Subcritical Water Hydrolysis of Citrus Pectin, Pea Protein Concentrate, and Their Mixture for Oligosaccharide and Peptide Production

by

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ABSTRACT

Bioactive oligosaccharides and peptides have drawn great attention due to reported health benefits such as antidiabetic, anti-obesity, anticancer, anti-hypertensive, and antioxidant. The most common methods to produce oligosaccharides and peptides are enzymatic and acid hydrolysis which have multiple disadvantages such as being environmentally harmful, time consuming, expensive, and using complicated procedures. Therefore, the main objective of this thesis was to evaluate subcritical water processing as an ecofriendly technology to hydrolyze pectin and pea protein concentrate to obtain oligosaccharides and peptides, respectively. In the first study, citrus pectin was hydrolyzed at 135-200^{o}C and 50 bar for 10-60 min using subcritical water, modified by either malic acid or citric acid, to obtain hexuronic acids and pectic oligosaccharides. The contents of hexuronic acids, pectic oligosaccharides, rhamnose, and the antioxidant activity of the obtained hydrolysates were determined. The results showed that with increasing temperature, the content of hexuronic acid significantly enhanced up to 0.63 g/g pectin at 200^{o}C/50 bar/10 min in aqueous citric acid media. The main oligosaccharide in the hydrolysates was arabino-oligosaccharide with the highest content obtained at 160^{o}C/50 bar/20 min with a molecular weight of 2.65 kDa. The main monosaccharide in the hydrolysates was rhamnose, which content increased at elevated temperature with a maximum value of 0.075 g/g pectin at 180^{o}C/50 bar/10 min. In the second study, citrus pectin was added as a catalyst for pea protein concentrate hydrolysis due to its ability of releasing hexuronic acids into the media to obtain bioactive peptides. Subcritical water at 160-240^{o}C and 50 bar for 10-60 min was employed. The degree of hydrolysis, the peptide size distribution, the hydrolysate antioxidant activity, the amino acid profile, the fluorescence intensity, and the total protein/peptide/amino acid content were determined. The degree of hydrolysis improved with increasing temperature with the highest value of 64.8% at 220^{o}C/50 bar/10 min

using pectin as the catalyst compared to citric acid (27.2%). The peptide with molecular weight of 4.1 kDa was the dominant peptide in the hydrolysate. The $DPPH^-$ scavenging activity of protein hydrolysates using citric acid was significantly higher than the ones obtained with pectin. The hydrolysates with pectin, on the other hand, had significantly higher ferric reducing antioxidant activity than the ones obtained with citric acid, indicating that pectin and citric acid catalyzed the hydrolysis reaction in different patterns to produce peptides capable of accepting and donating hydrogen atoms, respectively.

Keywords: Bioactive oligosaccharides, bioactive peptides, citrus pectin, pea protein concentrate, subcritical water hydrolysis.

PREFACE

This thesis is an original research work conducted by Ha Phuoc Hung Vo.

Chapter 3 of this thesis will be submitted as "Vo, H. and Saldaña, M.D.A. (2022). Hydrolysis of citrus pectin by subcritical water modified by carboxylic acids." to Food Hydrocolloids journal. I was responsible for designing and conducting the experiments, collecting and analyzing the data, and drafting the manuscript. Dr. Saldaña provided the research idea, advised on the experimental design, helped with data discussion, and provided the funding for the research.

Chapter 4 of this thesis has been presented as "H.P.H. Vo and M.D.A. Saldaña. (2022). Subcritical water hydrolysis of pea protein concentrate and its mixture with citrus pectin" at the 13th International Symposium on Supercritical Fluids in Montreal, Quebec, Canada. I was responsible for designing and conducting the experiments, collecting and analyzing the data, and preparing the manuscript. Dr. Saldaña provided the research idea, advised on the experimental design and experimental data, helped with data discussion, and provided the financial support. Part of this chapter has been filed for provisional patent with No. 63/340,088. This chapter 4 will also be submitted as "Vo, H. and Saldaña, M.D.A. (2022). Hydrolysis of pea protein concentrate in subcritical water media with addition of citrus pectin and citric acid." to the Journal of Supercritical Fluids.

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ABBREVIATIONS

λ_{max}	Maximum emission wavelength
ABTS	2,2'-Azino-bis(3-Ethylbenzothiazoline-6-Sulfonic acid)
ACE	Angiotensin converting enzyme
AG	Arabino-galacturonan
BSA	Bovine serum albumin
BSG	Brewer spent grains
CSF	Combined severity factor
DE	Degree of esterification
DH	Degree of hydrolysis
DP	Degree of polymerization
DPPH	1,1-Diphenyl-2-Picrylhydrazyl
E/S	Enzyme to substrate ratio
ELSD	Evaporative light scattering detector
EPG	Endopolygalacturonase
FRAP	Ferric reducing antioxidant power
FTIR	Fourier-transform infrared spectroscopy
GalA	Galacturonic acid
HexA	Hexuronic acids
HG	Homogalacturonan
HILIC	Hydrophilic interaction liquid chromatography
HM	High-methoxylated
HPLC	High performance liquid chromatography

HSS	High speed shearing
LDL	Low density lipoprotein
LM	Low-methoxylated
MW	Molecular weight
OligogalA	Oligogalacturonic acid
OligoRG	Oligorhamnogalacturonan
OPA	o-Phthalaldehyde
pK_a	Acid strength
PVA	Poly vinyl alcohol
RG-I	Rhamnogalacturonan-I
RG-II	Rhamnogalacturonan-II
sCW	Subcritical water
TPTZ	2,4,6-Tris(2-Pyridyl)-s-Triazine
UV/Vis	Ultraviolet/visible wavelength
XG	Xylo-galacturonan
XRD	X-ray diffraction

Chapter 1: Introduction

1.1. Rationale

Nowadays, the consumption of plant-based proteins has become more popular as it is of high quality, affordable, and a sustainable source of food for human consumption (Boukid et al., 2021; Kornet et al., 2020). According to Food and Agriculture Organization Expert Consultation, pulses, especially pea protein, meets almost all recommendations of essential amino acids for human consumption, except for methionine and cysteine. Canada is the world largest pea producer with a production yield of 2.5 million tonnes in 2021 followed by Russia, China, and the USA (Agri-Food Canada. 2021).

Pea, *Pisum sativum,* is one of the pulse crops that contains a relatively high amount of protein, 23-31% of pea seed (Boukid et al., 2021). The majority of protein found in pea are globulins and albumins accounting for 70-80% and 10-20% of total protein, respectively. Globulins can be further divided into three fractions, depending on the sedimentation coefficients: legumins (11S), vicilin (7S), and convicilin (7S) (Bogahawaththa et al., 2019). Several studies reported the potential bioactivities of pea protein hydrolysates. The angiotensin converting enzyme (ACE) inhibitory effect of pea protein hydrolysate was reported by Rudolph et al. (2017) where the inhibition capacity was 32mg/L obtained by the hydrolysis with an enzyme cocktail of thermolysin/chymotrypsin. Rudolph et al. (2017) mentioned that tryptophan- and tyrosine-containing dipeptides showed the strongest ACE inhibitory activity, particularly phenylalanine-trytophan and leucine-tyrosine. Moreover, the renin inhibitory activity of pea protein hydrolysates was reported by Chao et al. (2013) where the highest inhibition was 80% achieved after hydrolysis with 2% alcalase.

Enzymatic treatment is one of the most common methods to hydrolyze pea protein. Multiple types of enzymes have been employed such as alcalase, trypsin, chymotrypsin, pepsin, flavourzyme, papain, etc. (Awosika & Aluko, 2019; Barbana & Boye, 2010; Tamm et al., 2016). However, enzymatic hydrolysis requires various buffer systems, strict control temperature, pH, time, enzyme concentration, and ratio of enzyme and substrate. In addition, acids and alkaline hydrolysis have also been used to hydrolyze protein due to their effectiveness to achieve complete hydrolysis. Still, acids and alkaline hydrolysis can degrade and racemize amino acids due to the harsh conditions of concentrated acids or bases. The generated waste products would also require additional treatment before disposing due to the high content of corrosive reagents (Espinoza & Morawicki, 2012; Marcet et al., 2016).

In addition to pea protein, another type of biopolymer that has been extensively studied is pectin. Pectin is a heterogenous polysaccharide where D-galacturonic acid (GalA) is the predominant monomer (60%). Neutral saccharides are also present in pectic structure such as xylose, arabinose, fucose, and galactose. Pectin has a highly complex structure that can be categorized into three major structural fragments: homogalacturonan (HG), rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II (RG-II) (Valdivieso-Ramirez et al., 2021b). The most abundant fragment is HG accounting for 60% of the pectic polysaccharides, followed by RG-I and RG-II with 20-30% and 0.5-8%, respectively. Pectin is a potential source for the production of functional ingredients. The oligosaccharides derived from pectin such as arabinooligosaccharides, galactooligosaccharides, xylooligosaccharides, and mannooligosaccharides are known to have health-promoting and prebiotic effects (Khuwijitjaru et al., 2014). Moreover, rhamnose is a beneficial monosaccharide that constitutes the backbone of RG-I and RG-II regions in pectic structure. The reaction of rhamnose with fatty acids and amino acid can result in valuable

substances such as rhamnolipid (CA$166/10g) and furanone (CA$165/100g), respectively. Rhamnolipid and furanone are widely applied in food, pharmaceutical, and cosmetic industries (Illmann et al., 2009; Zhang et al., 2019).

Hydrolysis of pectin is a necessary step to generate beneficial compounds such as arabinooligosaccharide, galactooligosaccharide, oligogalacturonan, rhamno-monosaccharide, etc. Currently, the most common methods to hydrolyze pectin are acid and enzymatic treatment. Acid hydrolysis, however, requires extended time up to 72h using concentrated mineral acids such as H_2SO_4, trifluoroacetic acid (TFA), and HCl at high temperatures of 100-121^oC. In addition, without proper control of time and temperature, excessive hydrolysis can lead to the formation of lactones and furfural derivatives in different amounts (Li et al., 2017); these substances are known for being unsuitable for human consumption (Garna et al., 2006; Wikiera et al., 2015). Regarding enzymatic hydrolysis, multiple enzymes are required for the efficient depolymerization of pectin. The enzyme cocktail could include endo/exo-polygalacturonase, rhamnogalacturonan hydrolase, and rhamnogalacturonanlyase, among others. Babbar et al. (2015) used enzymes to produce pectic oligosaccharides with yields of 31.2% and 25.1% for sugar beet pulp pectin with ViscozymeL/40h/37^oC/pH 5 and orange peel pectin with Cellulase/45h/37^oC/pH 5, respectively.

Recently, subcritical water (sCW) has been used as a green technology to hydrolyze pectin and pectin-rich biomasses. The hydrolysis of passion fruit pectin was studied by Klinchongkon et al. (2017) using sCW at 80-160^oC/50 bar. The MW of pectin significantly reduced from 259 to 7 kDa when the temperature increased from 80 to 160^oC within only 5 min. They also mentioned that a higher temperature of 160^oC favored the release of uronic acids in the hydrolysate i.e., the content of uronic acids increased almost 6 times and reached the maximum value at 553 μmol/g at 160^oC/ 5 min. A different source of pectin isolated from apple pomace was studied by Eblaghi et al. (2021)

using sCW at 90-140°C up to 210 min. The authors reported that the content of uronic acids increased from 480 to 620 μmol/g as the temperature increased from 110 to 130°C at 30 min. Both studies investigated the hydrolytic potential of sCW towards pectin to produce uronic acids, however, none of them focused on the production of pectic oligosaccharides. Therefore, Valdivieso-Ramirez et al. (2021) conducted a study focusing on the yield of glucooligosaccharides derived from pea fiber hydrolysis. The yield of glucooligosaccharides with degree of polymerization of 2-6 was 20.4% at 135°C/120 min/100 bar.

Regarding protein hydrolysis, different biomasses has been treated with sCW to recover protein and obtain amino acids. Rice bran protein was hydrolyzed using sCW at 150-250°C up to 60 min (Sunphorka et al., 2012). The authors noticed that within the first 5 min, the protein aggregated and formed solid particle. Upon longer heating, the aggregates were decomposed to form amino acids with the highest content of 2g/L obtained at 250°C/60 min. Besides plant-based proteins, animal-based proteins such as tuna skin collagen was also hydrolyzed in sCW media (Ahmed & Chun, 2018). They reported that the highest degree of hydrolysis (DH) of 15% was obtained at 250°C/ 50 min. The DPPH scavenging and ABTS scavenging was found to be highest at 280°C/80 bar (21.32 and 50.68 vit C Eq/g of hydrolysate, respectively).

1.2. Hypothesis and objectives

To the best of our understanding, there is no study that employed sCW to hydrolyze either citrus pectin or pea protein concentrate to obtain beneficial compounds such as low MW bioactive oligosaccharides, rhamnose, hexuronic acids, and low MW bioactive peptides. Therefore, citrus pectin, pea protein concentrate, and their mixture were hydrolyzed with sCW in this study to achieve the objectives described below.

Study 1: Hydrolysis of citrus pectin by subcritical water modified with carboxylic acids.

Hypothesis: Subcritical water technology could promote the production of bioactive oligosaccharides, hexuronic acid, and rhamnose sugars from citrus pectin without generation of toxic chemicals such as furfural and furfural derivatives compounds.

Objectives:

- Study the effects of temperature (135, 145, 160, 170, 180, and 200ºC), reaction time (10, 20, 30, 40, 50, and 60 min), and solvents (water, aqueous citric acid (0.2% w/w), and malic acid (0.2% w/w)) on the production of pectic oligosaccharides, hexuronic acid, and rhamnose.
- Characterize the hydrolysates based on their physico-chemical properties, including the hexuronic acid content, rhamnose content, oligosaccharides degree of polymerization, the molecular weight distribution among others.
- Evaluate the functionality of the hydrolysates based on the antioxidant activity.

Study 2: Hydrolysis of pea protein concentrate in subcritical water with the addition of citrus pectin and citric acid.

Hypothesis: Hydrolysis of pea protein concentrate could be enhanced in subcritical water media with the supplementation of pectin-a biopolymer containing hexuronic acids.

Objectives:

- Study the effect of temperature (160, 180, 200, 220, and 240ºC), reaction time (10, 20, 30, 40, 50, and 60 min), mass ratio of protein:pectin (1:0.1, 1:0.5, 1:1, 1:2 and 1:3 w/w), and mass ratio of protein:citric acid (1:0.2, w/w) on the degree of hydrolysis of pea protein concentrate.

- Characterize the hydrolysates based on their physico-chemical properties, including the molecular weight distribution of peptides and amino acids profile.
- Evaluate the functionality of the hydrolysates based on the antioxidant activity.

Chapter 2: Literature review

2.1. Subcritical water

Subcritical water, also called superheated water, is hot liquid water at temperatures ranging from 100ºC (boiling point) to 374ºC (critical point) under pressure below 220 bar. **Fig. 2.1** shows the phase diagram of water which indicates the changes in physico-chemical properties at various pressures and temperatures. Under high temperature and pressure, many of the anomalous properties of water have been observed, such as low dielectric constant, low viscosity, and low density, all of which can affect the solvating power as well as the reactivity of subcritical water (Chakraborty et al., 2021; Saldaña & Valdivieso-Ramírez, 2015)

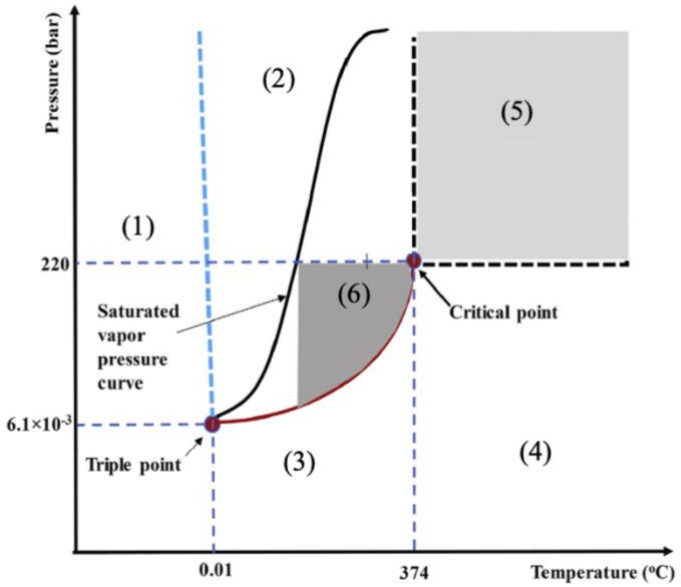


Fig. 2.1. Phase diagram of water: critical temperature of 374ºC and critical pressure of 220 bar. (1) Solid (ice), (2) liquid (water), (3) vapor, (4) superheated steam, (5) Supercritical water, and (6) subcritical water.

2.1.1. Physico-chemical properties of subcritical water

2.1.1.1. Dielectric constant

The dielectric constant, ε, is proportional to the polarity of a solvent and is determined by the ratio of absolute permittivity of a compound to the absolute permittivity of free space (Machmudah et

al., 2017). The higher the dielectric constant, the more polar a substance is, e.g., at ambient condition (25°C), the liquid water can dissolve most of the polar compounds due to high ε of 78.3. The dielectric constant of water is reduced drastically at increasing temperature, e.g., at 100°C, ε_{water}= 55.7 (Malmberg & Maryott, 1956). At temperatures near and above the critical point, the intermolecular interactions between water molecules are broken down because hydrogen bonding is exothermic, leading to the significant reduction of the dielectric constant as well as polarity of water (Abdelmoez & Abdelfatah, 2017; Moldoveanu & David, 2013). Due to the extensive breakdown of hydrogen bonds, the water becomes significantly less polar and shares the same behavior as organic solvents, such as methanol and acetone (Kus, 2012). Hence, it can dissolve organic compounds, forming a homogeneous fluid phase. Moreover, the single phase of sub/supercritical water can promote a fast and complete reaction for organic compounds (Machmudah et al., 2017).

2.1.1.2. Ionic strength

The ionic strength, I, measures the concentration of electrically charged species in a solution. It is determined as:

$$I = \frac{1}{2}\sum_i c_i Z_i^2 \quad (2.1)$$

where, c_i: the concentration of charge species i of charge Z_i.

The rate constant of reactions involving the same charge of ions proportionally increases with the ionic strength. Conversely, with the opposite charge ion, the reaction rate constant reduces as the ionic strength increases (Arnaut et al., 2007).

The ionic products (K_w) in sub/supercritical water increased by 10,000-fold when the temperature increases from 25°C to 300°C. At these temperature ranges, water acts as an acid or base catalyst due to the high concentration of H_3O^+ and OH^- ions. However, at temperatures higher than 300°C,

K_w value decreases, but increases with pressure e.g., K_w at 350°C/250 bar and 350°C/2500 bar are 1.69×10^{-12} mol^2 kg^{-2} and 1.23×10^{-10} mol^2 kg^{-2}, respectively (Kruse & Dinjus, 2007; Möller et al., 2011). The closer to the supercritical point, the sharper decrease is observed, particularly at 400°C, K_w value (3.72×10^{-20} mol^2 kg^{-2}) is even lower than the one at ambient condition (1.26×10^{-14} mol^2 kg^{-2}) (Marshall & Franck, 1981). Based on the ionic products, all reactions involving ionic species are generally enhanced in subcritical water media (Möller et al., 2011).

2.1.1.3. Mass transfer properties

The viscosity of water reduces substantially at subcritical temperature which can enhance the mass transfer rates, e.g., μ_{25^oC}= 884 μPa s and μ_{371^oC}= 50.4 μPa s. Because of the low viscosity and high diffusion rates, as well as high miscibility with non-polar substances, subcritical water is an outstanding medium for the fast and efficient chemical reactions.

