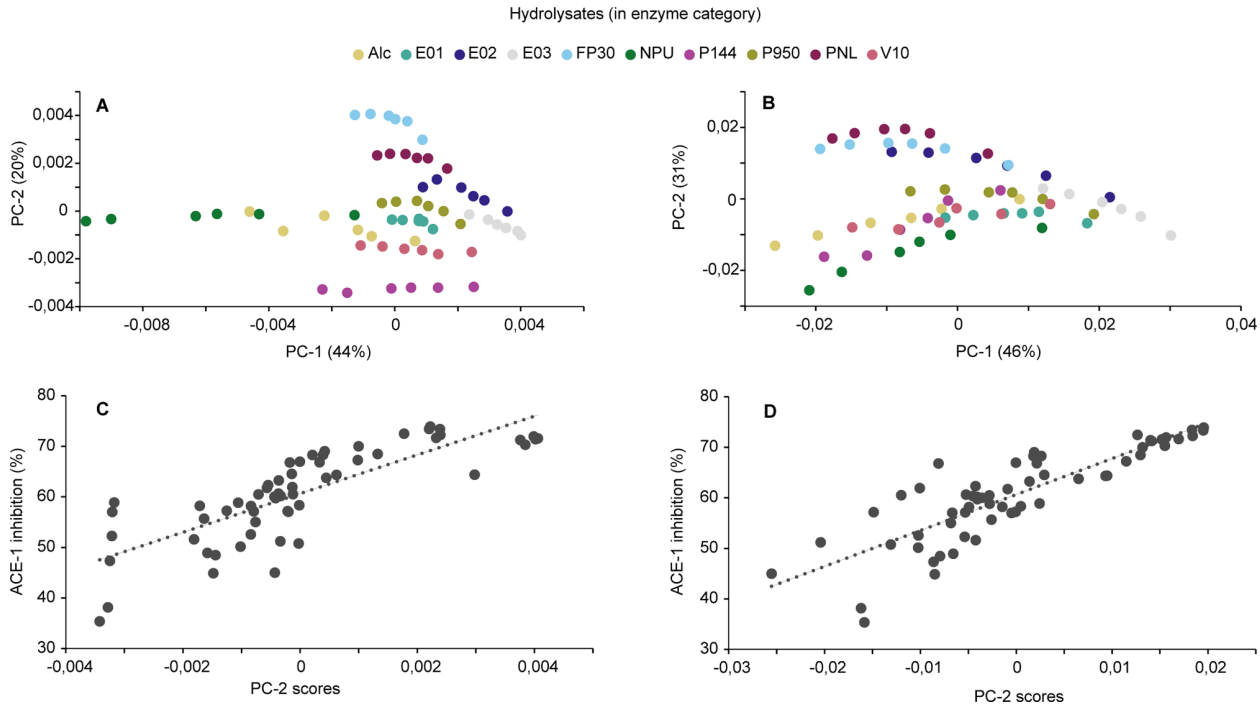

Fig. 4. PCA scores plot PC-1 vs PC-2 (A) for FTIR spectra and (B) for SEC. PC-2 scores in relation to the ACE-1 inhibition of the hydrolysates (C) for FTIR spectra and (D) for SEC.

type. The hydrolysates made by the enzymes P144 and V10, both produced from papaya, are grouped relatively together on one end of the axis in relation to the other hydrolysates made by enzymes produced by microorganisms (Table 1). The loadings for PC-2 (Fig. S2 A) show that the most prominent features are around 1678, 1643, 1155, 1120, 1070, 1049, 1040 and 1026 cm^{-1}. These features are characteristic for secondary structure (1678 and 1643 cm^{-1}), peptide backbone (1120 and 1049 cm^{-1}) and side chains of amino acids (1155, 1040, 1070 and 1026 cm^{-1}) (Barth, 2000; Böcker et al., 2017). Since there is a distinct enzyme-based grouping of hydrolysates along PC-2, the loadings for PC-2 could potentially be related to the specificity of protease. Interestingly, PC-2 scores were shown to have a correlation ($R^2 = 0.64$) with ACE-1 inhibitory activity of the hydrolysates (Fig. 4 C). This observation indicates that FTIR signatures can have a quantitative relationship with bioactivity of the hydrolysates.

3.2.2. SEC chromatograms of the hydrolysates

Representative SEC chromatograms of samples produced by the ten different proteases (hydrolysis time = 30 min) and samples hydrolysed for six time periods (enzyme = P950) are shown in Fig. 3 B and Fig. S2 B, respectively. The chromatograms are divided into five areas: (I) retention time (RT) 5–7.3 min corresponds to protein fragments larger than 2660 Da or 24 amino acids (calculated using M_w of 113); (II) RT 7.3–7.9 min corresponds to peptides of 2660–1500 Da or 24–13 amino acids; (III) RT 7.9–8.6 min – 1500–770 Da or 13–7 amino acids; (IV) RT 8.6–10.2 min – 770–230 Da or 7–2 amino acids and (V) RT 10.2–12 min – less than 230 Da or less than 2 amino acids. The absorbance intensities in each area are different depending on the enzyme type (Fig. 3 B) and the hydrolysis time (Fig. S2 B). For example, E03 has the most peptides at large MW (RT 5–7.9 min), while PNL has the most peptides at low MW (RT 7.9–10.2 min) compared to the other enzymes. When the chromatograms of hydrolysates produced by P950 are compared between different hydrolysis times (Fig. S2 B), the areas with high MW decreased and the areas with low MW increased as a function of hydrolysis time.

PCA of SEC chromatograms of the hydrolysates (Fig. 4 B) showed that PC-1 explained 46% of the sample variance, which could be attributed to the progress of hydrolysis or hydrolysis time. The loadings for PC-1 (Fig. S2 B) show that the main feature is the change in the areas' ratio of the highest MW (area I) and the lowest MW (area V). PC-2 explained 31% of the sample variance. An explanation for the sample grouping, as shown by the loadings for PC-2 (Fig. S2 D), is in the increase of the areas with peptides of 230–1500 Da (areas III and IV). In addition, PC-2 scores were found to have a strong correlation ($R^2 = 0.72$) with ACE-1 inhibition of hydrolysates (Fig. 4 D).

3.3. FTIR- and SEC-based prediction of bioactivity

PLSR models based on FTIR fingerprints and SEC chromatograms were developed for prediction of antioxidant and ACE-1 inhibition activity of the hydrolysates. The FTIR-based PLSR afforded an adequate model for prediction of DPPH radical scavenging activity with $R^2 = 0.74$ and RMSECV = 0.3 (Fig. 5 A, Table 2). The regression coefficients identified nine features as the most influential for the model (Fig. 5 C). These features can be attributed to peptide backbone (1049 cm^{-1}), protein secondary structure (1676, 1655, 1626 cm^{-1}) or amino acid side chains (1676, 1626, 1425, 1390, 1070, 1049, 1028 cm^{-1}) according to Barth (2000) and Böcker et al. (2017). FTIR spectra have previously been successfully used for prediction of antioxidant capacity of different products containing phenolic compounds (Leopold et al., 2012; Versari et al., 2010). The PLSR for prediction of ACE-1 inhibition afforded a model with R^2 of 0.91 and RMSECV of 2.7 (Fig. 5 B, Table 2). The regression coefficients indicated that five distinct features have the most influence in the model (Fig. 5 D). These features can be related to the peptide backbone (1412 cm^{-1}), protein secondary structure (1680, 1660 cm^{-1}) and amino acid side chains (1680, 1630, 1392 cm^{-1}) according to Barth (2000) and Böcker et al. (2017). The interpretations of the regression coefficients for DPPH radical scavenging and for ACE-1 inhibition suggest that the peptides' length and amino acid sequence are important for both activities.

PLSR models for prediction of bioactivities (i.e., DPPH radical scavenging and ACE-1 inhibition) were also developed using SEC chromatograms of the hydrolysates. The PLSR model for prediction of