The density of sub- and super-critical water are 0.8 g/cm^3 at 250°C and 0.17 g/cm^3 at 400°C, respectively. Because of its high density and high dissociation constant, subcritical water can induce ionic reactions while in supercritical water, radical reactions dominate. The ionic reactions can favor the hydrolysis reactions in subcritical water media due to having high K_w value of 1.23×10^{-10} mol^2 kg^{-2} at 350°C (Marshall & Franck, 1981; Toor et al., 2011).

Due to these properties, subcritical water is highly compatible with heterogeneous catalysts which can be well-dissolved in the reaction medium. Additionally, biomass hydrolysis reactions can be favored in subcritical water because of its superior transport properties of low viscosity and low density (Machmudah et al., 2017).

In subcritical water media, mass transfer rate of water is enhanced, however, water clusters phenomenon may occur. Near the critical point, an infinite network of hydrogen bonds is broken and water molecules cluster into each other to form a chain structure (Galkin & Lunin, 2005). The

density of the clusters (ρ = 1284 kg/m^3) can be up to 7 times higher than the density of the bulk solution (ρ = 167 kg/m^3) (Kalinichev & Churakov, 1999). At these "water clusters", the concentration of reactants may be different from the average one in the bulk solution due to a diffusion barrier. This phenomenon in subcritical water conditions results in inhomogeneous reaction products which may complicate the analysis (Möller et al., 2011).

In the subcritical water media, the solubility of certain types of salt, especially Type 2 salt like Na_2SO_4 is limited; therefore, it forms a fine-crystalline slimy precipitate which easily adheres to the wall of the system components, such as reactors or tubing, hence, causing folding or even blockage (Toor et al., 2011).

2.1.1.4. Severity factor

Severity factor (ln R_o) was first proposed by Overend & Chornet (1987) to combine the effect of time and temperature into a single factor that could evaluate the hydrolysis process. The severity factor is expressed in Eq (2.2).

$$R_o = t \times e^{\frac{T-100}{14.75}} \quad (2.2)$$

where: t is the reaction time (min) and T is the reaction temperature (°C).

A couple of years later, a new concept of combined severity factor was reported in the study of Chum et al. (1990) in which the authors used the pH after the hydrothermal treatment. The combined severity factor (CSF) is shown in Eq (2.3).

$$CSF = \ln R_o - pH \quad (2.3)$$

where: ln R_o is the severity factor and pH is the value obtained after the sCW treatment.

These factors have been widely employed in the sCW processes, especially for the hydrolysis of lignocellulosic biomasses using subcritical water. In the study of Kellock et al. (2019), where the authors used sCW as a pretreatment to remove hemicellulose and isolate lignin from spruce and

wheat straw, the severity factor varied from 3.4 to 3.9. There were up to 9% and 16% mass loss for spruce and wheat straw, respectively, at severity factor of 3.9. The effect of severity factor was again reported in the study of Batista et al. (2019) where the authors pretreated sugarcane straw with sCW to study the hemicellulose removal. The sugarcane straw had 96.47% and 97.01% of hemicellulose removed at severity factors of 4.7 and 4.99, respectively. Compared to severity factor of 3.23, the hemicellulose removal was 3 times lower than the one at a severity factor of 4.7. The authors mentioned that the severity factor and the residue yield was inversely proportional with a correlation of 0.989.

The severity factor is not only applicable in the sCW media, but it was also employed in the organic solvent media with acid as the catalyst. Eq. (2.4) shows the severity factor calculation in the acid modified organosolv media.

$$R^*_o = 10^{-pH} \times t \times e^{\frac{T-100}{14.75}} \quad (2.4)$$

where: log R^*_o is the severity factor, t is the reaction time (min), T is the reaction temperature (^oC). The concept of log R^*_o was used in the study of Salapa et al. (2018) where the authors used acetone media modified by sulfuric acid as a pretreatment to obtain xylose from barley straw at 140-160^{o}C within 20-40 min. At higher severity factor, the lignin removal rate was significantly enhanced. There was up to 60% of lignin was removed at severity factor of 1.5. A linear regression model was used to describe the relationship of lignin removal and severity factor (log R^*_o) (Eq (2.5)).

$$Removed\ lignin = 36.37 + 13.15\ logR^*_o \quad (2.5)$$

The relationship between xylose recovery and severity factor was described using third order empirical polynomial model which was expressed in Eq (2.6).

$$Total\ xylose = 35.40 + 30.48 logR^*_o - 4.85(logR^*_o)^3 \quad (2.6).$$

The maximum recovery of xylose was found to be 67.3% at 152 °C/25 min with 35 mol/m^3 of

sulfuric acid ($\log R^*_o = 0.78$).

2.1.2. Chemical reactions in subcritical water

2.1.2.1. Synthesis reactions

Alkylation is one of the major synthesis reactions that can be accomplished in subcritical water media. The reaction of phenol and propionaldehyde occurs without catalyst in the subcritical water media as indicated in the reaction scheme of **Fig. 2.2**. The results showed that the yield of alkylphenol increased with increasing water density, with a total yield of liquid products of 30% (Sato et al., 2003).

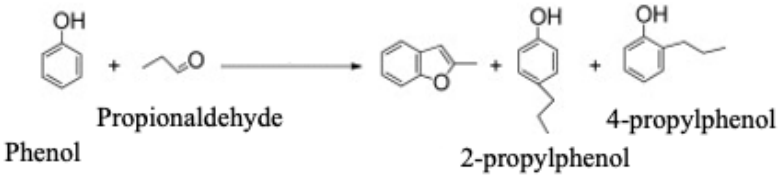


Fig. 2.2. Alkylation reaction of phenol with the production of alkylphenols.

The Friedel-Crafts alkylation reaction of phenol was studied in subcritical water at 275°C/20h/172 bar, up to 17% of alkylphenol (2-tert-butylphenol) was found. However, the closer to equilibrium, the reduction in alkylphenol yield was observed and only 10% of 2-tert-butylphenol was obtained at equilibrium (Chandler et al., 1998). It is believed that at subcritical temperature (275°C), the concentration of H_3O^+ and OH^- reach a maximum, hence, the acid- or base-catalyzed reactions occurs, leading to the formation of alkylphenol (Kruse & Dinjus, 2007).

Condensation reaction can also be favored by the H^+/OH^- rich environment of subcritical water. Condensation reaction involves the combination of two molecules to form one product with the loss of entropy (Raji et al., 2019). The reaction of benzaldehyde and 2-butanone was investigated in subcritical water without the addition of a catalyst. The yield of terminal enone is higher than other enones. Even though the efficiency of the reaction was low (20%), it demonstrated the

potential of subcritical water to conduct the condensation reactions without the addition of acids or bases catalysts (Kus, 2012; Pollet et al., 2014). **Fig. 2.3** shows the condensation reaction of benzaldehyde and 2-butanone.

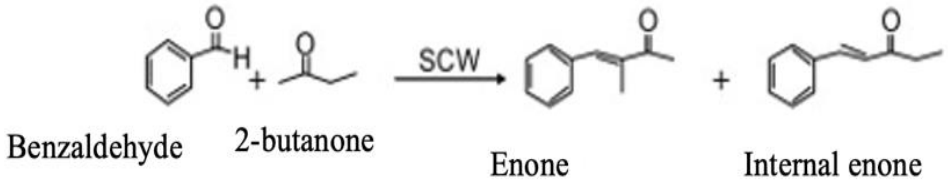


Fig. 2.3. Condensation reaction of benzaldehyde and 2-butanone to produce terminal enone and internal enone.

2.1.4.2. Cross-linking reaction

Cross-linking is one of the oxidative condensation reactions which can be accomplished in subcritical water. Chitosan and cassava starch were cross-linked with gallic acid in the study of Zhao & Saldaña. (2019). The authors detected new peaks in the FTIR spectra, indicating the formation of ester bonds between carbonyl and hydroxyl groups. The molecular weight of the starch/chitosan complexes was 1656 kDa, which was 5 times higher than the control sample of 331.4 kDa. The ester bonds were believed to form from the interactions of non-dissociated gallic acid with $-CH_2-OH$ groups of starch and chitosan.

After successfully crosslink starch and chitosan in subcritical water, a bioactive film was developed by crosslinking reaction of starch, chitosan, and gallic acid in sCW media with glycerol as a plasticizer in the study of Zhao et al. (2018) at the temperature from 75 to 150^{o}C under different pressures of 50-155 bar. Compared to the FTIR spectra of the chitosan, the amide-I peaks of the film shifted to the longer wavelength of 1640-1627 cm^{-1}, suggesting the linkages were formed between -OH groups of starch and $-NH_2$ groups from chitosan. Moreover, the peak at 1627 cm^{-1} was clearer indicating that starch, chitosan, and gallic acid were crosslinked via esters linkages. The XRD patterns showed a small peak at 20^{o} in the starch film which resulted from the

interaction of glycerol with amylose structure from starch.

Besides chitosan and starch, other polymer like polyvinyl alcohol (PVA) can also be formed by cross-linking reaction in subcritical water media. Polyvinyl alcohol synthesis was reported in the study of Purkayastha et al. (2022) where the authors used subcritical water at the temperature range from 140 to 170^{o}C up to 60 min of reaction time. Citric acid was used as the crosslinker and CO_2 as the acid catalyst for the reaction. They reported that with increasing temperature and time, the content of polyvinyl alcohol was enhanced with the highest content of 85% obtained at 30 min. The addition of CO_2 helped to increase the content of PVA which was increased approx. twice compared to the reaction without CO_2 due to the acid catalytic effect of H_2CO_3 formed by the dissolution of CO_2 in the sCW media. Moreover, the ester bonds found in the FTIR spectrum of PVA represented the linkage between -OH groups of PVA and -COOH groups of citric acid.

The use of crosslinker such as citric acid, gallic acid is necessary to form linkages between polymers. However, in the study of Esfahan et al. (2021), the crosslink networks within bovine serum albumin (BSA) were formed in subcritical water media without the addition of any crosslinker. The formation of such matrix was obtained at mild sCW temperature and short time of 120^{o}C/5 min. The BSA network had a highly porous structure that did not flow or fall in the inversion test, indicating that the protein network had well organized intermolecular interactions. The formation of BSA network was confirmed by gel electrophoresis. At 110^{o}C, the protein band at 116 kDa clearly showed up, meaning that the aggregation of BSA, which had molecular weight of 66 kDa in native form, was induced. The FTIR spectra showed that the intensity of -OH and -NH groups was stronger than the native protein, indicating that there was an enhanced interactions between -OH and -NH groups in the BSA matrix.

Again, the crosslinking reaction without the use of crosslinker was performed in the study of

Nkurunziza et al. (2021). They utilized sCW at 120 to 160^{o}C under constant time and pressure of 30 min and 30 bar, respectively, to crosslink gelatin and sodium alginate. The FTIR spectra confirmed the crosslinking between the two polymers. The amide A stretching of gelatin and -OH stretching of alginate disappeared, indicating the hydrogen bonding between the two polymers. Moreover, the peak at 1739 cm^{-1} was enhanced at 120^{o}C and 130^{o}C, indicating the crosslinking of gelatin and alginate via Maillard reaction. However, as the temperature increased to 140^{o}C and 150^{o}C, the peak at 1368 cm^{-1} disappeared, implying that sCW already hydrolyzed gelatin and alginate into smaller units. The X-ray diffraction pattern also indicated the crosslinking of gelatin and alginate. The native alginate and gelatin had 2θ peak at 13.5^{o} and 22^{o}, respectively. After the sCW treatment at 120 and 130^{o}C, the peak at 13.5^{o} disappeared and the intensity at 22^{o} was reduced. This result indicated that the crosslinking between two polymers was successful, and the partially crystal structure was the dominate arrangement in the crosslinked gelatin alginate chains. In addition, the hydrogel formed by the crosslinking of cellulose fibers was reported by Ciftci et al. (2017), where the authors employed ultrasound at 560W/40 min to form the nanofiber network without the addition of crosslinker. They noticed that the nanofibers were tangled to each other, forming a web-like structure. The interactions within the gel network were mainly inter- and intra-molecular hydrogen bonds.

2.1.4.3. Thermal hydrolysis

Hydrolysis in subcritical water media is usually referred as wet oxidation which involves high temperature (150-350^{o}C) and pressure (5-200 bar). In subcritical state, water molecules are highly ionized, the ionization constant of water is three orders higher than the value at ambient condition. Hence, subcritical water can act as an acid- or base- catalyst, favoring the hydrolysis reaction (Galkin & Lunin, 2005). There are several biomass materials that were hydrolyzed in subcritical

water. The most common ones are carbohydrates-rich, lipid-rich, and protein-rich biomasses.

- Carbohydrate-rich biomass hydrolysis

Carbohydrate-rich biomass has been extensively studied due to its potential to produce fermentable sugars, cellulose, and oligosaccharides (Khuwijitjaru et al., 2014; Novo et al., 2015; Prado et al., 2014; Valdivieso-Ramirez et al., 2021b). Fermentable sugars are the sugars that are directly involved in the bioethanol/biofuel production. They are commonly produced from lignocellulosic biomass hydrolysis by either acid or enzymatic digestion. However, those methods possessed several disadvantages such as high operational cost and environmental issues (Brethauer & Wyman, 2010). The subcritical water hydrolysis is a green alternative method that can effectively generate fermentable sugars from carbohydrate biomass.

In the study of Vedovatto et al. (2021), soybean hulls were subjected to subcritical water hydrolysis. The highest yield of fermentable sugars was obtained at 220^{o}C/250 bar with the value of 10.52g/100g soybean hull. With increasing temperature, the production of fermentable sugars subsequently increased due to the high concentration of ionic products, high diffusivity and the reduced viscosity of sCW; which favor the penetration of water into the lignocellulosic matrix, resulting in the better hydrolysis and higher yield of fermentable sugars. The hemicellulose sugars were produced using sCW hydrolysis of sweet lupin hulls in the study of Ciftci & Saldaña. (2015). The hydrolysis reaction was performed in the semi-continuous flow type system with temperatures ranged from 160-220^{o}C under 50-200 bar with flow rates of 2-10 mL/min. The yield of hemicellulose sugars was considerably influenced by temperature. When the temperature increased from 160 to 180^{o}C, the yield increased by 1.5-fold with the highest value of 85.5% at 180^{o}C/50 bar. However, at elevated temperatures of 200 and 220^{o}C, the yield was reduced, indicating the degradation of sugars into other substances such as furfural or hydroxymethyl

furfural. The effect of flow rate was investigated where increasing flow rate up to 5 mL/min increased the yield of hemicellulose sugars up to 85.5%. At higher flow rates of 7.5 and 10 mL/min, the yield was reduced due to the short residence time of sCW with the material hence the hydronium ions were not given enough time to hydrolyze the hull.

Cellulose is a polymer of glucoses which are linked by β-(1 → 4)-glycosidic bonds. Cellulose has strong intra- and intermolecular hydrogen bonds which limit the cellulose solubility in water and hence resist the attack of enzymes toward glycosidic bonds. In the study of Rogalinski et al. (2008), pure cellulose and corn starch were hydrolyzed in subcritical water under pressures of 200-250 bar and temperatures from 210^{o}C to 310^{o}C. At 280^{o}C, the conversion rate of cellulose reached 100% only within 2 min. With the addition of CO_2, the conversion rate was enhanced significantly due to the formation of H_2CO_3 that resulted from the dissolution of CO_2 in sCW media, which later acted as an acid catalyst. However, the effect of CO_2 became less pronounced at the temperatures above 260^{o}C.

Starch hydrolysis occurred faster than cellulose hydrolysis because the β-1,6-glycosidic bonds are more prone to hydrolysis than the β-1,4-glycosidic bonds (Rogalinski et al., 2008). The reaction rate increased 50 times when the temperature raised from 210^{o}C to 270^{o}C. Upon further heating above 270^{o}C, the glucose concentration in the hydrolysate significantly reduced and dropped to undetectable level, which could be due to the further degradation of glucose to carboxylic acids and furfural compounds.

Starch is one of the most abundant carbohydrate biomasses, which is widely used in food, nutraceutical, and pharmaceutical industries. Hydrolysis of starch to reducing sugars and low molecular weight starch has been studied (Li et al., 2016; Mohd-Thani et al., 2019; Nagamori & Funazukuri, 2004). Zhao & Saldaña. (2019) employed subcritical water under moderate conditions

with temperatures from 75 to 150°C under 50-155 bar for 10 min to obtain low molecular weight starch from cassava starch hydrolysis. The authors reported that amylose content of the starch hydrolysates gradually reduced at increasing temperature, indicating that the starch was hydrolyzed at higher extent. The depolymerization sequence possibly occurred in the order of long amylose chains → short amylose chains → dextrins. The reducing end content of the hydrolysates increased at higher temperature, indicating that low MW compounds were formed during hydrolysis. However, they noticed that no reducing sugars were found, therefore, low MW starch was produced during the hydrolysis. The low MW starch (172.5 kDa) was obtained at 150°C/50 bar/10 min.

Another type of carbohydrate-rich biomass is straw derived from barley and canola. The hydrolysis of barley and canola straw was investigated in the study of Huerta & Saldaña (2018). The authors extracted phenolic compounds that were linked to lignin and hemicellulose from the biomass using high temperatures of 140-220°C under 50-200 bar for 10-40 min. The content of phenolic compounds increased with increasing temperatures. At 220°C, total phenolic content was 3.5 times higher than its content at 140°C. The authors argued that the glycosidic linkages between phenolics, glucose, and lignin were broken down, leading to the more effective extraction process at a high temperature of 220°C. The fermentable sugar is one of the compounds that can also be obtained from the canola straw. The hydrolysis of canola straw using sCW was performed in the study of Oliveira et al. (2022). The hydrolysis was conducted at a constant temperature of 230°C from 5 to 35 min. The highest yield of fermentable sugars (arabinose, xylose, cellobiose, glucose) was 11.5% obtained at 30 min. Among them, xylose was the dominant sugar which had the content of 6.1%. Under the effect of sCW, the lignocellulosic matrix was dissolved, leading to the release of monomeric sugars and xylose was originated from the hydrolysis of hemicellulose structure.

Pectin is the second most important carbohydrate hydrocolloids material after starch. Pectin hydrolysis has been investigated due to the potential of obtaining multiple beneficial compounds such as uronic acids, neutral sugars, and oligosaccharides (Gómez et al., 2016; Onumpai et al., 2011; Pińkowska et al., 2019; Valdivieso-Ramirez et al., 2021b). The properties of pectin and technologies for pectin hydrolysis were later discussed in detail in section 2.2.

- Protein hydrolysis

Peptide bonds are aimed to break down during protein hydrolysis reaction to produce oligopeptides and amino acids. The peptide bonds are quite stable under high temperature; hence the reaction rate is slow at a temperature below 230^{o}C (Rogalinski et al., 2005; Toor et al., 2011). Compared to acid hydrolysis, the amino acid yield in subcritical water is lower due to the thermal sensitivity of amino acids under high temperature conditions. In the study conducted by Abdelmoez & Yoshida (2013), bovine serum albumin was hydrolyzed in subcritical water to produce amino acids with the highest yield of 10% obtained at 225^{o}C. Upon further heating, the yield dropped to 5.6% at 300^{o}C. Four types of protein: α-globin, β-globin, bovine serum albumin (BSA), and β-casein, were hydrolyzed in sCW conditions at 160-300^{o}C up to 20 min, to study the specificity towards the production of peptides (Powell et al., 2016). They found that the highest peptide yields were 97% and 96% for α-globin and β-globin hydrolysis, respectively, obtained at 160^{o}C/20 min. As the temperature increased to 253^{o}C, there was a drastic reduction in peptide yield and eventually, no peptide was detected at 300^{o}C. Similarly, the highest yield of peptides derived from BSA and β-casein were 69% and 100%, respectively also obtained at 160^{o}C/20 min. The peptide yields from trypsin hydrolysis were comparable to sCW hydrolysis, particularly, 89%, 93%, 86%, and 40% for α-globin, β-globin, BSA, and β-casein, respectively. The authors also mentioned that sCW treatment might not be effective in breaking down disulfide bonds, hence, the pretreatment to

reduce the disulfide bonds was necessary to obtain high yield of peptides. Therefore, they used two reducing agents: 10 mM dithiothreitol and 55 mM iodoacetamide prior to the sCW treatment and reported that the peptide yield significantly enhanced up to 71.1% obtained at 160°C. The peptides are known to have strong bioactivities. Bioactive compounds are defined as the compounds that exhibit the therapeutic potential such as reducing the inflammatory, oxidative stress, and metabolic disorders (Santos et al., 2019). Moreover, bioactive compounds also offer many health benefits such as free radical scavenging, anti-inflammatory, antimicrobial, and anticancer activity (Saldaña et al., 2021).