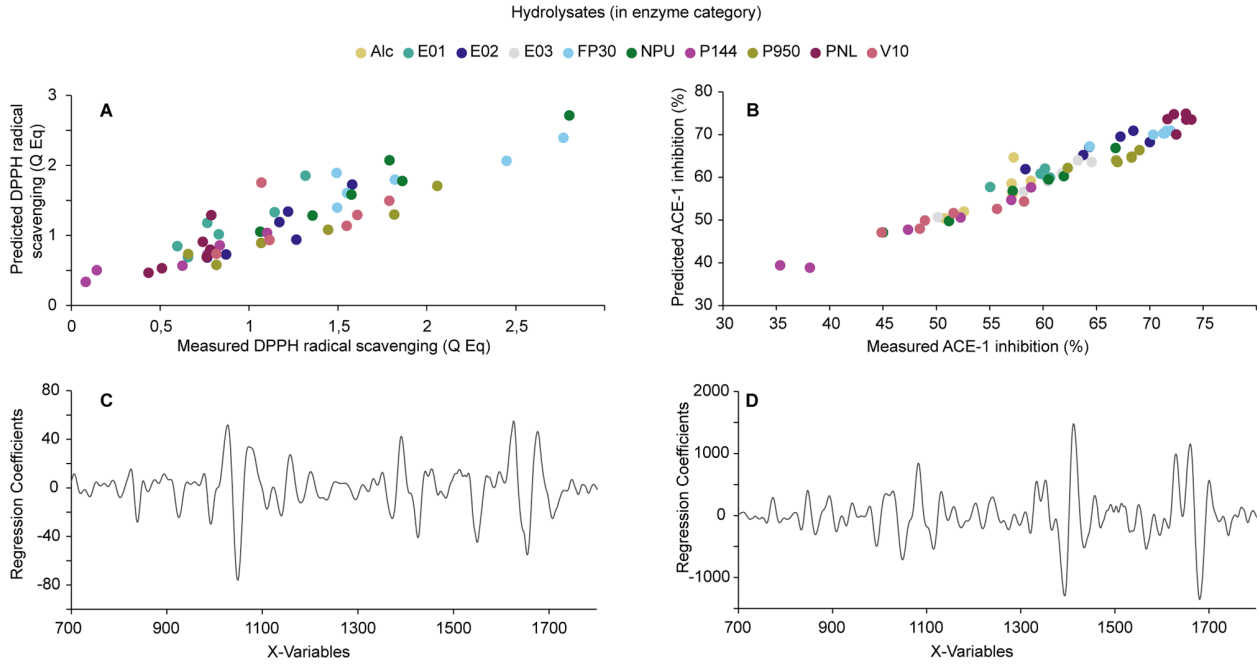

Fig. 5. PLSR models based on FTIR fingerprint of the hydrolysates: (A) PLSR model for prediction of DPPH radical scavenging, (B) PLSR model for prediction of ACE-1 inhibition and regression coefficients for prediction of (C) DPPH radical scavenging and (D) ACE-1 inhibition.

Table 2
Parameters of the PLSR models based on FTIR fingerprint and SEC chromatogram for prediction of DPPH radical scavenging and ACE-1 inhibition. PLSR models for DPPH radical scavenging were made using the results from 48 samples and PLSR models for ACE-1 inhibition were made using the results from 60 samples.

	FTIR fingerprint				SEC chromatogram			
	DPPH radical scavenging		ACE-1 inhibition		DPPH radical scavenging		ACE-1 inhibition	
	LOOCV	LOGOCV	LOOCV	LOGOCV	LOOCV	LOGOCV	LOOCV	LOGOCV
R-square	0.74	0.57	0.91	0.83	0.75	0.33	0.85	0.73
RMSECV	0.3	0.4	2.8	3.9	0.3	0.5	3.5	5.0
Number of factors	3	7	6	5	6	4	5	5

DPPH radical scavenging performed relatively similar to FTIR-based model with R^2 = 0.75 and RMSECV = 0.3 (Fig. 6 A, Table 2). The regression coefficients showed that the peak of the area III (i.e., 1061 Da) and the second peak of area IV (i.e., 405 Da) were the main variables with the largest influence on the prediction model (Fig. 6 C). Similarly, the SEC-based PLSR for prediction of ACE-1 inhibition afforded an adequate model with R^2 of 0.85 and RMSECV of 3.5 (Fig. 6 B, Table 2). The regression coefficients indicated that the peak in area III has the highest influence on prediction of ACE-1 inhibition (Fig. 6 D). A link between peptide M_w and ACE-1 inhibitory activity (Hernández-Ledesma et al., 2011) or antioxidant activity (Centenaro et al., 2014; Fernando et al., 2020; Liu et al., 2015) has previously been indicated, but this is the first study presenting a direct prediction of bioactivities from SEC chromatograms.

The number of factors, R^2 and RMSECV for all PLSR models are summarized in Table 2. The performance of models based on FTIR and SEC to predict the bioactivities of hydrolysates were relatively similar, when LOOCV was used. LOGOCV was performed to further test the robustness of the models. When comparing model performances after LOGOCV, the model based on FTIR fingerprint had higher R^2 compared to the models based on the SEC chromatograms, indicating a higher robustness of the FTIR-based models. FTIR fingerprints contain information on secondary structure, peptide backbone and side chains of amino acids (Barth, 2007). While SEC chromatograms contain information on hydrodynamic volume of a peptide, this volume is a function of molar mass, conformation and molecular configuration (Lubomirsky et al., 2021). Our results indicated that FTIR fingerprints possess more valuable information for prediction of bioactivities than the SEC chromatograms. The prediction models for ACE-1 inhibition showed a slightly better performance than for DPPH radical scavenging. This difference is likely due to the lower number of samples and lower range of values in the DPPH radical scavenging data set in comparison to ACE-1 inhibition data set.

3.4. General discussion

In the present study we demonstrated direct multivariate correlation between chemical fingerprints (SEC and FTIR) and bioactivities (ACE-1 inhibition and DPPH radical scavenging) of poultry by-product protein hydrolysates. Moreover, promising PLSR models for predicting bioactivity of the protein hydrolysates from their chemical fingerprints were developed. Such models can provide a quick insight into variables (a reflection of chemical constituents) important for a given activity of a hydrolysate. Both SEC and FTIR were in several previous studies used for chemical characterization of protein hydrolysates (Lindberg et al., 2021; Wubshet et al., 2017) and were used to predict parameters such as DH% (Kristoffersen et al., 2020). The current study, for the first time, directly predicted the bioactivities of protein hydrolysates using the two chemical fingerprints. Such prediction models (especially the FTIR-based models) can serve as an industrially relevant analytical solution to control quality of a given bioactive product. However, the reported models in this study are based on one type of raw material and two types

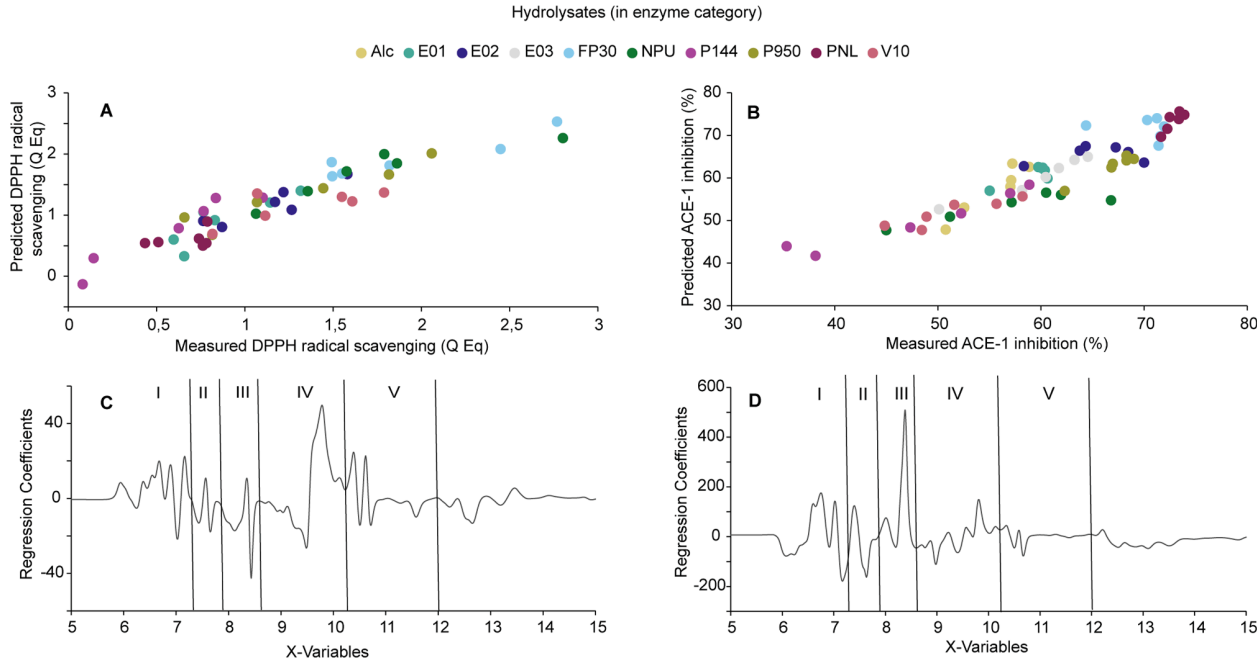

Fig. 6. PLSR models based on SEC chromatograms of the hydrolysates: (A) PLSR model for prediction of DPPH radical scavenging, (B) PLSR model for prediction of ACE-1 inhibition and regression coefficients for prediction of (C) DPPH radical scavenging and (D) ACE-1 inhibition.

of bioactivities. Further studies with larger calibration- and validation data sets, incorporating relevant raw material variations, are needed to make the model more robust. The present study suggests a potential that PLSR models of FTIR and SEC fingerprints can be expanded to other proteinaceous materials, such as by-products from marine products (e.g., fish) or novel protein sources (e.g., insects and algae) to predict bioactivities of their resulting hydrolysates/peptides.