The defatted rice bran was hydrolyzed by subcritical water in the study of Watchararuji et al. (2008) to recover protein from the biomass, where a major portion of protein (84%) could be recovered in the hydrolysates at 220°C/30 min. Under subcritical water conditions, the high concentration of H_3O^+ and OH^- could break down peptide bonds to form peptides and amino acids which are soluble in water. However, when the temperature increased to 220°C, the protein yield reduced because of the further degradation of amino acids to carboxylic acids. The hydrolysis of protein derived from plant-based biomasses in sCW are summarized in **Table 2.1**.

Table 2.1. Hydrolysis of protein-rich biomass in subcritical water.

Material	Hydrolysis conditions	Remarks	Reference
Rice bran (1g)	- T: 100-220°C - P: 1-39.7 bar - t: 5-30 min - Material:water ratio: 1:5 (w/v)	- The highest protein (219 mg/g) and amino acid (8 mg/g) yields were obtained at 200°C/30 min. - Hydrolysis time did not significantly affect the protein and amino acids yields. - Alkali hydrolysate had less protein and amino acids than sCW. - Hydrolysate at 200°C/30 min showed the highest antioxidant activity and was the favorable medium for yeast growth.	Sereewatthanawut et al. (2008)

Table 2.1. *Continued*

Rice bran protein (weight not reported)	- T: 150ºC-250ºC - P: 4.7-39.7 bar - t: 5-60 min - Material:water ratio: 0.167:100, and 0.416:100 (w/v)	- Protein aggregates reduced; polypeptides and amino acids yield increased after 5 min of heating. - Maximum amount of polypeptides was 3.7 g/L obtained at 250ºC for 35 min. - Amino acid content increased from 0.2 to 3.9 g/L at temperatures from 150 to 250ºC, without an observable maximum.	Sunphorka et al. (2012)
Rice bran (3g)	- T: 100-360ºC - P: not reported - t: 5 min - M/W: 3:18 (w/v)	- The highest total soluble nitrogen was reached at 280ºC. - Lysine and glutamic acid had the highest contents of 0.9 and 2.7 mg/g, respectively at 127ºC then decreased upon further heating; being degraded to carboxylic acids and gaseous products. - Sucrose was detected with the highest concentration of 85 mg/g at 140ºC then degraded to fructose and glucose which contents were 37 and 17, respectively, mg/g at 200ºC. - Organic acids: formic, acetic, citric, glycolic, and levulinic acids were the degradation products of amino acids; which had the highest content at >190ºC.	Pourali et al. (2009)
Red algae (*Pyropia yezoensis)* (35g)	- T: 120-230ºC - P: 30 bar - t: 30-50 min - M/W: 1:20 (w/v)	- The highest hydrolysis efficiency (73.62%) at obtained 210ºC - Maximum DPPH (16.63 mg/g), ABTS (19.45 mg/g), and total phenolic content (14.29 mg/g) obtained at 210ºC. - Increasing temperature resulted in lower amino acids content, with the highest content obtained at 120ºC (6273 mg/100g).	Park et al. (2019)
Microalgae (*Scenedesmus sp.*) (50g)	- T: 240-320ºC - P: 207 bar - t: 6-12s - M/W: 1:10 (w/v)	- Only arginine presented in significant amount after hydrolysis with maximum value of 127.8 mg/g at 320ºC/ 6s. - Highest soluble peptides (674.7 mg/g) obtained at 320ºC/9s. - Gallic acid and Maillard reaction products could form in the hydrolysates. - Phenols in the hydrolysates was 200-1000 times less than conventional process (300-350ºC/1h)	Garcia-Moscoso et al. (2015)

Table 2.1. *Continued*

Material	Hydrolysis conditions	Remarks	Reference
Brewer's spent grains (BSG) (weight not reported)	- T: 105-135ºC - t: 15-120 min - P: not reported - M/W: 2.5, 4, 5, 6.67:100 (w/v)	- BSG protein did not degrade to soluble peptides and amino acids under the investigated temperatures. - Instead, more carbohydrates were soluble in the hydrolysates.	Qin et al. (2018)
Soybean protein isolate (83% protein) (0.35-1.65g)	-T: 40-160ºC -P: 103 bar -t: 4-14 min - M/W: 1:100, 4:100, and 7:100 (w/w)	- Highest isoflavones yield (61%) obtained at 114ºC, 2 min with a ratio of solid/liquid of 1%. - Conversion of malonyl to glucosides forms occurs at >60ºC, glucosides to aglycones at >160ºC. - Temperatures did not significantly affect isoflavones recovery but could alter the structure of its derivatives. - Highest amount of malonyl (23%) and glucosides (42%) obtained at 80ºC and 160ºC, respectively.	Benjamin et al. (2017)
Soy protein isolate (85.79% protein) (weight not reported)	- T: 90, 120ºC - P: 0.7/1.9 bar - t: 20 min - M/W: 1:100, 2:100, and 4:100 (w/v)	- Hydrodynamic diameter of protein increased with increasing temperature and protein concentrations. - Partial hydrolysis also occurred in sCW condition. - Partial unfolding led to the exposure of the molecule's interior containing hydrophobic fragments. - Less exposed hydrophobic sites at 120ºC than 90ºC. Suggesting larger soluble aggregates. - Heat treated proteins had more flexible confirmation, hence higher surface activity at air/water or oil/water interfaces.	Wang et al. (2019)
Soybean meal (weight not reported)	- T: 200-220ºC - P: 1-39.7 bar - t: 10-30 min - M/W: 1:5 and 2:5 (w/v)	- Protein content increased with increasing temperature and time, maximum content of 165.72 mg/g at 210ºC for 30 min. - Further heating at 220ºC could decrease protein yields. - Highest amino acids yield (18.62 mg/g) was obtained at 210ºC/ 30 min. - At 220ºC, reaction time did not greatly affect the amino acids content.	Watchararuji et al. (2008)

Table 2.1. *Continued*

Material	Hydrolysis conditions	Remarks	Reference
Soy meal (1.5g)	Enzyme pre-treatment for hydrolysis - Protease M - E/S: 4:100 (w/w). - T: 50°C - t: 10-120 min sCW extraction - T: 120°C - P: not reported. - t: 20 min - M/W:1:10 (w/v).	- Protein yield, 59.3%, considerably higher than conventional methods. - Large protein aggregates were disrupted and eventually improved solubility. - sCW treated soy protein had higher neutral (Gly, Ser, Thr, Tyr, Cys) and hydrophobic (Ala, Ile, Leu, Met, Phe, Val, Pro) amino acids than the native one. - Long hydrolysis time, high content of hydrophobic amino acids. - Plenty of aggregates were found, which were bigger than the native protein. - Red-shifted in fluorescence spectrum indicated a more polar environment. Reduction in fluorescence intensity showed the partial unfolding of protein.	Lu et al. (2016)
Okara extract (140 mg)	- T: 170-260°C - P: not reported - t: 2-120min - M/W: 2:100 (w/v)	- Highest protein content (7g/L) was obtained at 240°C meanwhile the time had insignificant effect on the protein content. - sCW could disrupt the strong aggregation of protein which mainly resulted from hydrophobic interactions. - Hydrolysate at 240°C/5 min had the highest antioxidant activity of 70%. - Antioxidant activity was proportional to protein content.	Wiboonsirikul et al. (2013)
Flaxseed meal (2g)	- T: 130-190°C - P: 340 bar - t: 180-420 min - M/W: 1:90, 1:150, 1:210 (w/v)	- Protein yield reached a maximum of 225 mg/g meal at 160°C/400 min and pH 9. - Polar protein is more soluble at lower temperature, less polar protein is more soluble at higher temperature.	Ho et al. (2007)
Bean dregs (weight not reported)	- T: 200-240°C - P: 18-34 bar - t: 5-30 min - M/W: 1:10 (v/v)	- Arg and Ala were dominant amino acid, highest content were 16.23 and 13.54 mg/L obtained at 240°C/5 min and 220°C/5 min, respectively. - The optimal condition to produce amino acids was 200°C/20 min with the yield of 52.9%.	Zhu et al. (2011)

T: temperature, P: pressure, t: time, M/W: ratio of material and water, E/S: ratio of enzyme and substrate.

The animal-based biomass was also investigated to recover protein and amino acids in subcritical water conditions. The fish gelatin was hydrolyzed in the study of Ueno et al. (2015) using subcritical water at temperatures from 160°C to 240°C under constant pressure of 20 bar. The highest amino acids concentration of 20 mM was obtained at 220°C. After further heating to 240°C, the amino acids content was reduced, indicating that 240°C was too harsh for amino acid production. The authors also reported the formation of lysinoalanine which is toxic for human consumption in the hydrolysates obtained at 160 and 180°C. However, at higher temperatures of 220 and 240°C, lysinoalanine was undetectable, hence, the hydrolysates at these conditions did not pose any risk for human use.

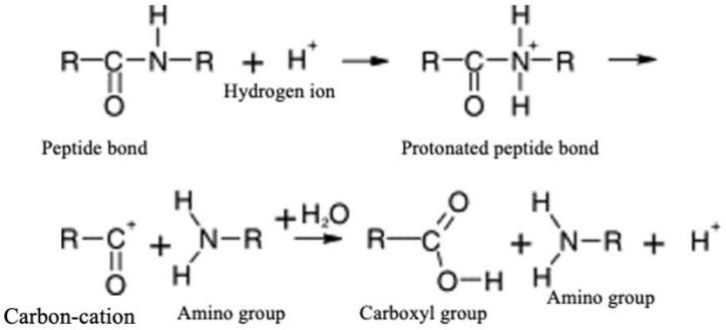


Fig 2.4. Mechanism of peptide bond cleavage in subcritical water media.

The possible mechanism of protein hydrolysis is proposed in **Fig. 2.4**. Firstly, a hydrogen ion attaches to a nitrogen atom in peptide bond which make it becomes positively charged. Leading to the cleavage of the bond to form carbon-cation and an amino group. Later, a hydroxyl group, dissociated from a water molecule, binds with the carbocation forming a carbonyl group (Brunner, 2009).

- Lipid hydrolysis

Hydrothermal liquefaction of lipids has also been widely studied in subcritical water processing. The solubility of organic compounds quickly increases in the subcritical region of water due to the

low dielectric constant. As aqueous media returns to ambient conditions, the dielectric constant consequently increases which no longer favors the dissolution of oil in water, therefore, the extractions can be efficiently completed due to the separation of water and oil phases (Machmudah et al., 2017; Reddy et al., 2014). The hydrolysis of oil normally occurs in three-step reactions: triglyceride → diglyceride → monoglyceride →glycerol where fatty acids are released in each step of the reaction. Sunflower oil was hydrolyzed by subcritical water using a continuous flow system to produce biodiesel from fatty acids in the study of Alenezi et al. (2009). Temperature had the most pronounced effect in fatty acid production, especially at temperatures above 300^{o}C, the highest yield of fatty acids was 90% obtained at 350^{o}C/8 min/200 bar. The authors explained that fatty acids can act as an acid catalyst in the hydrolysis reaction, hence, the reaction rate was significantly enhanced at high temperatures (>300^{o}C) without catalyst addition. The dominant fatty acid was C18:2 which had the highest yield of 52% obtained at 300^{o}C/25 min/150 bar. Yedro et al. (2014) investigated the recovery of bio-oil from the hydrolysis of grape seeds. The yield of bio-oil significantly increased at higher temperature and reached the maximum value of 32% at 340^{o}C. Among them, light and heavy bio-oil accounted for 8.1–15.7 wt% and 10.6–16.2 wt%, respectively.

2.2 Pectin

2.2.1. Structure

Pectin is the third important hydrocolloid after gelatin and starches that has been used in various industries: food and beverage, pharmaceutical, nutraceutical, and biomedical (Ciriminna et al., 2022). Pectin is a soluble polysaccharide abundantly found in plant cell walls and intercellular layers, which acts as a type of glue for cell adhesion and separation. Industrially, pectin is mainly produced from citrus processing waste (56% from lemons, 30% from limes, and 14% from

oranges), apple pomace (~14%), and a small portion (~1%) from sugar beet pulp (Das & Arora, 2021). The structure of pectin is still under debate, up to date, there are 5 polymeric structures derived from pectic polysaccharides: homogalacturonan (HG), rhamnogalacturonan-I (RG-I), rhamnogalacturonan-II (RG-II), arabinogalacturonan (AG), and xylogalacturonan (XG). However, three pectic regions which are well-established are HG, RG-I, and RG-II (Basak & Annapure, 2022).

Homogalacturonan (HG) is a straight-chained homopolysaccharide which accounts for 60-65% of pectin structure. HG is composed of galacturonic acid (GalA) units linked together by α-1,4-glycosidic bonds. GalA residues can be partially esterified at C-6, O-2, or O-3 positions (Picot-Allain et al., 2020). The percentage of carbonyl groups esterified by methyl is referred as degree of esterification (DE). The commercial pectin is classified into two categories based on its degree esterification: high-methoxylated (HM with DE>50%) and low-methoxylated (LM with DE<50%) pectin. HM pectin are commonly used in jams and jellies production due to the ability to form gel in the presence of cosolutes such as sucrose, under acidic environment (pH<3.5). On the other hand, the LM pectin requires divalent cations such as Ca^{2+} to form gels due to the formation of calcium bridges that connect HG regions (Fraeye et al., 2010). Hence, LM pectin are utilized as fat replacers in ice cream, yogurt, and cake glaze productions (Robledo & Vázquez, 2019).

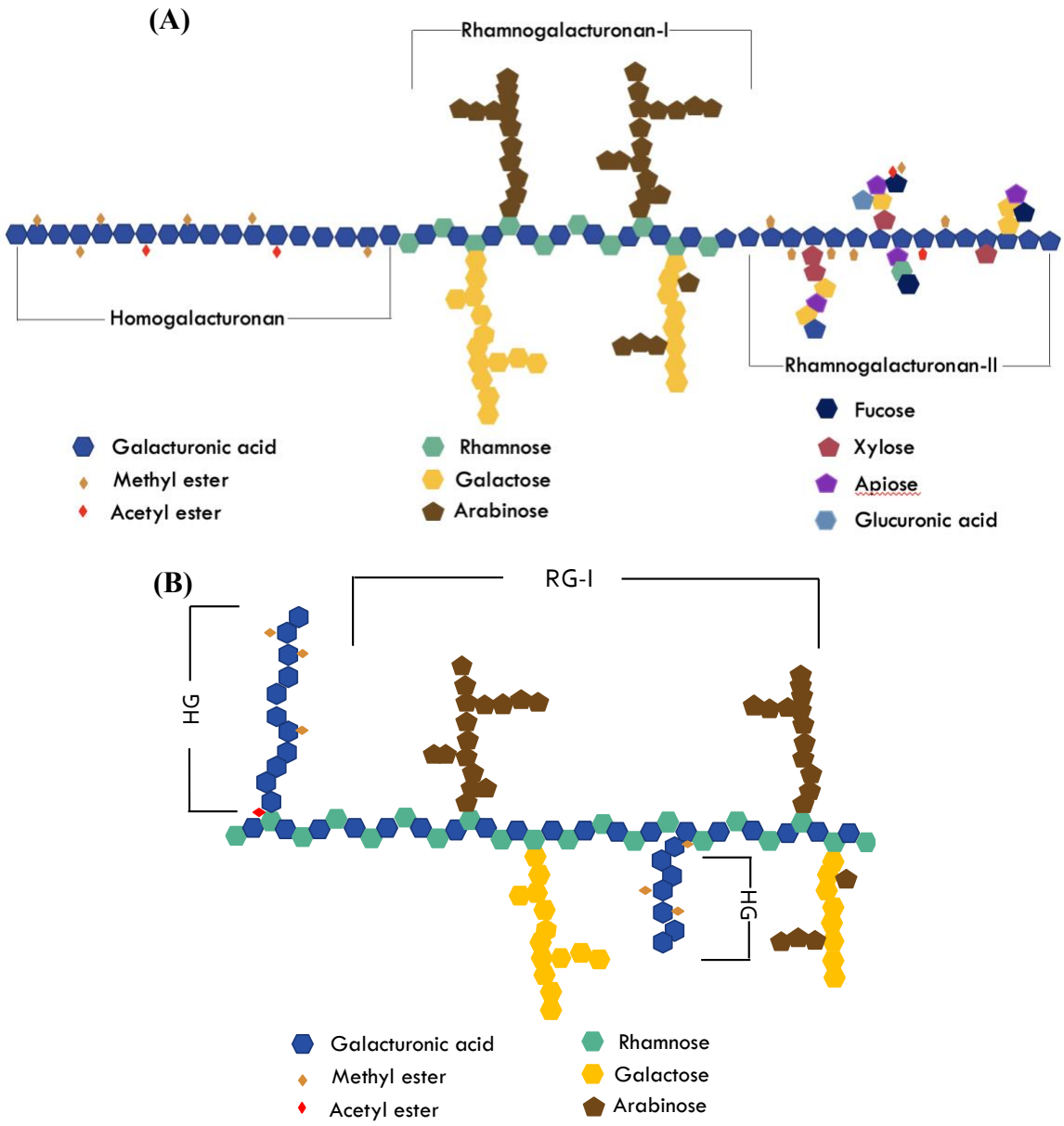


Fig. 2.5. (A) Schematic structure of pectin and (B) New proposed pectin model composed of repeating units of Rha and GalA as the backbone and linear HG, and various oligosaccharides (Ara and Gal) as the side chains, (adopted from Lin et al. (2022); Wang et al. (2018)).

Rhamnogalacturonan-I (RG-I), highly branched pectic polysaccharide, accounts for approx. 20-

30% of the pectin structure. The backbone of RG-I consists of alternating D-rhamnose and GalA residues. The RG-I and HG are linked to each other by covalent bonds, and they cannot be separated unless enzymes or chromatographic fractionation are employed (Robledo & Vázquez, 2019; Valdivieso-Ramirez et al., 2021b). Rhamnogalacturonan-II (RG-II) is the most complex pectic polymer which accounts for only a small portion, 0.5-8%. Similar to HG, linear chains of GalA is the backbone of RG-II, however, there are various hetero-oligomeric side chains attached to the backbone. The compositions of RG-II branches include 11 to 12 rare saccharides such as 2-O-methyl fucose, aceric acid, 2-keto-3-deoxy-D-lyxo heptulosaric acid, etc. (Picot-Allain et al., 2020). The structure of pectin with 3 main fragments is shown in **Fig. 2.5A**.

The structure of the pectin, however, could be more complex than the existing motifs indicated by other study (Yapo, 2011). Therefore, a new model has been developed and predicted that new motif has linear side chains of HG attached to the main chain of RG-I, as shown in **Fig. 2.5B**. Various types of carbohydrates are linked to RG-I as the side chain; hence, this new model would have the combined characteristics of HG and RG-I (Lin et al., 2022). However, due to the limitation of the current analytical methods, the characterization of new pectic model could not be yet certainly confirmed and the pectin structure is yet to be clearly understood.