Ensuring stable quality over time is an essential aspect for products with health-promoting effects. Due to the inherent raw material variation, bioactive products based on enzymatic hydrolysis of by-products are prone to product quality variations. This aspect is one of the major technological hurdles hampering development of bioactive peptides from complex by-products such as poultry residues. Therefore, analytical technologies to monitor variations in bioactivities of protein hydrolysates are essential elements in process and quality control. The FTIR-based model presented here can serve as such technology by providing a quick prediction tool for bioactivity. A recent study by Måge et al. (2021) based on a database of more than 1300 FTIR spectra of hydrolysates demonstrated that FTIR signatures can serve as an industrial tool to capture and monitor quality variations. The authors used the FTIR signature as a "quality" specification without direct correlation to attributes such as bioactivity. Our study suggests that such databases can further be expanded by providing a direct measure of the desired characteristics (i.e., bioactivities) and, hence, serve as quality control tool. However, the presented models must be expanded to include larger calibration datasets and independent validation sets before they can be used as a robust technology for quality control in the industry.

4. Ethics statement

Ethical approval was not required for this study.

CRediT authorship contribution statement

Liudmila Sorokina: Investigation, Methodology, Formal analysis, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Anne Rieder:** Conceptualization, Investigation, Software, Writing – review & editing, Supervision. **Shiori Koga:** Conceptualization, Investigation, Writing – review & editing, Supervision. **Nils Kristian Afseth:** Conceptualization, Investigation, Software, Writing – review & editing. **Rita De Cássia Lemos Lima:** Conceptualization, Methodology. **Steven Ray Wilson:** Writing – review & editing. **Sileshi Gizachew Wubshet:** Conceptualization, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2022.105170.

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Supplementary material

TABLES

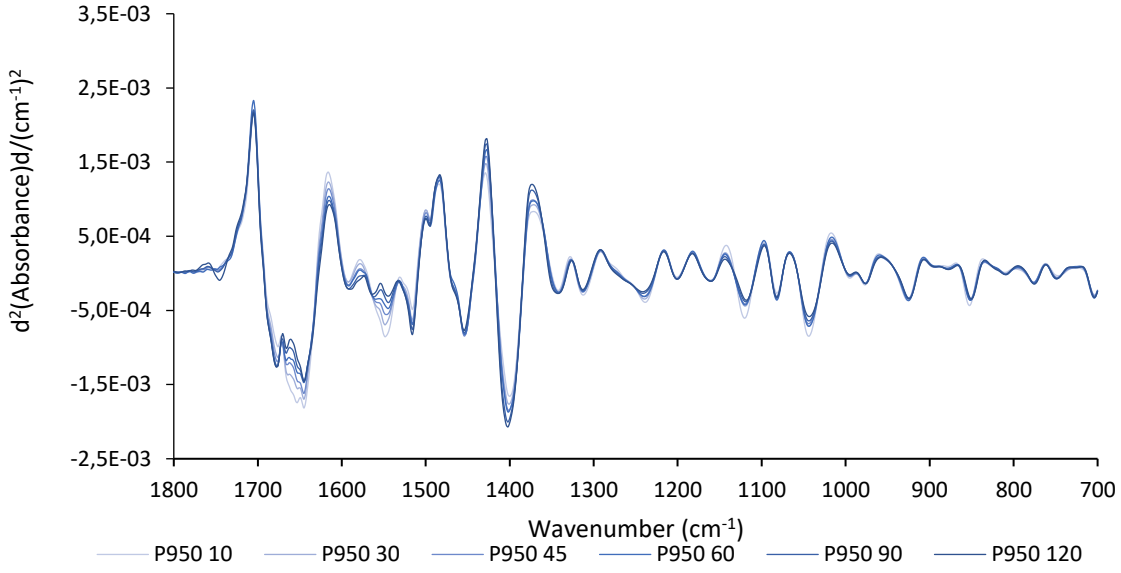
Table S1 – Protein content, moisture content, M_w and areas of the SEC chromatograms of hydrolysates.

Sample name		Protein content (%)	Moisture content (%)	M_w (Da)	Total area	area 1	area 2	area 3	area 4	area 5
protease	hydrolysis time (min)					(5-7,325 min)	(7,325-7,875 min)	(7,875-8,625 min)	(8,625-10,25 min)	(10,25-12 min)
Alcalase	10	81,6	4,2	2305,4	835	221	126	221	199	67
Alcalase	30	83,9	3,6	2029,7	832	180	107	217	219	110
Alcalase	45	85,2	4,0	2061,5	842	175	100	212	225	130
Alcalase	60	85,2	3,8	1719	831	144	94	210	233	150
Alcalase	90	85,1	3,9	1439,8	810	113	85	199	240	174
Alcalase	120	85,9	3,6	1301,2	805	94	76	191	249	195
Endocut 01	10	81,5	4,3	2965,6	803	287	120	195	151	51
Endocut 01	30	83,9	3,4	2443,1	861	255	129	222	186	69
Endocut 01	45	85,6	3,4	2347,2	863	245	127	221	192	78
Endocut 01	60	86,7	3,6	2224,6	876	235	127	223	203	87
Endocut 01	90	88	3,4	2041,7	897	219	128	229	216	105
Endocut 01	120	88,1	2,9	1902,1	898	201	125	227	225	119
Endocut 02	10	89,4	3,1	2726,1	979	344	147	236	212	40
Endocut 02	30	90,6	2,6	2196,8	996	275	147	261	259	53
Endocut 02	45	90,9	2,6	1943,2	998	236	145	271	282	64
Endocut 02	60	91	2,7	1760,5	1027	211	146	285	310	75
Endocut 02	90	90,6	3,2	1526,2	995	161	137	281	325	91
Endocut 02	120	90,4	3,2	1369,1	997	131	133	282	343	108
Endocut 03	10	87,3	3,1	4290,5	961	460	123	187	156	35
Endocut 03	30	89,7	3,2	3345,1	1005	412	139	218	194	41
Endocut 03	45	90,2	2,9	3001,6	1010	383	143	228	208	47
Endocut 03	60	90,4	2,7	2760,5	1031	365	148	241	224	53
Endocut 03	90	90,4	2,8	2447,8	1020	322	148	249	239	63
Endocut 03	120	90,1	3,0	2250,9	1046	301	152	262	258	74
FoodPro 30L	10	78,3	6,8	2028,1	828	185	127	236	233	47
FoodPro 30L	30	81,6	5,6	1647	844	138	118	247	276	64
FoodPro 30L	45	83,9	7,1	1485,3	874	119	117	257	302	79
FoodPro 30L	60	84,1	7,2	1388,5	870	103	110	254	314	88
FoodPro 30L	90	84,2	6,7	1223,9	864	79	102	250	328	104
FoodPro 30L	120	84	7,0	1124,7	860	65	93	245	337	119
FoodPro PNL	10	77,4	5,1	1824,6	841	165	133	257	250	37
FoodPro PNL	30	79,7	4,6	1508,1	867	120	127	273	297	51
FoodPro PNL	45	80,2	3,6	1395,3	881	104	123	277	316	60
FoodPro PNL	60	80	6,7	1308,1	862	89	115	271	319	68
FoodPro PNL	90	80,9	5,9	1205,7	851	73	106	263	326	84

FoodPro PNL	120	80,2	5,9	1136,7	841	62	98	256	328	97
MaxiPro NPU	10	77,4	10,9	2619,5	692	216	101	180	134	61
MaxiPro NPU	30	77,3	9,6	2111,6	667	156	90	178	147	96
MaxiPro NPU	45	78,4	9,4	1898,7	704	149	92	184	158	122
MaxiPro NPU	60	78,1	7,8	1829,6	711	144	91	180	159	137
MaxiPro NPU	90	77,6	7,9	1607,5	683	118	77	163	159	166
MaxiPro NPU	120	77,3	6,8	1517,5	673	109	70	151	155	188
Promod 144P	10	75,9	5,5	1803,5	767	160	136	234	190	47
Promod 144P	30	77,3	5,2	1617,7	793	139	130	235	208	81
Promod 144P	45	78	5,5	1607,8	770	137	122	216	198	98
Promod 144P	60	78,5	5,6	1554,2	744	125	111	201	194	112
Promod 144P	90	79,2	5,9	1612,5	723	129	94	174	187	140
Promod 144P	120	80,4	6,0	1376,7	734	102	93	180	202	157
Promod 950L	10	84,9	3,5	2915,3	869	310	129	205	182	43
Promod 950L	30	87,3	4,2	2409,2	929	272	137	235	223	62
Promod 950L	45	87,9	3,7	2149,3	963	250	140	250	247	76
Promod 950L	60	90,9	4,5	2013	924	221	131	242	247	82
Promod 950L	90	88,7	3,8	1776,9	942	190	127	251	270	104
Promod 950L	120	88,9	3,7	1623,3	937	165	122	250	281	120
Veron L10	10	75,1	3,7	2092,2	779	212	141	218	166	43
Veron L10	30	76,6	3,6	1934	766	186	129	207	175	69
Veron L10	45	77,8	3,7	1696	786	156	128	220	199	84
Veron L10	60	78,4	3,6	1765	760	158	112	198	195	98
Veron L10	90	79,8	3,6	1743,3	774	147	102	194	211	120
Veron L10	120	80,2	3,9	1400,4	777	111	102	200	228	136