2.2.2. Hydrolysis of pectin

2.2.2.1. Enzymatic hydrolysis

The modification of the pectin structure is well-known to be achieved by enzymatic treatment. The pectinolytic enzymes can be classified into 3 categories: (1) pectinesterases which facilitate the cleavage of methoxyl group in pectin, (2) polygalacturonases that induce the breakdown of α-1,4-glycosidic bonds between nonesterified galacturonic acid residues, and (3) lyases that catalyze the pectin depolymerization by transelimination (Bélafi-Bakó et al., 2007). These enzymes have

different affinities to different sites in the pectin chain, leading to the cleavage at different spots, hence resulting in different fragments with varied molecular weights and structures (Basak & Annapure, 2022).

Polygalacturonase is one of the most popular enzymes to hydrolyze pectin. This enzyme was employed by Bélafi-Bakó et al. (2007) to study the kinetics of pectin hydrolysis. The reaction time and substrate concentration had the most pronounced effect on the yield of reducing sugars. With longer time, the reducing sugars content significantly increased with the maximum value of 1.8 g/L at 150 min. The concentration of reducing sugars increased 3 times as the substrate concentration increased from 2 to 8 g/L. However, the authors noticed that during the reaction, the inhibitory product led to the reduction of reaction rate. The inhibitory product was later determined to be galacturonic acid which was formed due to the breakdown of pectin by polygalacturonase. They concluded that using the membrane bioreactor, the efficiency of pectin hydrolysis reaction can be greatly enhanced due to the elimination of inhibitory product in the hydrolysate.

Oligosaccharide is a valuable product derived from pectin that exhibits multiple beneficial functionalities such as anti-ulcer, anti-cancer, and anti-obesity (Gullón et al., 2013; Matsumoto et al., 2008). Moreover, pectic oligosaccharide is a potential prebiotic that has proven to be able to increase the populations of beneficial bacteria in human gastrointestinal tract (Manderson et al., 2005). Combo et al. (2012) investigated the production of pectic oligomers using 6 types of commercial pectinases. The authors noticed that endopolygalacturonase randomly attacked polygalacturonic acid and releases long chain of oligogalacturonic acid (oligogalA) which were later hydrolyzed into smaller oligomers. The highest yield of oligogalA was obtained by EPG-M2 from Megazyme with DP3, DP2, DP1 yields of 58%, 18%, and 13%, respectively, after 2h of reaction. Other enzymes produced mainly monogalacturonic acid with a small amount of tri- and

di-galacturonic acid in the hydrolysate. They concluded that Pectinase 62L, Viscozyme L, Pectinase, Macer8 FJ and Pectinex Ultra SP-L are more favorable to produce galacturonic acid, while the hydrolysate obtained from EPG-M2 are richer in oligogalA with DP3.

Besides oligogalA, another type of oligosaccharide derived from pectin was reported to have a strong prebiotic effect that can selectively promote the growth of beneficial bacteria in the gut and reduce the risk of colon cancer. Galacto(arabino)-oligosaccharides was produced from potato pulp using single or multiple types of enzymes (Depol 670L, Gamanase 1.5 L, Iogen HS 70, Newlase II, Pectinex Ultra SPL and Viscozyme L) in the study of Khodaei & Karboune (2016). They observed that the higher yield of oligosaccharides was 93.9% obtained from the treatment with Depol 670L with DP 2-12. They also mentioned that Depol 670L showed the strongest enzymatic activity of galactanase + arabinanase/rhamnogalacturonase but very low polygalacturonase activity, therefore, the catalytic activity had higher affinity toward RG-I side chain compared to its backbone indicated by high content of oligosaccharides and low yield of monosaccharides (0.0–14.0% w/w).

2.2.2.2. Ultrasound-assisted hydrolysis

Ultrasound is a well-known physical method to hydrolyze pectin. Ultrasonication is normally performed by bath or probe systems, where pectin suspension is subjected to high energy and low frequency ultrasound. Under the effect of acoustic energy, cavitation and microstreaming phenomenon occurs, leading to the increase in local temperature and pressure oscillation which eventually favor sonolysis of glycosidic linkages (Muñoz-Almagro et al., 2017). The combination of ultrasound and enzyme was widely employed to depolymerize pectin due to the improvement of enzymatic activity under high-intensity ultrasound. Sonoenzymolysis can improve the hydrolysis rate by: (1) enhancing the interaction between enzyme and substrate due to violent

mixing effect, (2) breaking up the enzyme clusters and altering the enzyme structure hence exposing more active sites, (3) degrading the substrate structure therefore providing larger contact area for enzyme binding and (4) increasing the reaction temperature to the optimum temperature of enzymes (~50^{o}C) due to the cavitational effect (Ma et al., 2016).

Larsen et al. (2021) employed ultrasound together with enzymatic treatment to study the hydrolysis of pectin obtained from apple and sugar beet. Compared with the enzymatic treatment alone, ultrasound-assisted hydrolysis significantly increased the content of medium MW oligosaccharides by 14% after 60 min treatment. The authors argued that ultrasonication could influence the pectin degradation in several aspects: (1) due to the violent mixing, the interaction between enzymes and pectin was enhanced, (2) ultrasound alone generates violent shear force and microjets that can alter the structure of pectin, exposing more active sites that can be easily approached by enzymes, and (3) the linkages in the structure of enzymes such as hydrogen bonds, and van der Waals forces are disrupted by ultrasound, leading to the unfolding of enzyme structure and increase in β-sheet conformations eventually, exposing more active binding sites in the enzyme hence its catalytic activity increased.

The modified structure of citrus and apple pectin was explored in the study of Muñoz-Almagro et al. (2017) using the combination of ultrasound (bath and probe) and enzymatic digestion (Viscozyme L) to develop value-added foods. The authors reported that ultrasonic treatment showed no effect on the degradation of pectin regardless of the use of the bath or probe system. The main hydrolytic action was contributed by pectinase with 2 major fragments identified as 2.8 kDa and 0.7 kDa. The higher pectin concentration, the lower hydrolysis was detected. Probably due to the aggregation of the polysaccharide chains where the energy transfer was limited, leading to the reduction of the enzyme-substrate interaction.

The synergistic effect of ultrasound and enzymatic treatment was investigated in the study of Ma et al. (2016) for pectin hydrolysis. In contrast to the previously study, the hydrolysis rate of pectin increased with increasing ultrasound intensity and reach the highest value of 23.9% at 5 W/mL. The hydrolysis rate later decreased at higher power of 9 W/mL. Due to the large amount of free radicals and violent shear forces generated during sonication, the structure of pectinase could be destroyed hence decrease its catalytic activity. Though ultrasonic treatment could favor complete pectin degradation, denaturation of pectinase structure significantly lowered hydrolysis rate. As the sonication time exceeded 15 min, the authors noticed that the enzyme was inactivated as per the lower hydrolysis rate than the control. The sonoenzymolysis and enzymolysis rate were 37% and 51%, respectively. They reported that the optimal temperature for pectinase was 20-50^{o}C as the hydrolysis rate increased from 23.74 to 43.30 %, respectively. However, at temperatures higher than 50^{o}C, thermal inactivation of pectinase and reduced cavitation forces occurred, leading to the lower sonoenzymolysis rate of 8% at 70^{o}C.

2.2.2.3. High speed shearing depolymerization

High speed shearing (HSS) is a relatively new physical method that was employed to disintegrate the structure of pectin. Due to the strong mechanical forces generated by HSS, the complex pectin structure and its glycosidic bonds can be disrupted and produce smaller fragments. However, the depolymerization of pectin cannot be achieved within a short period of time, on the other hand, only prolonged shearing time up to 8h can produce noticeable effects on pectin structure. Chen et al. (2014) used fig seeds pectin as the raw material. The microscopic structure was clearly modified after HSS treatment, the flake-like structure was disintegrated to form silk-like structure after 24h of HSS. The authors noticed that the molecular weight of sheared pectin was significantly reduced from 2566.3 to 212.7 kDa after 24h treatment, they believed that the reduction in MW could be

due to 2 reasons: disruption of the pectin aggregates and breakdown of the glycosidic bonds. However, the disruption of glycosidic bonds is more favorable under HSS treatment indicated by the content of reducing sugars that dramatically increased from 0.075 to 0.25 mM after 24h shearing. They concluded that energy provided by HSS could effectively disrupt the covalent bonds and hydrophobic forces in pectin structure, resulting in smaller fragment in the sheared samples.

The high-speed shearing was employed by Lin et al. (2021) to extract pectin from passion fruit peel. The HSS pectin had MW of 12.7 kDa which was 2-fold lower than the conventional extracted pectin of 24.3 kDa. They argued that under the strong shearing action, the pectin chain could be partially hydrolyzed into average MW (12.7 kDa) polymers. Low degree of linearity and high degree of branching was observed in the structure of high-speed sheared pectin, due to the random cleavage in the main and side chains under the effect of high-speed shearing.

2.2.2.4. Subcritical water (sCW) hydrolysis

Green technology is defined as the technology which activity has minimal impact on the natural environment (Oxford Languages, 2022). Therefore, sCW can be considered as a green alternative method to hydrolyze pectin due to its non-hazardous nature and simple operation. At temperatures higher than 100^{o}C up to 374^{o}C under high pressure condition, liquid water has a strong hydrolytic potential because of its distinct properties: low dielectric constant and high ionic products (H_3O^+ and OH^-) which could promote acid-, base-, or bi-catalyzed reactions (Zhao & Saldaña, 2019). The mechanism of pectin hydrolysis can be briefly described as the H_3O^+ and OH^- ions initiate the hydrolysis of pectin to yield hexuronic acids which later donate the proton and act as the catalyst for pectin self-hydrolysis (Shao et al., 2022). Various studies have utilized sCW to hydrolyze pectin from multiple sources, as summarized in **Table 2.2**.

Table 2.2. Subcritical water hydrolysis of pectin from various biomasses.

Material	Hydrolysis conditions	Remarks	Reference
Citrus pectin (15g)	- T: 90-110°C. - P: 5-50 bar. - t: 60-240 min. - M/W: 5:100 (w/w).	- The molecular weight (MW) of pectin reduced from 648.3 to 205.7 kDa with prolonged heating time of 240 min, additional pressure of 50 bar helped to reduce the MW to 30 kDa. - Smallest MW was 30 kDa obtained at 100°C/50 bar/240 min. - The reducing end content was reversely proportional to the MW. - The increase in reducing end during hydrolysis followed zeroth order reaction kinetics law. - Under ambient pressure, hydrolysis reaction hardly occurred.	Shao et al. (2022)
Citrus pectin (weight not reported)	- T: 75-110°C. - P: not reported. - t: 0-300 min. - M/W:1:100 (w/w).	- Under both acidic and alkaline environment, pectin breakdown was mainly due to β-elimination, though, at alkaline pH, pectin is less stable. - Formation of reducing groups increased at higher pH, primarily by β-elimination pathway. - The rate of β-eliminative reaction increased by 3.5 times for every 10°C rise in temperature. - Molecular weight of pectin reduced almost 80% at pH 8.5 and 95°C/30 min. - Arrhenius plot was used to determine activation energy of β-elimination, acid hydrolysis and demethylation which were 136, 95, and 98 kJ/mol, respectively.	Diaz et al. (2007)
Citrus pectin (1g)	- T: 140°C - P: not reported - t: 35 min - M/W: 1:25 (w/v) - Enzyme: endo-polygalacturonase	- Hydrothermal pre-treatment could reduce pectin MW by 73.9% from 93.9 kDa to 24.5 kDa. The degree of esterification (DE) was slightly reduced from 35% to 30 % after the hydrothermal treatment. - The hydrothermal treatment did not play a significant role in reducing the DE. - Combination of hydrothermal (140°C/35 min) and enzymatic treatment increased the yield of oligogalacturonides (54.5%) by 5 times higher than hydrothermal treatment alone (10.7%). - Optimal pectinolysis time was 36h which yielded the highest amount of oligogalacturonic acid of 75.3%.	Wang et al. (2021)
Passion fruit pectin (weight not reported)	- T: 80-160°C. - P: 50 bar. - t: 0.5-1, 2-4, 5-25 min. - M/W: 0.4:100 (w/v).	- MW and dispersity of mass distribution reduced with increasing temperature; the lowest MW was 7 kDa obtained at 160°C/5 min. - Emsley equation was used to describe the reduction in degree of polymerization of pectin. - The content of reducing end increased with longer reaction time and was described by zeroth order kinetics. - During hydrolysis, the pectin chains were randomly cleaved which made the pectin structure less complex and hydrolysis progressed easier.	Klinchongkon et al. (2017)

Table 2.2. *Continued*

Material	Hydrolysis conditions	Remarks	Reference
Passion fruit pectin (weight not reported)	- T: 100-160°C - P: not reported - t: 0-50 min - M/W: 80:125 (w/v)	- The viscosity of the hydrolysates initially decreased due to the electrostatic repulsions and then increased because of pectin-pectin interaction among aggregations. - At 160°C, MW of hydrolyzed pectin was 3 kDa, reduced 98.4% compared to native pectin, 197 kDa. - Addition of NaCl could reduce the viscosity of the native and hydrolyzed pectin by 14 and 43 times, respectively, suggesting that NaCl can prevent pectin aggregations. - Reduction of MW did not affect moisture adsorption characteristics of pectin.	Klinchongkon et al. (2018)
Apple pectin (weight not reported)	- T: 90-140°C. - P: not reported. - t: 0-210 min. - M/W: 1:2 (w/w)	- Content of hexuronic acids significantly increased at a temperature higher than 120°C, the highest content was 9.8 μmol GalUA/g pomace at 140°C/20 min. - Acid hydrolysis and β-elimination occurred at the same time. - Thermal hydrolysis of pectin is better described by first-order kinetics. - At temperature higher than 110°C, pectin is more likely to be hydrolyzed into GalA, indicated by hexuronic acid content that increased from 0.2 to 7 μmol/g pomace at 90 and 130°C, respectively.	Eblaghi et al. (2021)

T: temperature, P: pressure, t: time, M/W: ratio of material and water.

Another type of biopolymer, which hydrolysate has been proven to have many health benefits, is pea protein hydrolysate. The bioactive peptides found in pea protein hydrolysate was confirmed to be capable of lowering cholesterol level, regulating blood pressure, and modulating immune activities (Boukid et al., 2021; Ndiaye et al., 2012). Therefore, the properties of pea protein and pea protein hydrolysate were reviewed in detail in the following section.

2.3. Pea protein

2.3.1. Structure

Pea, *Pisum sativum L.*, is the second biggest pulse crop cultivated on more than 30% of the total dry pulse area (Boukid et al., 2021). In 2019, the global production of pea seeds was reported to be more than 14 million tons. Canada is the world largest pea producer, its production yield was

2.5 million tons in 2021, followed by Russia, China, and the USA (Agri-Food Canada, 2021). The protein content in pea seeds can range from 23.3 to 31.7%, depending on the genotypes and ecophysiological factors (Reinkensmeier et al., 2015). Gorissen et al. (2018) stated that pea protein possesses a well-balanced amino acid profile, its essential amino acids content (except for methionine) satisfies the recommendations of the World Health Organization and Food and Agricultural Organization.

The majority portion of pea protein is globulin which accounts for 70-80%, and a minor portion of albumin was also found in pea seed which has 10-20% of total protein (Acquah et al., 2020). Globulin is a salt soluble globular protein which is made up of three different fractions: legumin, vicilin, and convicilin which are different in sedimentation coefficient and molecular mass. Legumin (11S, ~360 kDa) is a hexameric protein which consists of 6 subunits linked together non-covalently. Each of these subunits constitute of a α-acidic chain and a β-basic chain linked together by one disulfide bond. Even though there is a large variation in the amino acid profiles of α- and β-chains, glutamic acid is the predominant amino acid in α-chain with an N-ternimal amino group of leucine. Whereas N-terminal amino group of β-chain is glycine and β-chain contains a high number of alanine, valine, and leucine residues (Lam et al., 2016). The α-acidic chain is hydrophilic therefore it is exposed to the surface, meanwhile β-basic chain is buried in the molecule interior due to its high hydrophobicity (Boukid et al., 2021).

Vicilin (7S, 150-170 kDa) is a trimer that contain low level of cysteine hence cannot form disulfide bonds, the subunits are mainly held together by hydrophobic interactions (Shewry et al., 1995). The subunits of vicilin are estimated approx. of ~47-50 kDa, due to having two possible cleavable sites, the proteolytic cleavage can result in fractions of 12.5–33 kDa (Mession et al., 2012). Vicilin is dominated by basic (arginine, lysine) and acidic (aspartic acid, glutamic acid) amino acids but a

low level of sulfur containing amino acids (methionine, cysteine) and tryptophan (Sikorski, 2001). Both structures of legumin and vicilin are dominated by β-sheets secondary conformation. The properties of amino acids present in pea flour is shown in **Table 2.3.**

Table 2.3. Profile and properties of amino acids found in pea (*Pisum sativum*) protein.

Amino acid	**Content in pea flour (g/100g)**		**Melting point (°C)**	**Water solubility (g/L)**	**Isoelectric point**
	Ref[A]	Ref[B]			
Essential amino acids					
Threonine[5]	2.5	4.5	228-256	97	5.6
Methionine[3]	0.3	1.6	280-282	56.6	5.7
Phenylalanine	3.7	5.2	270-284	26.9	5.9
Histidine[2,4]	1.6	2.3	287	45.6	7.6
Lysine[2,4]	4.7	6.25	224	1000	9.5
Valine[3]	2.7	5.1	293-315	58.5	6.0
Isoleucine[3]	2.3	3.9	285	34.4	6.0
Leucine[3]	5.7	7.8	269-293	21.5	6.0
Non-essential amino acids					
Serine[5]	3.6	5.7	228	425	5.7
Glycine[5]	2.8	4.8	262-290	249	6.1
Glutamate[1,4]	12.9	18.5	213-224	8.5	3.1
Proline[3]	3.1	4.6	205-222	162	6.3
Cysteine	0.2	0.4	220-260	277	5.0
Alanine[3]	3.2	4.8	300	164	6.1
Tyrosine[5]	2.6	3.3	344	0.5	5.6
Arginine[2,4]	5.9	7.9	222-244	182	10.8
Aspartate[1,4]	NR	11.2	272	5.4	2.9

Ref[A]: Gorissen et al. (2018), Ref[B]: Boye et al. (2010), [1]: acidic amino acid, [2]: basic amino acid, [3]: hydrophobic amino acid, [4]: hydrophilic amino acid, [5]: uncharged polar amino acid. NR: not reported.

Lastly, convicilin (7S, ~210±70 kDa) is a trimeric fraction which molecular weight can increase to ~290 kDa with N-terminal extension. Although the amino acid profiles of vicilin and convicilin are quite similar, convicilin is richer in sulfur containing amino acids and its polypeptide N-terminal extension is highly charged and hydrophilic (Bogahawaththa et al., 2019; González-Pérez & Arellano, 2009).The structure of legumin, vicilin, and convicillin are shown in **Fig. 2.6**.

Albumin (2S, 5-80 kDa) is a water soluble protein which consists of enzymes, protease inhibitors, amylase inhibitors, and lectins (Boye et al., 2010). The essential amino acids content in albumin is higher than globulin regarding tryptophan, lysine, threonine, and methionine residues (Boukid et al., 2021). Within the structure of albumin, two major fractions were identified: bigger albumin constituted by two polypeptides with MW of 25 kDa and a smaller albumin 6 kDa (Boye et al., 2010).

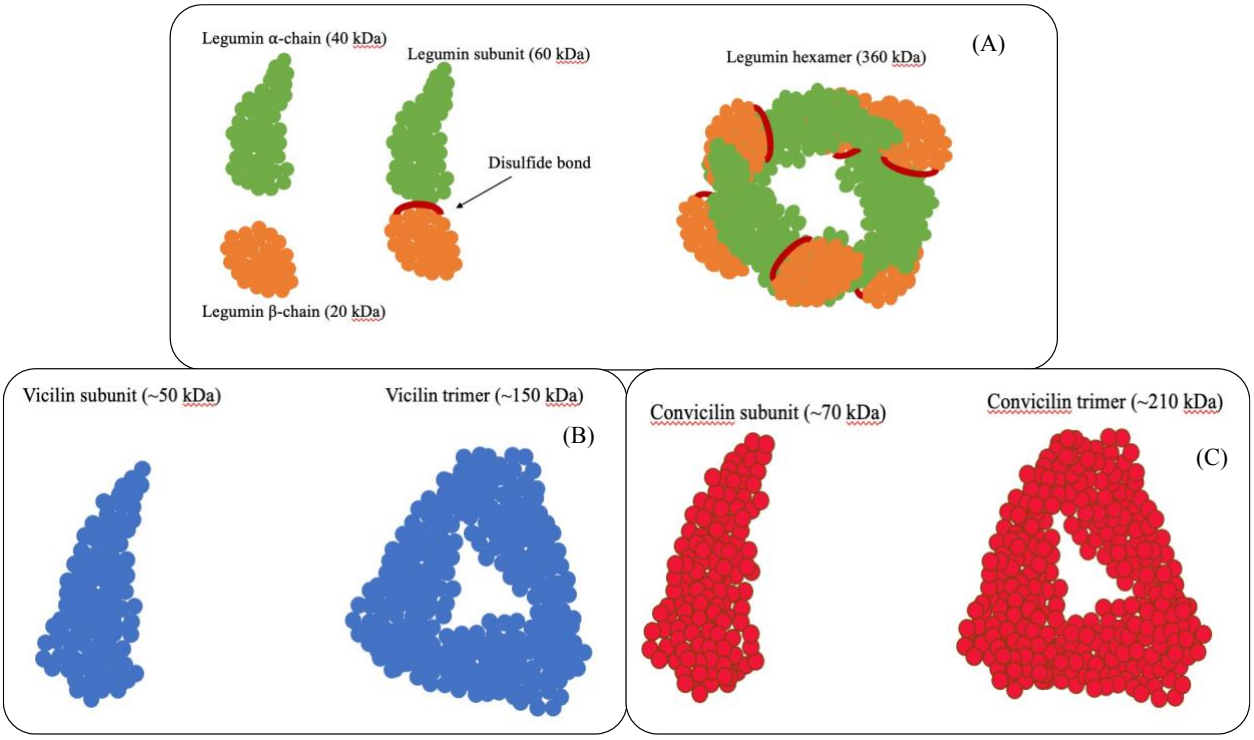


Fig. 2.6. Native conformation of pea (*Pisum sativum*) globulin: (A) hexameric legumin (360 kDa), (B) trimeric vicilin (~150 kDa), and (C) trimeric convicilin (~210 kDa).

2.3.2. Hydrolysis of pea protein

2.3.2.1. Microwave assisted acid hydrolysis

Microwave is a green technology that emit electromagnetic radio in a frequency range of 300 MHz and 300 GHz. The electromagnetic energy can be converted to thermal energy in food due to two mechanisms: dipolar rotation and ionic conduction (Barba et al., 2016).

Microwave assisted hydrolysis has been employed in various types of protein such as chia seed (Urbizo-Reyes et al., 2019), black soybean (Li et al., 2018), cricket (Hall & Liceaga, 2020), etc. Kroll et al. (1998) employed hot and concentrated hydrochloric acid and microwave to hydrolyze pea protein. The authors noticed that microwave hydrolysis could obtained a comparable amount of amino acids to the conventional method (110^{o}C/24h) at significantly shorter time (130^{o}C/2h). For example, the content of glutamate after the conventional and microwave hydrolysis were 130 and 129 mg/g, respectively. Microwave hydrolysis took only 2h at 130^{o}C, while the conventional

method required 24h at 110°C to recover the same yields of amino acids which were 827 and 832 mg/g protein, respectively.

2.3.2.2. Enzymatic hydrolysis

Enzymatic digestion is a well-known method to cleave peptide bonds in the protein, hence, obtain protein hydrolysates or peptides (Ndiaye et al., 2012). However, different proteolytic enzymes can specifically cleave at different binding sites of the protein, therefore, the released peptides can be varied in size and composition, leading to differences in the bioactivities (Chalamaiah et al., 2018). Numerous enzymes obtained from plants, animals and microbes have been employed to produce protein hydrolysates or peptides with potent bioactivities.

In the study of Ding et al. (2020), alkaline protease (2-5%, w/w) was utilized to produce bioactive peptides derived from pea protein isolate. The highest DPPH scavenging activity was 27.95% obtained at enzyme to substrate ratio of 10% w/w, increasing enzyme to substrate ratio resulted in the reduction of antioxidant activity. The authors argued that with higher enzyme concentration, competitive inhibition occurred which hindered the binding of enzyme to protein, leading to insufficient hydrolysis and eventually reduced antioxidant activity. They reported that the optimal conditions for complete hydrolysis were pH 9 and 50°C. Within the protein hydrolysate, they identified 3 peptide sequences that primarily contributed to the antioxidant activity: Tyr-Ser-Ser-Pro-Ile-His-Ile-Trp (YSSPIHIW), Ala-Asp-Leu-Tyr-Asn-Pro-Arg (ADLYNPR), and His-Tyr-Asp-Ser-Glu-Ala-Ile-Leu-Phe (HYDSEAILF).

Other antioxidative properties of pea protein hydrolysate such as ferric reducing power, superoxide scavenging, H_2O_2 scavenging, and OH• scavenging activities were investigated by Pownall et al. (2010). Thermolysin was employed to initiate the hydrolysis, resulting in the hydrolysate with high concentration of hydrophobic amino acids. The most hydrophobic fraction showed the highest

activity to scavenge OH•, superoxide radicals and H_2O_2. They hypothesized that with high concentration of tryptophan (1.16%), which was reported as a potent hydrogen donor, was able to neutralize the free radicals. On the other hand, phenylalanine (16.44%), with the aromatic structure, can easily react with OH• free radicals to form stable hydroxylated derivatives. However, pea protein hydrolysate and its fractions exhibited quite low ferric reducing power, due to the low content of sulfur-containing amino acids as sulfhydryl groups are effective reducing agents.

Besides antioxidative property, peptides derived from pea protein showed several inhibitory effects for diabetes and obesity (diabesity). Awosika & Aluko. (2019) employed 4 types of enzymes alcalase, pepsin, trypsin, and chymotrypsin to obtain pea peptides that are capable of inhibiting α-amylase, α-glucosidase and lipase, which are 3 main enzymes contributing to diabesity. Chymotrypsin hydrolysate had the highest inhibitory activity against α-amylase (30.52%) and α-glucosidase (53.35%). Regarding lipase inhibitory effect, alcalase and trypsin showed the highest activity with the lowest IC_{50} values of 3.98 and 3.95 mg/mL, respectively. The fractionation did not show significant effect on α-amylase inhibitory activity of chymotrypsin hydrolysate which was likely due to the synergistic effect of the peptides. However, <1 kDa fraction of chymotrypsin hydrolysate had significantly higher inhibitory activity against α-glucosidase and lipase than the unfractionated one. Because peptides with smaller size can easily bind to the active sites of enzymes and exert the inhibitory effect, meanwhile, the unfractionated are bigger and wider, hence, they are limited in binding to the enzymes, resulting in lower inhibitory activity.

Apart from the forementioned enzymes, other types of proteases have been employed to produce bioactive peptides from pea protein, namely papain, and flavourzyme (Barbana & Boye, 2010), thermoase (Liao et al., 2019), α-chymotrypsin (Barbana & Boye, 2010; Rudolph et al., 2017), and

L. plantarum 299v fermentation (Jakubczyk et al., 2013).

Besides being a popular yet effective method to produce bioactive protein hydrolysates or peptides from pea protein, enzymatic treatment can also modify the protein structure, resulting in hydrolysates with enhanced techno-functionalities. Pea protein is known as having low solubility due to the folded structure of its globular proteins. Hence, Klost & Drusch (2019) utilized trypsin to hydrolyze pea protein concentrate to enhance its functional properties: solubility and emulsifying activity. After enzymatic digestion, the solubility improved from 30% (degree of hydrolysis of 0) to 60% (degree of hydrolysis of 4) at pH 4 to 6 due to the higher amount of terminal carboxyl- and amino-groups in the hydrolysates. Compared to the untreated protein, the interfacial shear modulus of hydrolyzed pea protein significantly increased from 62 to 121 mN/m at the DH of 0 and 4%, respectively. This indicated that at a higher DH of 4%, the intermolecular interactions were enhanced which limited the aggregation of oil droplets.

Pea protein isolate was hydrolyzed by 11 types of enzymes (alcalase, flavourzyme, Neutrase, Protamex, Savinase, Trypsin, Bromelain, Esperase, Chymotrypsin, Corolase, and Papain) in the study of García-Arteaga et al. (2020) to understand the techno-functionalities of pea protein hydrolysates. The solubility was significantly increased after enzymatic hydrolysis, the highest solubility was 78% obtained after 120 min hydrolysis with Esperase at pH 7. Esperase is a serine endopeptidase which can cleave peptide bonds at tyrosine, phenylalanine, or leucine at the carboxyl side, therefore, increased the solubility. Trypsin hydrolysate had the highest foaming capacity of 2271% but papain hydrolysate was the most stable foam (97%) after 120 min storage. The hydrolyzed protein produced peptide with higher surface hydrophobicity, leading to a more stable foam. Chymotrypsin and trypsin hydrolysate showed the highest emulsifying capacity of 727 mL/g and 711 mL/g, respectively. The authors explained that these enzymes could facilitate

the unfolding of protein to expose the hydrophobic side chains, hence improve the amphiphilic nature of the hydrolysates.

Tamm et al. (2016) hydrolyzed pea protein isolate using trypsin and alcalase to obtain partially hydrolyzed protein which was used as a stabilizer to encapsulate rapeseed oil. They noticed that trypsin hydrolysate could produce smaller oil droplet size with significantly more stable emulsions. There was no phase separation observed after 24h of storage, while the alcalase hydrolysates showed a noticeable aggregation, the particle size increased from 8.32 to 32.09 μm after 24h storage. They explained that trypsin hydrolysates had low content of low MW peptides, the formation of thick interfacial films was more favorable hence effectively stabilized the oil droplets after homogenization. At DH of 6%, the higher zeta potential due to the exposure of hydrophobic segments from the protein core to the surface resulted in the improved hydrophobicity.

2.3.2.3. High pressure assisted enzymatic hydrolysis

High pressure processing is a non-thermal technology that operates under isostatic pressure from 1000 to 10000 bar. This technology attracts the interest of the scientific community because bioactive compounds are well preserved due to low or moderate temperatures applied in the process (Ulug et al., 2021). Recently, high-pressure processing has been employed as a pre-treatment for enzymatic digestion of pea protein. Under high pressure, the disruptions of non-covalent bonds occur, leading to the rearrangement and unfolding of protein conformation which favor the enzymatic cleavage due to the exposure of more binding sites from the protein core. Chao et al. (2013) pretreated pea protein isolate at 2000-6000 bar before subjecting the protein to alcalase hydrolysis. The high-pressure treatment resulted in the red shifting of maximum emission wavelength, suggesting that tryptophan residues were more exposed to the surface of the protein. However, under 6000 bar treatment, the fluorescence intensity reduced which was due to the

shielding effect from excessive protein-protein interactions. After enzymatic treatment, the obtained peptides had strong bioactivities. Among which, 6000 bar pre-treatment produced peptides with the highest ACE and renin inhibitory activities. Under high pressure, the protein unfolded and exposed the hydrophobic regions which increased the binding sites for enzymes, hence, resulting in higher amounts of bioactive peptides.

Other antioxidant activities such as DPPH, oxygen radical absorbance, metal chelating, superoxide, and hydroxyl scavenging activities of high-pressure treated pea protein were investigated in the study of Girgih et al. (2015). Pea protein isolate was subjected to high-pressure pre-treatment of 2000-6000 bar then hydrolyzed by alcalase to obtain the protein hydrolysates. The high-pressure treatment showed significant effect in improving the antioxidant activities of the hydrolysates. The scavenging activities against DPPH and oxygen radicals had the highest values of 18% and 897 μM TE/g, respectively, at 4000 bar treatment. The authors concluded that pressurization enhanced the exposure of peptide bonds, resulting in the increased production of peptides with strong proton donating capacity. On the other hand, superoxide, hydroxyl, and metal chelating activities had maximum values of 41%, 17%, and 12%, respectively, at 2000 bar treatment which was due to the slightly change in protein conformation at 2000 bar that favored the release of peptides with potent superoxide and hydroxyl radical scavenging activity.

The structural and functional changes of high-pressure treated pea protein was evaluated in the study of Chao et al. (2018). They observed that after high pressure treatment, the non-covalent bonds were shortened, leading to the enhanced protein-protein interactions and eventually protein aggregates. The protein fluorescence intensity significantly reduced after 6000 bar treatment, suggesting that the protein conformation was highly denatured and exposed to the aqueous environment. The droplet size of the emulsions stabilized by 6000 bar treated pea protein had the

most significant decrease from 92 μm to 68 μm as the protein structure was unfolded, resulting in the enhanced interactions of hydrophobic regions to the oil droplets, leading to the smaller emulsified particles. In contrast, the foaming capacity of 6000 bar treated protein was the lowest, instead the highest foaming capacity was obtained at 2000 bar treatment. The authors explained that, at higher pressure, the aggregated protein was less flexible, and their ability to form air bubble and foam stability were therefore reduced.

2.4. Summary

Overall, the technologies discussed in this literature review (e.g., high pressure processing, microwave, ultrasound, high speed shearing, etc.) were able to produce protein and pectin hydrolysates with enhanced functionalities and techno-functionalities. However, most of the technologies such as high pressure processing, microwave, and ultrasound processing are commonly employed as the pre-treatment prior to enzymatic hydrolysis. Hence, the procedure to generate protein hydrolysates can be costly and complicated. Though, enzymatic hydrolysis is an effective method to hydrolyze pectin and pea protein concentrate, the high cost of enzyme prolonged procedure, and hazardous reagents limits its applications for large-scale productions. Moreover, the high speed shearing technology requires extensively long time up to 24h to disintegrate pectin, and the hydrolyzed pectin still had high molecular weight of 212 kDa. In addition, the high pressure technology was used as the pretreatment for protein hydrolysis, in which the obtained hydrolysates had relatively low antioxidant activities with $DPPH^-$ scavenging and metal chelating activities of 18 and 12%, respectively. Therefore, it is necessary to employ a technology that is capable of directly producing the pectin and protein hydrolysates with high degree of hydrolysis and satisfactory functionalities. As a result, subcritical water processing has been used as a green alternative method to hydrolyze some sources of protein (soy protein, rice

bran protein, microalgae, etc.) and pectin (passion fruit, apple, pea hull fiber, etc.) to obtain beneficial compounds such as reducing sugars, oligosaccharides, amino acids, and peptides. For that reason, sCW was employed in this research as an effective method to depolymerize citrus pectin and pea protein concentrate, which were presented and discussed in Chapters 3 and 4 of this thesis, respectively.

Chapter 3: Hydrolysis of citrus pectin by subcritical water modified with carboxylic acids[1]

3.1. Introduction

Pectin, a heteropolysaccharide accounting for approximately 30% of the plant primary cell walls (Cui et al., 2021), is mainly produced from citrus processing waste (56% from lemons, 30% from limes, and 14% from oranges), apple pomace (~14%), and a small portion (~1%) from sugar beet pulp (Das & Arora, 2021). Commercial pectin is commonly produced by acid extraction using strong mineral acids such as sulfuric, nitric, or phosphoric acid at high temperatures from 80 to 100^{o}C (Dranca & Oroian, 2018). Even though the acid extraction method generates high yields from 11.1% to 21.3%, the pectin structure is altered to small molecular weight pectin fractions (Marić et al., 2018). Alkali and enzymatic treatments have also been used to obtain pectin from plant biomass. The alkali extraction produces pectin with high yields of 17.9–24.5%. However, under alkaline conditions of pH 9-11, the structure of pectin can be degraded via β-elimination pathways and debranched especially in the arabinose side chain (Khodaei & Karboune, 2014; Mao et al., 2019). The enzymatic extraction, on the other hand, employs moderate conditions of pH 3.7-4.5 but the process can be costly due to the enzyme's price and therefore unsuitable for large scale hydrolysis process (Khodaei et al., 2016).

Pectin is a highly complex biopolymer in which D-galacturonic acid (GalA, approx. 65% of pectin) is the predominant constituent (Picot-Allain et al., 2020). Several types of neutral sugars were also found in pectin, such as galactose, arabinose, rhamnose, and xylose (Das & Arora, 2021). Depending on the side chains, the pectin structure can be classified into three structural fragments: homogalacturonan, rhamnogalacturonan-I, and rhamnogalacturonan-II (Picot-Allain et al., 2020).

[1] A version of this chapter will be submitted as "Vo, H. and Saldaña, M.D.A. (2022). Hydrolysis of citrus pectin by subcritical water modified by carboxylic acids." to Food Hydrocolloids journal.

Homogalacturonan (HG) is an unbranched homopolysaccharide, accounting for 60-65% of pectin structure. HG is composed of GalA units linked together by α-1,4-glycosidic bonds. GalA residues can be partially methyl-esterified at C-6 carbonyl or acetyl-esterified at O-2 or O-3 positions (Picot-Allain et al., 2020). Rhamnogalacturonan-I (RG-I) is a highly branched pectic polysaccharide that accounts for approx. 20-30% of pectin. The backbone of RG-I consists of alternating D-rhamnose and GalA residues where the side chains of D-galactose and L-arabinose residues are linked at O-4 positions. Rhamnogalacturonan-II (RG-II), which accounts for only a small portion of 0.5-8% of pectin, is the most complex polymeric form of pectin. The backbone of RG-II is composed of linear chains of GalA with various hetero-oligomeric side chains.

From the hydrolysis of pectin, various types of oligosaccharides can be produced: oligogalacturonides, oligo-rhamnogalacturonides, galacto-oligosaccharides, and arabino-oligosaccharides (Babbar et al., 2016; Singh & Tingirikari, 2021). Pectic oligosaccharides have been known for many health benefits such as anti-inflammatory (Yeung et al., 2021), anti-obesity (Choi et al., 2016), anticancer (Kapoor & Dharmesh, 2017), antioxidant (Ogutu & Mu, 2017) and prebiotic potential (Gómez et al., 2016).

One of the most common methods to produce pectic oligosaccharides is enzymatic hydrolysis. Polygalacturonase has been used to breakdown pectin due to its potential to cleave α-1,4-glycosidic bonds (Silva et al., 2019). Apple pectin was hydrolyzed by polygalacturonase (Nikolić & Mojovic, 2007). The results showed that with longer incubation time up to 24h, the concentrations of octa-, hepta-, and hexa-galacturonic acid was reduced, while tetra-, tri-, di-, and mono-galacturonic acid contents increased in the hydrolysate. When apple pectin was hydrolyzed by polygalacturonase and pectin esterase, the hydrolysate had mostly tri-galacturonic acid (34.1%), followed by di-galacturonic acid (18.6%), and mono-galacturonic acid (6.84%) after 24h

hydrolysis. In the study of Sabater et al. (2019), ViscozymeL and Glucanex200G were used to hydrolyze apple and citrus pectins to produce pectic oligosaccharides. The highest yield of oligosaccharide was 652 mg/g (65.2%) achieved at 30 min of hydrolysis with ViscozymeL where the molecular size ranged from 0.8 to 2.5 kDa. Besides di- and tri-galacturonic acids as the dominant oligosaccharides, the authors also detected various acidic peaks which had degree of polymerization (DP) from 2, 3, and >3. Though the peaks' identity were not clarified, they believed these unknown acidic compounds were oligosaccharides with one galacturonic acid linked to one or more neutral sugars residues based on the elution patterns (Sabater et al., 2019).