FIGURES

A



B

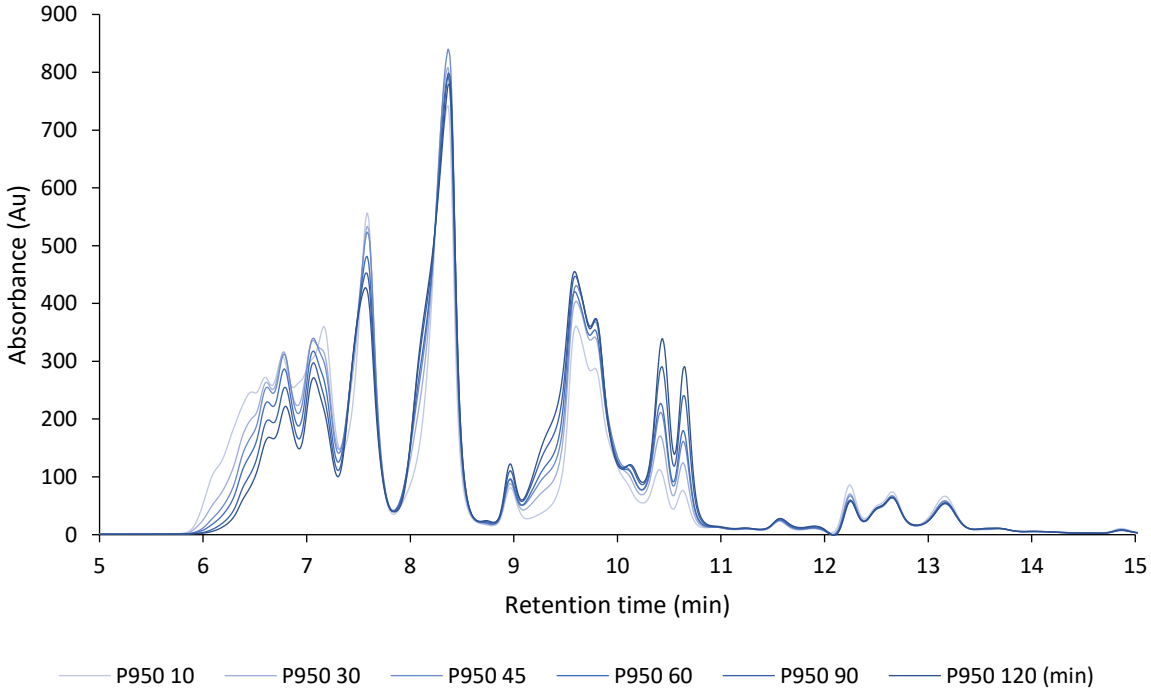
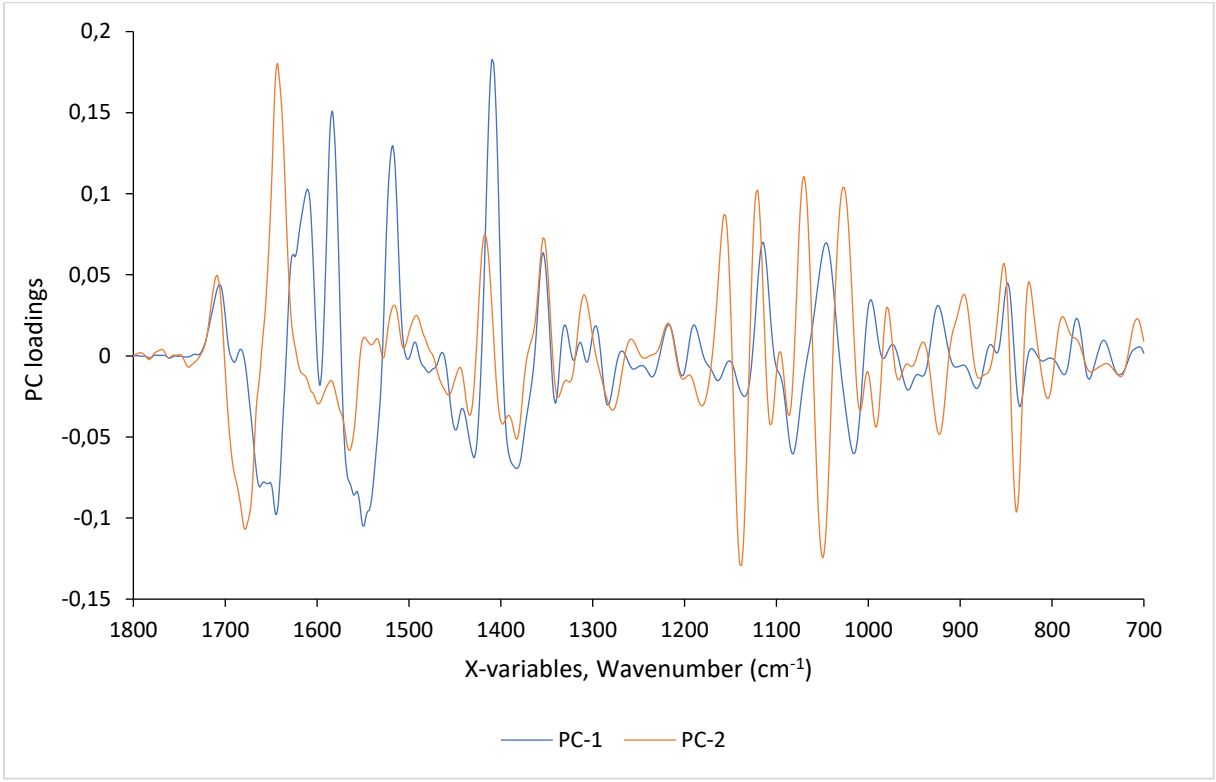