Onion skin pectin was hydrolyzed in the study of Babbar et al. (2016) to obtain oligogalacturonides using three types of enzymes: viscozyme, pectinase, and EPG-M2. The hydrolysis was performed at 45^{o}C for 120 min. Among the three, EPG-M2 was the best enzyme to produce oligogalaturonides with DP2-4. The highest yields of DP2 and DP3 were 18.7% and 37.1%, respectively, obtained at 120 min of hydrolysis. Meanwhile, DP4 had the highest yield of 6.5% at a shorter time of 5 min. Babbar et al. (2016) suggested that EPG-M2 had the behavior of endo-polygalacturonase due to the random formation of different oligomers.

Besides pectic oligosaccharides, rhamnose is also a valuable monosaccharide (C$482/100g, Sigma-Aldrich at https://www.sigmaaldrich.com/CA/en/product/sigma/r3875) that can be produced from pectin hydrolysis. Rhamnose is used in food, pharmaceutical, and cosmetic industries.

Furanone is a cyclic compound that results from the reaction of rhamnose with amino acids. It has been used as a food additive due to its caramel and fruity flavor and sweet taste (Illmann et al., 2009). In addition, furanone also exhibits multiple therapeutic effects, such as anticancer, antiviral, and anti-inflammatory (Husain et al., 2019).

Moreover, rhamnose can be used as the substrate sugar for biofuel production particularly 1,2-propanediol. van der Wal et al. (2013) reported that rhamnose was efficiently utilized by *Clostridium beijerinckii* to produce 1,2-propanediol with considerably high content of 9.7 g/L. Recently, the anti-ageing effect of rhamnose has been reported in the study of Pageon et al. (2019). They mentioned that rhamnose can provide beneficial effect on the papillary dermis and dermal epidermal junction which can slow down the anti-ageing process.

To date, there are limited studies that obtained rhamnose from pectin hydrolysis. In the study of Valdivieso-Ramirez et al. (2021a), rhamnose was one of the monosaccharides obtained from the hydrolysis of soybean rhamnogalacturonan among arabinose, fucose, and xylose. They reported that at 125ºC, rhamnose was undetectable, however, as the temperature increased, the yield of rhamnose significantly increased and reach the highest value of 86.6% at 135 °C/100 bar/60 min. In addition, apple pectin was hydrolyzed in the study of Wikiera et al. (2015) using 2M trifluoracetic acid. The content of rhamnose increased significantly as the time increased with the highest value of 1.12 g/100g (1.12%) at 100ºC/2.5h. The temperature, on the other hand, had negative effect on the content of rhamnose, which substantially decreased from 1.12 to 0.39 g/100g as the temperature increased to 120ºC as degradation of neutral sugars occurred, leading to reduction in the rhamnose content. However, the degradation products were not quantified (Wikiera et al., 2015).

Even though enzymatic and acid hydrolysis are well-known methods to depolymerize pectin, the process involves one or several hazardous and environmentally harmful reagents (e.g., concentrated mineral acids, furfural, and lactones) that requires extensive treatment before disposing. Moreover, the protocol does not only require long time, costly reagents, complex buffer systems, strictly controlled temperature, and enzyme/substrate ratio, but the depolymerization of

pectin may also be incomplete. For those reasons, subcritical water (sCW) treatment has recently become an ecofriendly alternative process for pectin hydrolysis. sCW is water in aqueous state at high temperatures from 100°C to 374°C under high pressure conditions. sCW has unique properties which could favor the hydrolysis process: low dielectric constant and high ionic products (H_3O^+ and OH^-) which could promote acid-, base, or bi-catalyzed reactions (Zhao & Saldaña, 2019). There are some studies employing sCW to hydrolyze pectin which are summarized in **Table 2.2** where mono- and oligosaccharides were produced from pectin hydrolysis. Additionally, uronic acid, a product derived from the oxidation of -OH group on C6 of aldose sugar was obtained from the hydrolysis of pectin (Moreno & Peinado, 2012b; Pińkowska et al., 2019). The properties of uronic acids derived from pectin were summarized in **Table 3.1.**

Table 3.1. Uronic acids obtained from pectin hydrolysis (Adopted from Donati et al., 2009; Kohn & Kovác, 1978; Pińkowska et al., 2019)

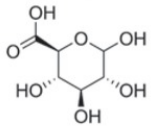
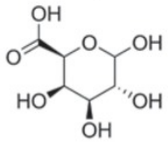
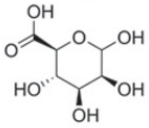
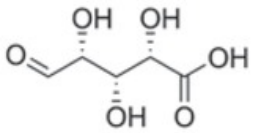
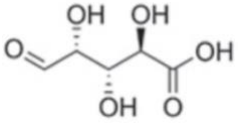
Neutral sugar	**Uronic acid**	**Uronic acid structure**	**Uronic acid solubility in water (g/L)**	**Uronic acid pKa**
Hexose				
Glucose	Glucuronic acid		295	3.21
Galactose	Galacturonic acid		100	3.24
Mannose	Mannuronic acid		295	3.38

Table 3.1. *Continued*

Neutral sugar	Uronic acid	Uronic acid structure	Uronic acid solubility in water (g/L)	Uronic acid pKa
Pentose				
Xylose	Xyluronic acid		NR	NR
Arabinose	Arabinuronic acid		NR	NR

NR: not reported.

The selectivity of the sCW media is quite low, therefore, several studies modified the reaction environment to obtain the desirable product with high yield. Brunner (2009) reported the increase in selectivity by adding 96% CO_2 to the media, as a result, the yield of glucose obtained from corn starch hydrolysis was improved from 5 to 60% higher than sCW alone. Recently, Valdivieso-Ramirez et al. (2021b) modified the reaction media using carboxylic acids (malic and citric acid) in the hydrolysis reaction of pea hull fiber. The authors found that citric and malic acid enhanced the selectivity of sCW towards cleavage of glycosidic bonds. Specifically, the content of gluco-oligosaccharides (2–6 DP) was approximately 5-fold higher compared to the media with sCW alone.

Carboxylic acids are organic acids that contain one or more carboxyl groups (-COOH) in their structure. Similar to other acids, carboxylic acids are able to donate hydrogen atoms to water to form hydronium ions (Moreno & Peinado, 2012a), the mechanism is shown in **Fig. 3.1**.

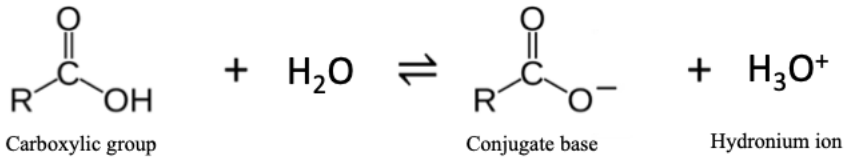


Fig 3.1. Schematic description of proton donation by carboxylic acid.

In the study of Gomes et al. (2020), citric acid, a tricarboxylic acid, was used as a biocatalyst to pre-treat sugarcane bagasse prior to the enzymatic hydrolysis. The purpose of the pre-treatment was to increase the cellulose content, remove lignin and hemicellulose from the sugarcane bagasse biomass. The lignin removal was not significantly changed in the short reaction time (<60 min), however at longer time of 90 min with high concentration of citric acid (12% w/w), the lignin content in the biomass slightly decreased from 24.1 to 22.7%. The hemicellulose fraction, on the other hand, was efficiently removed in a short period of time (<60 min) with citric acid concentration of 6.0% w/w, resulting in a low content of 13.57% in the pretreated biomass. Due to the reduction of lignin and hemicellulose in the biomass, the content of cellulose increased. The highest content of cellulose was 36.9% obtained at 60 min in 6% w/w citric acid.

Another type of biomass, rice straw, was used in the study of Amnuaycheewa et al. (2016). The material was firstly pre-treated with acetic, oxalic, and citric acid to remove lignin to obtain the substrate for anaerobic fermentation. Compared to other carboxylic acids, oxalic acid was the most effective to remove lignin which content reduced approx. 3 times from 13.27% to 4.89%, compared to the untreated material. The optimal condition for oxalic acid pre-treatment was found to be at 136^{o}C/31 min with 5% w/w oxalic acid.

Generally, carboxylic acids were used as a green and effective catalyst for the hydrolysis of lignocellulosic biomasses and pectin. However, to the best of our knowledge, there are no previous

studies that investigated the optimization of rhamnose and oligosaccharides production from citrus pectin hydrolysis using carboxylic acids as catalysts.

Therefore, the objectives of this study were to: (1) evaluate the reaction parameters (temperature, time, and solvent) on the production of rhamnose sugar and oligosaccharides, (2) determine functionality of the hydrolysates based on the antioxidant activity, and (3) elucidate the catalytic effect of citric acid and malic acid towards the pectin hydrolysis reaction.

3.2. Materials and methods

3.2.1. Materials

Citrus pectin (73% DE) was kindly provided by CP Kelco (Atlanta, GA, USA). The reagents for the sCW hydrolysis were citric acid and malic acid (> 99.5%, ACS grade) from Sigma Aldrich (Oakville, ON, Canada), water from the Milli-Q system (18.2 MΩ cm, Millipore, Billerica, MA, USA) and nitrogen gas (99.9% purity) from Praxair (Edmonton, AB, Canada).

For sample characterization, all chemicals used were of analytical grade. Sodium acetate, copper (II) sulfate, sodium chloride, sodium nitrate, ammonium formate, ferric (III) chloride hexahydrate, ethanol, acetonitrile, 12M sulfuric acid, galacturonic acid monohydrate (≥ 97%), L-rhamnose (≥ 98%), D-arabinose (≥ 98%), ascorbic acid, Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl, and 2,4,6-Tris(2-pyridyl)-s-triazine were purchased from Sigma Aldrich (Oakville, ON, Canada). Ethanol and methanol (99.9%) were purchased from Fisher Scientific (Ottawa, ON, Canada). The calibration standards for the oligosaccharide size distribution (5, 12, 25 and 72 kDa dextran) were obtained from the American Polymer Standards Corporation (Mentor, OH, USA).

3.2.2. Subcritical water hydrolysis of citrus pectin

The hydrolysis was conducted using a Parr 4590 system (Parr Instrument Company, Moline, IL, USA) as shown in **Fig. 3.2**. The system was equipped with a 600mL batch reactor, a 780W heating

mantle, a stirrer, a nitrogen tank, a pressure gauge, and a temperature controller (Valdivieso-Ramirez, Sanchez Gallego, et al., 2021). The stirring speed was estimated to be approximately 660 rpm (1.5 knob position on the controller's panel). The optimized proportional, integral, and derivative parameters for the temperature controller were 21, 500, and 71, respectively.

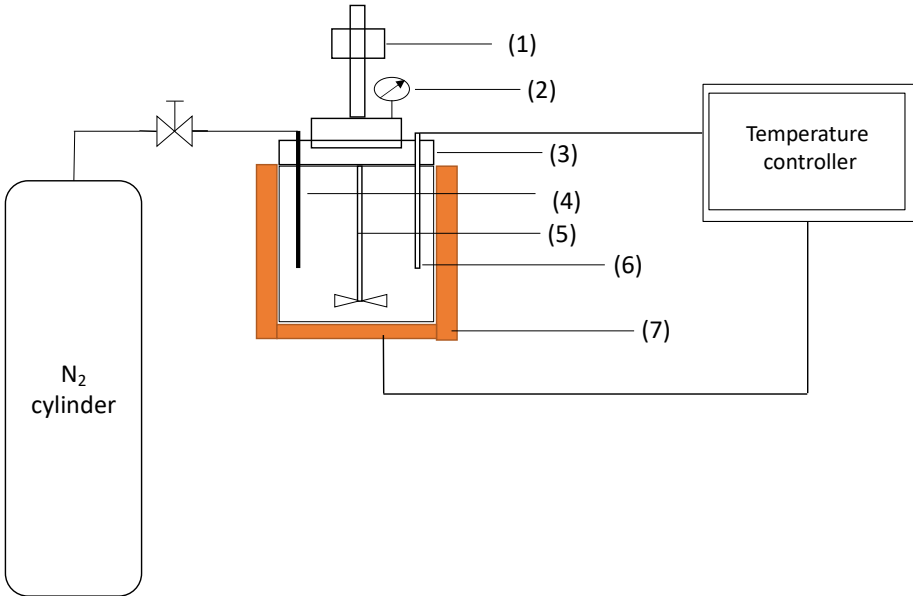


Fig. 3.2. Schematic description of subcritical water system in batch mode. (1) magnetic driver, (2) pressure gauge, (3) fixed head for sealing, (4) dip tube, (5) stirrer, (6) thermocouple, and (7) heating jacket.

Citrus pectin (10g) prior to the sCW treatment was washed with 2L of 80% ethanol to remove any interfering sugars, according to the method described by Eblaghi et al. (2021). The pectin suspension at 1% (w/w) in water was prepared by mixing 1g of pectin with 100g of miliQ water. Similarly, 1g of pectin was mixed with 0.2g of citric acid and 100g of miliQ water to obtain pectin suspension in 0.2% (w/w) aqueous citric acid. The pH values of pectin suspensions with water, citric acid, and malic acid were 4.6, 2.6, and 2.5, respectively. After, the suspensions were loaded into the 600 mL reactor and then purged with N_2 gas for 12 min under constant stirring. The pectin suspensions were very viscous because pectin is a natural gelling agent, therefore, prolonged purging time of 12 min was used to remove any dissolved air oxygen in the suspensions to prevent

any undesirable oxidation reactions. The reactor was then pressurized with N_2 gas to a certain level (e.g., use of 30 bar for 180°C, use of 25 bar for 200°C, etc.) so that after reaching the desired temperature, a final pressure value of 50 bar was achieved. The reactions were conducted at different temperatures (135, 145, 160, 170, 180, 200) and times (10, 20, 30, 40, 50, and 60 min) under a constant pressure of 50 bar. To reach the desired temperature, the heating time ranged from 6-7.5 min (e.g., use of 6 min for 160°C, use of 7.5 min for 200°C, etc.). The reactor was immediately cooled down to 40°C within approximately 3-5 min, depending on the reaction temperature used (e.g., use of 3 min for 160°C, use of 5 min for 200°C, etc.), employing a water bath. After cooling the reactor, the system was depressurized within 4 min. The resulting hydrolysates were collected and stored at -18°C until further characterization. The hydrolysis of pectin was performed in duplicate, and the processing conditions are summarized in **Table 3.2.**

Table 3.2. Hydrolysis conditions of citrus pectin in subcritical water media.

<table>
<tr><th>Temperature (°C)</th><th>Time (min)</th><th>Solvent</th></tr>
<tr><td>135</td><td rowspan="6">10</td><td rowspan="12">Water</td></tr>
<tr><td>145</td></tr>
<tr><td>160</td></tr>
<tr><td>170</td></tr>
<tr><td>180</td></tr>
<tr><td>200</td></tr>
<tr><td rowspan="6">180</td><td>10</td></tr>
<tr><td>20</td></tr>
<tr><td>30</td></tr>
<tr><td>40</td></tr>
<tr><td>50</td></tr>
<tr><td>60</td></tr>
<tr><td>135</td><td rowspan="6">10</td><td rowspan="6">Citric acid
0.2% (w/w)</td></tr>
<tr><td>145</td></tr>
<tr><td>160</td></tr>
<tr><td>170</td></tr>
<tr><td>180</td></tr>
<tr><td>200</td></tr>
</table>

Table 3.2. *Continued*

180	10	Citric acid 0.2% (w/w)
	20	
	30	
	40	
	50	
	60	
160	10	Malic acid 0.2% (w/w)
	20	
180	10	

3.2.3. Hexuronic acid content

As hexuronic acid is uronic acid derived from hexose, the content of hexuronic acids can reflect the total amount of acidic sugars in the hydrolysate. The spectrophotometric method was used to determine to content of hexuronic acids in the hydrolysates, the protocol was adopted from Klinchongkon et al. (2017), with slight modifications. First, copper (II) solution was prepared by mixing 23.3g NaCl, 5.4g NaAc, and 0.32g $CuSO_4$, which were then dissolved in 100 mL of miliQ water. Later, 0.3 mL of copper (II) solution was added to 0.3 mL of the pectic hydrolysate. The mixture was then heated to 100ºC/15 min, followed by the addition of 2.4 mL of diluted Folin-Ciocalteu reagent (1:40 v/v with miliQ water), and mix well for 10s. The absorbance was read at 750 nm using the UV-Vis spectrophotometer (Rose scientific, Edmonton, AB, Canada) with galacturonic acid (0.1-1g/L) as the standard for the calibration curve (See **Fig. A1** in Appendix A).

3.2.4. Rhamnose determination

Rhamnose is one of the constituents of the backbone of rhamnogalacturonan region. The higher content of rhamnose in the pectic hydrolysate reflects the higher extent rhamnogalacturonan hydrolysis. The content of rhamnose was determined using the HPLC method, previously described by Valdivieso-Ramirez et al. (2021a). The analysis was performed using the Shimadzu

10-A HPLC pump (Shimadzu Corp., Kyoto, Japan) with a refractive index detector RID-10A, an Aminex HPX-87H column (300 × 7.8 mm, 9 µm particle size) (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada), and a column heater. The separation involved 0.005M H_2SO_4 as the mobile phase with the flow rate of 0.3 mL/min at 45ºC. The rhamnose concentration in the hydrolysates was calculated against the calibration curve obtained from different concentrations of L-rhamnose from 0.1 to 1.5 mg/mL (See **Fig. A2** in Appendix A**)**.

3.2.5. Oligosaccharide determination

Pectic oligosaccharides obtained from the hydrolysis reaction was determined by hydrophilic interaction liquid chromatography. The characterization was conducted using the method described by Valdivieso-Ramirez et al. (2021) with minor modifications. The separation was performed using Shimadzu SPD-20A HPLC pump (Shimadzu Corporation, Kyoto, Japan) connected to the evaporative light scattering detector (3300 Alltech ELSD, BÜCHI Labortechnik. AG, Flawil, Switzerland) with N_2 as the carrier gas at the flow rate of 1.5 L/min and the drift tube temperature was 50ºC. The oligosaccharides separation were carried out using XBridge Amide column (4.6 × 250 mm, 3.5 µm particle size, Waters Corporation, Milford, MA, USA) at 35ºC and the flow rate of 1 mL/min. The eluents were composed of two solvents: (A) 80:20 (v/v) acetonitrile/10 mM ammonium formate at pH 3 and (B) 20:80 (v/v) acetonitrile/ammonium formate at pH 3. The calibration curve was determined by D-arabinose at the concentration of 0.1 to 1 µg/mL (see **Fig. A9** in Appendix A). The pectic hydrolysates were diluted by 2-fold with acetonenitrile:water 1:1 (v/v) then filtered before injecting to the column.

3.2.6. Oligosaccharide size distribution

The changes in pectin molecular weight was monitor by high performance size exclusion chromatography using the procedure described by Valdivieso-Ramirez et al. (2021). The

oligosaccharide size distribution was determined by a Shimadzu 10-A HPLC pump coupled with a refractive index detector RID-10A. The 4-fold diluted hydrolysates was filtered and injected through Ultrahydrogel 250 column (7.8 × 300 mm, 6 μm particle size) (Waters, Milford, MA, USA). The size exclusion was conducted using 0.05 M sodium nitrate at pH 6.8 as the mobile phase at the flow rate of 0.5 mL/min with column temperature of 40°C. The estimation of oligosaccharide molecular weight was calculated based on the dextran standards with different molar weights of 5, 12, 25, and 72.7 kDa. The calibration curve was generated using third order polynomial regression equation of the retention time vs. log (molecular weight) (see **Fig. A3** in Appendix A).

3.2.7. Antioxidant activity of pectic hydrolysates

The antioxidant activity was determined by the ability to scavenge $DPPH^-$ free radicals and reduce ferrous ions. The protocol was adapted from Ekaette & Saldaña. (2021) with minor modifications. Briefly, 0.1 mM DPPH solution was prepared with absolute methanol. The diluted hydrolysate (1 mL) was mixed with 3 mL of DPPH solution then incubated in the dark at room temperature for 35 min. After that, the solution was centrifuged at 7000 g for 5 min to collect the supernatant. The absorbance of the supernatant was measured at 514 nm using the UV-Vis spectrophotometer. The scavenging ability was expressed as the percentage of scavenging ability using Eq. (4.1):

$$\text{DPPH scavenging ability (\%)} = \frac{Ac-As}{Ac}\text{x}100 \qquad (4.1)$$

where: A_c is the absorbance of the DPPH solution, and A_s is the absorbance of the sample.

The ferric reducing activity was determined based on the complex of Fe^{3+} and tripyridyltriazine that has maximum absorbance at 593 nm (Benzie & Strain, 1996). The pectic hydrolysates were diluted by 4-fold with deionized water before the measurement. Briefly, 0.4 mL of the diluted

hydrolysates were mixed with 3 mL of TPTZ solution which is composed of 0.3 M sodium acetate, 10 mM TPTZ in 40 mM HCl, and 20 mM ferric (III) chloride hexahydrate at the ratio of 10:1:1 (v/v/v). The mixture was incubated in the water bath at 37^{o}C for 30 min then centrifuged at 7000g for 7 min to collect the supernatant. The absorbance of the mixture was measured at 593 nm using the UV-Vis spectrophotometer. The calibration curve was determined using vitamin C as the standard at the concentrations from 50 to 300 μM (See **Fig. A4** in Appendix A).

3.2.8. Statistical analysis

The subcritical water hydrolysis of citrus pectin was performed in duplicate. The results were reported as mean ± standard deviation. The effect of temperature, reaction time, and solvents were statistically evaluated using one-way analysis of variance (ANOVA). The p-value of 0.05 was used to determine the significant difference between treatments. The differences between means were determined by t-test and Tukey's multiple range test using Minitab software version 17 (Minitab Inc., State Collegue, PA, USA).

3.3. Results and discussion

The pectic hydrolysates were characterized based on the content of hexuronic acids which could be used as an indicator of the extent of pectin hydrolysis. Moreover, the rhamnose content, the oligosaccharides degree of polymerization, and the antioxidant activities of the hydrolysates were also determined.

3.3.1. Hexuronic acid content

Hexuronic acid (HexA) is any uronic acid that is derived from the oxidation of hexose. These include glucuronic acids, galacturonic acids, fructuronic acids, and mannuronic acids (Moreno & Peinado, 2012). The reducing end of hexuronic acids can react with copper ions and Folin–Ciocalteau reagent to form blue color complex which can be used to monitor the pectin

depolymerization reactions. The more pectin hydrolyzed, the higher content of hexuronic acids. **Fig. 3.3** shows the effect of time and temperature on the content of hexuronic acid in the pectic hydrolysates.

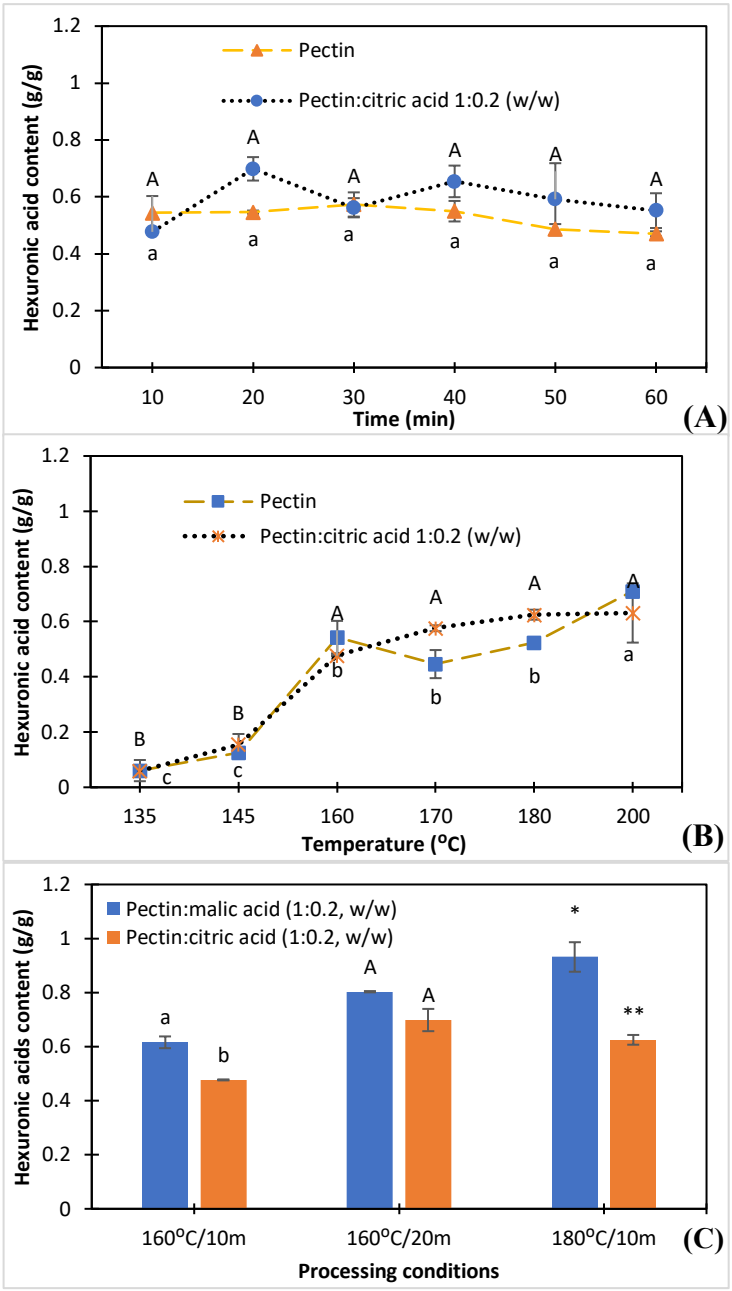


Fig. 3.3. Hexuronic acid content of citrus pectin hydrolysates in sCW and sCW modified with citric acid as a function of time at 160°C and 50 bar (A), temperature at 50 bar and 10 min (B), and types of solvent (0.2% (w/w) malic acid, and 0.2% (w/w) citric acid) (C). The averages that do not share the same letter or symbol are statistically different.

The reaction time did not have significant effect on the content of hexuronic acid (HexA), the highest value was 0.57g HexA/g pectin obtained at 160°C/30 min/50 bar (**Fig. 3.3A**). The similar content of HexA obtained could be attributed to the dissociation constant (K_w) of water, which depends on temperature and pressure of the media (Martinez-Monteagudo & Saldaña. 2014) i.e., the concentration of H_3O^+ and OH^- ions do not change under constant pressure and temperature conditions. For this reason, the HexA content did not increase after the pectin was hydrolyzed by the available ionic species in the sCW medium. Meanwhile, with the addition of citric acid, a significant increase in HexA content with reaction time from 10 to 20 min was observed, which had the maximum value of 0.69g HexA/g pectin at 160°C/20 min. Previously, in the study of Valdivieso-Ramirez et al. (2021b), citric acid could effectively enhance the hydrolysis reaction of pea hull fiber, resulting in 5-fold increment of glucooligosaccharide compared to the media with water alone. However, the production of HexA declined from 20 min to the end of the hydrolysis of 60 min, which could be due to the further degradation of HexA into acetic acid, formic acid, and lactic acid. Kühnel et al. (2011) employed sCW at 140-170°C for the pretreatment of sugar beet pulp and reported that the content of degradation products increased with increasing temperature. The highest concentrations of acetic, lactic, and formic acids were 27.8%, 18%, and 7.5%, respectively, obtained at 170°C

Eblaghi et al. (2021) reported that the formation HexA decreased after reaching the maximum value at 140°C/20 min, when using sCW treatment for apple pectin hydrolysis. They produced HexA at high temperatures ≥ 120°C (optimum at 140°C, with the yield of 0.12g HexA/g pectin) in the first 20 min of the reaction, however, this content could rapidly reduce at prolong times up to 210 min. Shao et al. (2022) noticed a similar increment of HexA content with longer times up to 100 min when hydrolyzing citrus pectin in sCW. However, they did not notice the reduction of

HexA content upon longer heating, probably due to the low temperature of 110°C employed in the study, leading to the steady increase of HexA content instead of the fast release under higher temperatures (≥ 120°C). Particularly, after 100 min of hydrolysis at 110°C/50 bar, the content of HexA reducing end only increased 10% compared to the initial HexA content of untreated sample. On the other hand, temperature significantly affected the concentration of HexA in the hydrolysates (**Fig. 3.3B**). The HexA content of pectic hydrolysate increased with increasing temperatures and reached a maximum value of 0.71 g HexA/g pectin at 200°C/10 min. Regarding the pectin + citric acid hydrolysates, HexA content also increased with temperature, the highest value of 0.63 g HexA/g pectin was obtained at 200°C/10 min. At temperatures above 145°C, there was more HexA produced which might have occurred via three possible pathways: acid, base, and bi-catalyst (combination of acid and base catalysts) because of the H_3O^+ and OH^- ions in sCW media could catalyze the hydrolysis of pectin. Valladares-Diestra et al. (2022) employed subcritical water at 120-180°C modified with 0.5-2.3% (w/v) citric acid as a pretreatment of cocoa pod husk. They noticed that with the high citric acid concentration of 1.25% (w/v) the concentration of xylose significantly enhanced up to 69.75 mg/g. Valladares-Diestra et al. (2022) explained that citric acid was be able to enhance the solubilization of pectin to facilitate the cleavage of covalent bonds, hydrogen bonds, and van der Waals interactions of pectin structure, therefore, leading to the escalation of HexA content in the hydrolysates.

Several authors reported a similar effect of temperature on apple, passion fruit, and sugar beet pectin hydrolysis with the highest HexA yields of 0.12g/g pectin, 0.11g/g pectin, and 0.01g/g pectin, respectively. The yield of HexA in this study was 0.71g/g pectin which was considerably higher than the reported values in the previous studies (Eblaghi et al., 2021; Klinchongkon et al., 2017; Pińkowska et al., 2021) due to the differences in processing temperature used in this study

compared to the others. The temperature used in this study was up to 200°C, meanwhile other study employed a relatively low temperature of 80-160°C. For that reason, the hydrolytic potential of sCW was lower, leading to the lower content of HexA in the hydrolysates. Several studies hydrolyzed citrus pectin using sCW, however, the authors did not quantify the content of HexA, hence the comparisons between studies were not possible (Diaz et al., 2007; Wang et al., 2021)

The pectin hydrolysis in this study was enhanced at increasing temperature regardless of the reaction media (either water at pH 4.5 or aqueous citric acid at pH 2.6), which indicated that the depolymerization reaction of pectin is more temperature dependent than pH dependent. Moreover, the hydrolysis of pectin may occur in both pathways: acid hydrolysis and β-elimination indicated by the dramatically increment of HexA content from 0.16 g/g pectin at 145°C to 0.63 g/g pectin at 180°C. However, β-elimination could be more favorable than acid hydrolysis because β-eliminative degradation could happen at either acidic or alkaline conditions which was reported in the study of Diaz et al. (2007). The authors employed high temperature of 110°C to hydrolyze citrus pectin at different pH from 4.5-8.5 and found that the amount of reducing sugars stayed almost unchanged in either alkaline or acidic pH conditions.

Besides citric acid, malic acid was also used as a biocatalyst to breakdown citrus pectin. The content of HexA in the hydrolysates with malic acid is shown in **Fig. 3.3C.** Generally, malic acid hydrolysates had significantly higher content of HexA than the ones obtained with citric acid. The highest HexA content (0.93 g/g pectin) was obtained at 180°C/10 min. This behavior could be due to the acid strength of citric and malic acid as per their pK_a values. As reported by Papagianni (2007), the pK_a values of citric acid are 3.1, 4.7, and 6.4, meanwhile malic acid has pK_a values of 3.4 and 5.13 (Uslu & Kirbaşlar, 2010) . Because of the lower pK_a values, malic acid has higher tendency to donate hydrogen atoms than citric acid. Therefore, the hydrolysis was more effective

with malic acid, which eventually led to the higher content of HexA. A similar observation was reported in the study of Amnuaycheewa et al. (2016) where the authors used carboxylic acids such as acetic, oxalic, and citric acid to hydrolyze rice straw at 100^{o}C-140^{o}C for 30-60 min prior to the enzymatic hydrolysis. The authors noticed that oxalic acid, which had the lowest pKa values (1.25 and 4.28), had the highest catalytic potential indicated by the sugar yields in the hydrolysates. They reported that the hydrolysates with oxalic acid (4.56%) had significantly higher yield of sugars than the ones obtained with acetic (2.54%) and citric acid (2.31%).

3.3.2. Rhamnose content

The content of rhamnose in the hydrolysates can provide information about the structural changes of pectin, especially in the rhamnogalacturonan regions. Rhamnose was detected using the Aminex HPX-87H column and the content is shown in **Fig. 3.4.** Also, the more rhamnogalacturonan hydrolyzed, the more rhamnose released in the hydrolysates.

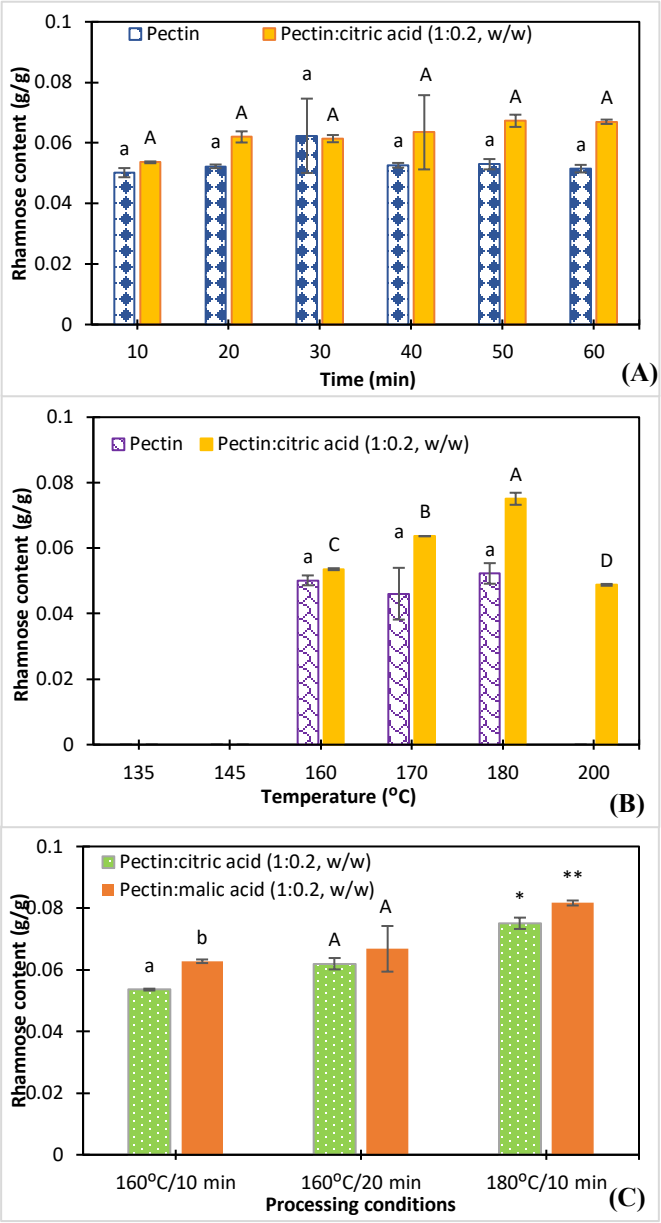


Fig. 3.4. Rhamnose content of pectic hydrolysates in sCW and sCW modified with citric acid as a function of time at 160°C and 50 bar (A), temperature at 10 min and 50 bar (B), and types of solvent (0.2% (w/w) malic acid or 0.2% (w/w) citric acid) (C). The averages that do not share the same letter or symbol are statistically different.

The reaction time did not significantly influence the amount of rhamnose, as shown in **Fig. 3.4A**. As mentioned earlier, the concentrations of H_3O^+ and OH^- ions do not change under constant temperature and pressure conditions. Therefore, pectin could not be further hydrolyzed by the

available ionic species, leading to the insignificant effect of reaction time on rhamnose content. However, there was a slight increase from 0.05 to 0.06 g/g pectin as the time increased from 10 to 30 min, respectively. The hydrolysates with 0.2% citric acid, on the other hand, required less time, 20 min, to obtain the same amount of rhamnose. There was a significant improvement from 0.05 to 0.06 g/ g pectin of rhamnose content as the time increased from 10 to 20 min. Valdivieso-Ramirez et al. (2021a) also noticed the increase of rhamnose content with longer time in the aqueous citric acid media. They reported that the maximum yield of 86.6% was obtained at 145^{o}C/100 bar/60 min. Ramirez et al. (2021a) performed the hydrolysis at a temperature of 145^{o}C, while this study was conducted at 160^{o}C. The higher temperature used in this study could lead to the faster release of rhamnose from the pectic structure. The release of rhamnose from pectic structure could be potentially favored by three factors: (1) the ionic products (H_3O^+ and OH^-) from the dissociation of water, (2) the hexuronic acids released from the hydrolysis of pectin, and (3) the H^+ atoms dissociated from carboxylic groups of citric acid.

The sCW temperature played an important effect on the rhamnose content. At lower temperatures of 135^{o}C and 145^{o}C, rhamnose sugar was undetectable (**Fig. 3.4B**) regardless of the addition of citric acid. However, at 160^{o}C or higher, its content drastically increased and reached the maximum value of 0.075g/g pectin at 180^{o}C/10 min then decreased upon further heating to 200^{o}C. The results agreed well with findings in **Fig. 3.3B** that at 135 and 145^{o}C, the content of HexA was quite low, indicating that pectin was hydrolyzed to a lower extent. At 180^{o}C, the HexA content reached the value of 0.63g/g pectin, suggesting that pectin was extensively hydrolyzed and broken down into smaller units, which was confirmed by the rhamnose concentration at 180^{o}C (0.08g/g pectin). Similar observations were reported in the study of Zhang et al. (2019) using sCW from

140 to 240°C for the hydrolysis of green algae *Enteromorpha prolifera* to obtain rhamnose. The addition of citric acid significantly enhanced the rhamnose concentration by 1.4-fold compared to the hydrolysates with only water at 180°C/10 min. They noticed that the yield of rhamnose gradually improved from 1.59% to 10.92% as the temperature increased from 140 to 180°C. With further increase of temperature higher than 200°C, rhamnose was no longer detectable in the hydrolysates probably due to the decomposition of monosaccharides under high temperature to form hydroxymethyl furfural, acetic acid and lactic acid. Zhang et al. (2019) recommended 180°C as the most suitable temperature for monosaccharide production. Additionally, they studied the effect of formic acid as a catalyst on the yield of rhamnose monosaccharide and found that the rhamnose yield was approximately 8 times higher than the hydrolysate without formic acid. In addition, the result also agreed with findings of Xu et al. (2020) where poplar wood was hydrolyzed using sCW at 160-180°C to obtain reducing sugars (xylose, rhamnose, and arabinose). With increasing temperature, the yield of rhamnose was significantly enhanced at 180°C/30 min which was 1.5 times higher than the one obtained at 160°C/30 min (Xu et al., 2020).

It was interesting that at 200°C, the concentration of rhamnose was undetectable in the hydrolysates with only water, however, with citric acid added, the rhamnose content was 0.05 g/g pectin. These results demonstrated that the release of rhamnose catalyzed by citric acid was significantly higher than the rhamnose degradation rate. The cleavage of glycosidic bond results from the hydrogen ions obtained from the ionization of water, hexuronic acids, and citric acid. Firstly, H^+ ions would protonate O atoms in the glycosidic bond. The positive charge was then transferred to the C atom, resulting in the breaking of C-O-C bonds. Finally, positively charged C atoms attached with one -OH group and negatively charged -C-O- groups attached with one

hydrogen atom result in the formation of two new monosaccharides (Zhang et al., 2019). The possible mechanism of glycosidic bond cleavage is described in **Fig. 3.5**.