Figure S1 – Chemical characteristics of the hydrolysates produced by P950 at varied time of processing. (A) Second derivative of FTIR spectra (1800-700 cm^{-1}). (B) SEC chromatograms of the samples measured at 214 nm (from 5 to 15 min).

A



B

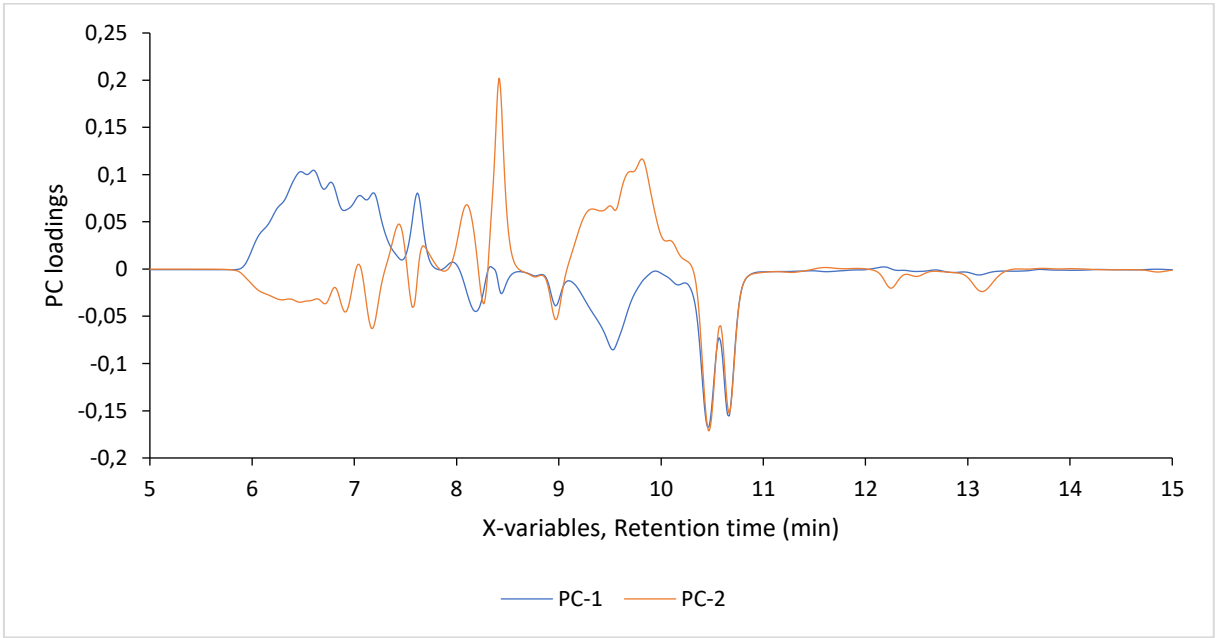


Figure S2 – Loadings for PC-1 and PC-2: (A) FTIR spectra and (B) SEC chromatograms.

Paper II

Paper III