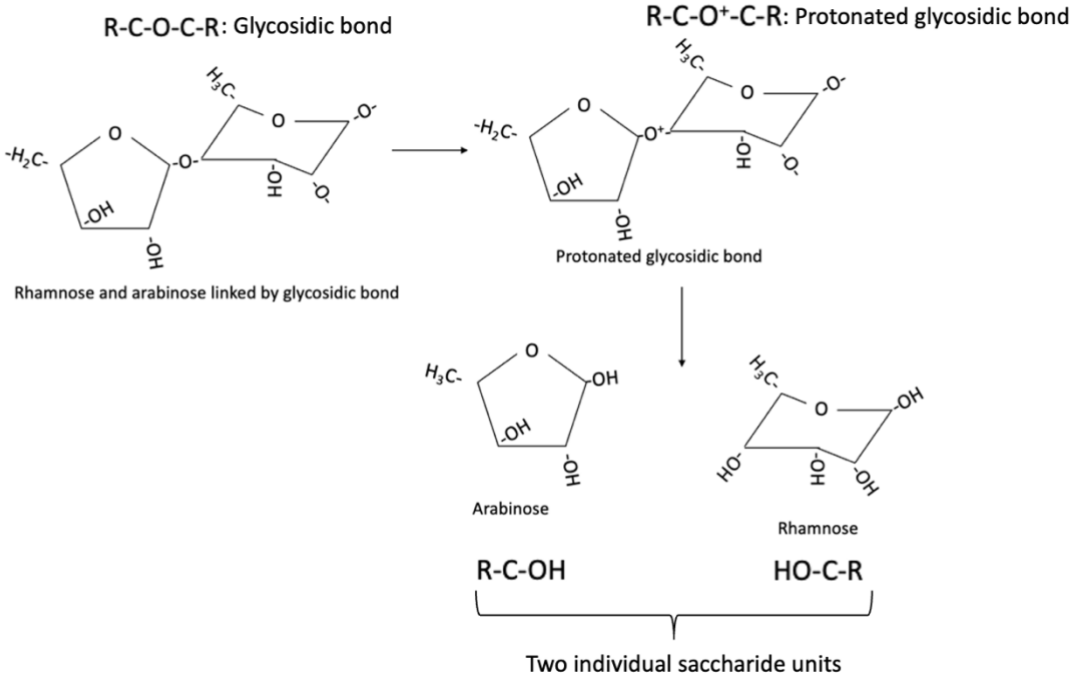


Fig. 3.5. Schematic representation of glycosidic bond hydrolysis in sCW.

The catalytic effect of citric acid was reported by Valladares-Diestra et al. (2022) where the content of glucose was up to 38.2 mg/g in citric acid media, suggesting that citric acid can act as a biocatalyst for the hydrolysis of cocoa pod husks. In addition, Gomes et al. (2020) reported that hydrothermal pretreatment with citric acid could increase the content of reducing sugars (28.2g/L) from sugarcane bagasse hydrolysis.

The effect of different solvents aqueous citric and malic acids, on rhamnose content is shown in **Fig. 3.4C.** Compared to the citric acid hydrolysates, the hydrolysates with malic acid had slightly higher rhamnose content. As reported by Valdivieso-Ramirez et al. (2021b), the catalytic pattern of malic acid was random, meaning that side chains and backbone of pectin were hydrolyzed simultaneously. On the other hand, stepwise hydrolysis is the catalytic pattern of citric acid, indicating that the side chains were broken down prior to the backbone. Therefore, the higher

content of rhamnose in the hydrolysates could be due to the random catalytic pattern of malic acid. The highest rhamnose yield in this study (0.075g/g citrus pectin) was higher than the yield reported in the study of Gómez et al. (2013) where the authors had 0.058g rhamnose/g pectin in the lemon peel pectin hydrolysate obtained by sCW hydrolysis. However, it was lower than the yield obtained in the soybean rhamnogalacturonan (0.112g/ g RG) and macroalgae hydrolysates (0.417g/g algae), (Valdivieso-Ramirez et al., 2021a; Zhang et al., 2019).

3.3.3. Oligosaccharide determination and molecular weight estimation

The hydrolysis of pectin results in different kinds of oligosaccharides depending on the hydrolyzed regions in the pectic structure (Onumpai et al., 2011). The homogalacturonan hydrolysis can produce oligogalacturonides as it contains galacturonic acid as the monomer (Chung et al., 2017). On the other hand, hydrolyzed rhamnogalacturonan region can generate various types of oligosaccharides such as rhamnogalacturonides, galacto-oligosaccharides, and arabino-oligosaccharides due to its nature of being heteropolysaccharides (Gómez et al., 2014; Singh et al., 2020; Valdivieso-Ramirez et al., 2021a). The type of oligosaccharides, in this study, was determined by hydrophilic interaction liquid chromatography, which are shown in **Fig. 3.6.**

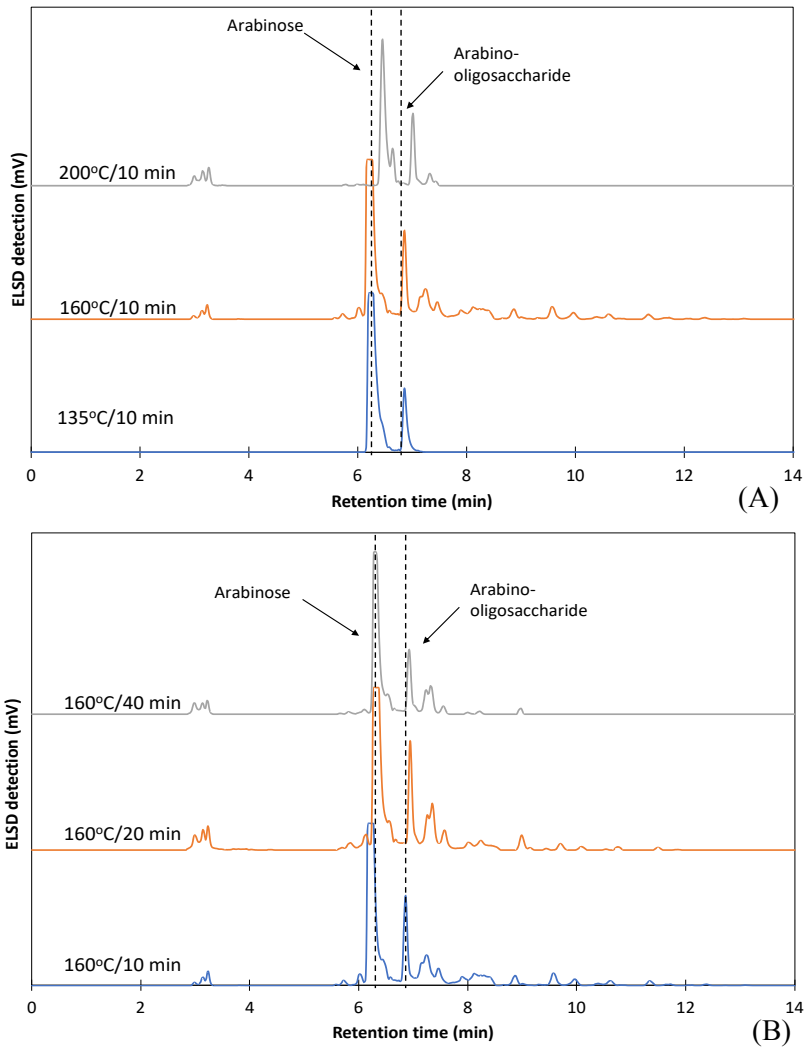


Fig. 3.6. Elution patterns of arabinose and arabino-oligosaccharides from HILIC-ELSD analysis of pectin hydrolysates under the effect of (A) temperatures and (B) reaction time in 0.2% (w/w) citric acid media.

The predominant oligosaccharide in the hydrolysates was arabino-oligosaccharides eluted at the retention time of 6.8 min. Together with arabino-oligosaccharides, arabinose was also detected in the hydrolysates at the retention time of 6.3 min. The temperature and reaction time significantly influenced the content of arabinose. When the temperature increased from 135 to 160°C, arabinose content increased from 1.4 to 1.5 mg/g pectin, respectively. However, it reduced to 0.8 mg/ g pectin upon further heating at 200°C. Compared to the content of rhamnose (**Fig. 3.4B**), the highest

rhamnose content was 0.08 g/g pectin obtained at 180°C, while the highest content of arabinose was 1.5 mg/g pectin obtained at lower temperature of 160°C. This could be contributed by the thermal stability of rhamnose and arabinose. Xu et al. (2020) reported that arabinose was more prone to hydrolysis than rhamnose where the removal of arabinose could be achieved at 160°C, however, rhamnose removal required a higher temperature of 170°C. The contents of arabinooligosaccharides also had increased as the temperature increased from 135 to 160°C but reduced upon further heating at 200°C, as indicated by the peak's height and area in the chromatograms of **Fig 3.6A.** At 135, 160, and 200°C, the peaks' area of arabinooligosaccharide were 3.6×10^6, 4.7×10^6, and 3.3×10^6, respectively. Earlier Gómez et al. (2013) hydrolyzed lemon peel to obtain pectic oligosaccharides using subcritical water at 150−180 °C. They reported that the content of arabinooligosaccharide improved with increasing temperature and reached the maximum value of 4.91% at 160°C but reduced to 3.72% at 180°C.

Rhamnose and arabinose are two monosaccharides presented in the region of rhamnogalacturonan-I (Yapo, 2011), as shown in **Fig. 2.5A**. The release of rhamnose and arabinose in the hydrolysates clearly indicated rhamnogalacturonan-I hydrolysis. Due to having rhamnose and arabinooligosaccharide as the main mono- and oligosaccharide (**Figs. 3.4-3.6**), respectively, it is very likely that the citrus pectin used in this study was composed of mainly rhamnogalacturonan regions.

The influence of reaction time on the arabinose and arabino-oligosaccharides contents is shown in **Fig. 3.6B.** The chromatograms showed a similar elution pattern as in **Fig. 3.6A**. The concentration of arabinose slightly improved from 1.5 to 1.6 mg/g pectin as the time increased from 10 to 20 min, however, it reduced to 1.1 mg/ g pectin upon longer heating of 40 min at 160°C. Interestingly, the peaks' areas at t_R=6.8 min which represents arabino-oligosaccharides, exhibited a similar trend.

The peaks' areas slightly increased from 4.7×10^3 to 5.4×10^3 as the reaction time increased from 10 to 20 min but it decreased to 3.4×10^3 at longer reaction time of 40 min. In general, the hydrolysis of pectin in this study released arabino-oligosaccharides as the predominant pectic oligosaccharides, the content of arabinose and arabino-oligosaccharides were the highest at 160ºC/20 min. The molecular weight of pectic oligosaccharides obtained at 160ºC/20 min was determined and is shown in **Fig. 3.7**.

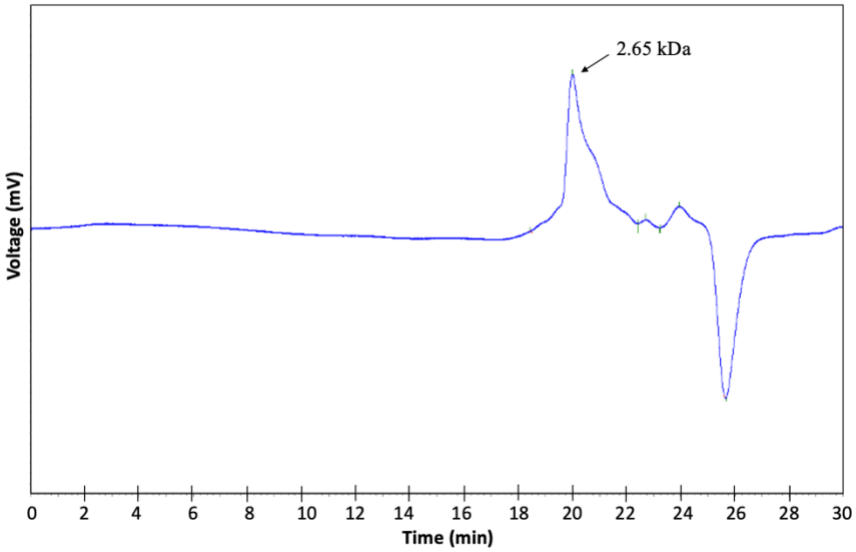


Fig 3.7. The molecular weight of oligosaccharide in the citric acid hydrolysate obtained at 160ºC/50 bar/20 min.

The dominant oligosaccharide was eluted at 20.01 min with the molecular weight of 2.65 kDa. Because oligoarabinan (AraOS) is the dominant oligosaccharide in the hydrolysates, it can be interpolated that AraOS had the degree of polymerization from 17 DP-18 DP, corresponding to the molecular weight of 2.55 and 2.7 kDa, respectively. Moreover, as rhamnose is the main monosaccharide in the hydrolysates (**Fig. 3.4**), it is very likely that oligo-rhamnogalacturonan (OligoRG) was also presented after hydrolysis of pectin. Based on the molecular weight, the degree of polymerization of oligoRG could be from 7 DP-8 DP, corresponding to the molecular weight of 2.38 and 2.72 kDa, respectively. The production of AraOS from lemon peel hydrolysis

was reported by Gómez et al. (2016) where the authors employed subcritical water at 160ºC/326 min. They noticed that the lowest molecular weight of pectic oligosaccharide was 5.9 kDa which was larger than the one identified in this study (2.65 kDa). Though the authors used a substantially longer time of 326 min, they employed only water for the hydrolysis without any catalyst added, which likely produced the oligosaccharides with considerably larger MW of 5.9 kDa.

3.3.4. Antioxidant activity

The functionality of the pectic hydrolysates was determined by its ability to neutralize the $DPPH^-$ free radicals and reduce the ferric ions. The $DPPH^-$ scavenging activity determines the ability of a substance to donate protons to the negatively charged radicals (Kedare & Singh, 2011) which is shown in **Fig. 3.8.**

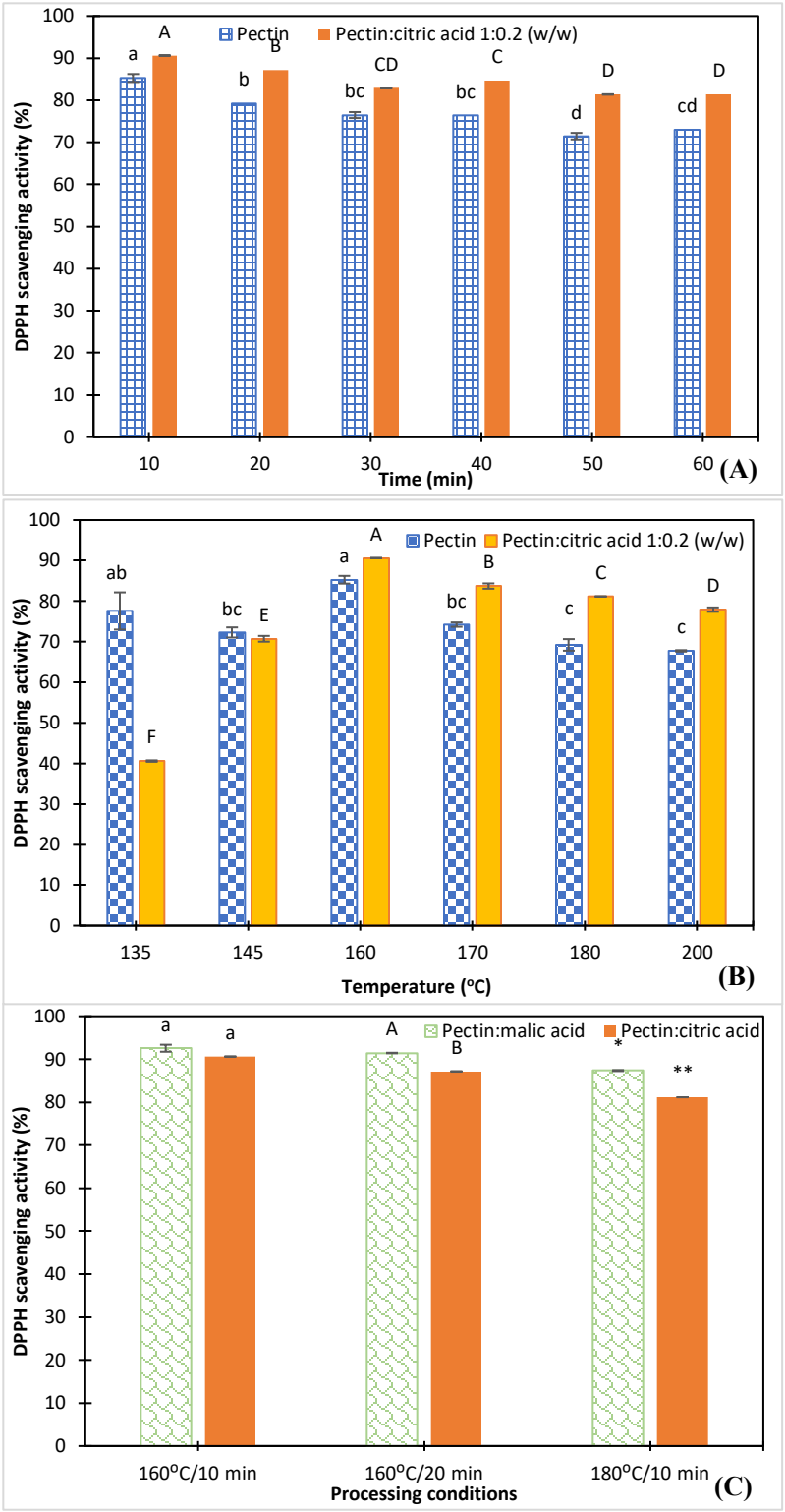


Fig. 3.8. DPPH· scavenging activity of pectic hydrolysates in sCW and sCW modified with citric acid as a function of reaction time at 160°C and 50 bar(A), temperature at 50 bar and 10 min (B), and types of solvent (0.2% (w/w) malic acid and 0.2% (w/w) citric acid) (C). The averages that do not share the same letter or symbol are statistically different.

There was a significant reduction of the scavenging activity of pectic hydrolysates from 85.3% to 72.9% when the time increased from 10 to 60 min (**Fig. 3.8A)**, respectively. Similarly, the hydrolysates with 0.2% (w/w) citric acid had scavenging activity reduced from 90.6% to 82.9% as the time increased from 10 to 30 min, respectively. However, from 30 min to the end of the hydrolysis of 60 min, the antioxidant activity remained constant. The highest scavenging activity was 90.6% obtained at 160^{o}C/10 min in aqueous citric acid media. Despite the reduction in the $DPPH^{-}$ scavenging activity, the hydrolysates with citric acid were significantly higher than the control samples. As mentioned earlier, citric acid could act as a biocatalyst which hydrolyzed pectin at a higher extent as shown in **Fig. 3.3A**. Therefore, the hidden functional groups such as carbonyl and hydroxyl in the pectic structure were now exposed to the cleavage sites. These functional groups acted as protons/electrons donor hence neutralized the negatively charged radical species, which greatly contributed to the scavenging activity (Wang et al., 2018).

The temperature influenced the scavenging activity of pectic hydrolysates in a different pattern (**Fig. 3.8B).** When the temperature increased from 135 to 160^{o}C, the scavenging activity significantly enhanced for both hydrolysates with and without citric acid. At lower temperature of 135 and 145^{o}C, the hydrolysates with citric acid, in fact, had lower scavenging activity than the ones obtained without citric acid. However, from 160 to 200^{o}C, the addition of citric acid enhanced the scavenging activity of the hydrolysates which were significantly higher than the ones obtained with only water. The highest value was 90.6% obtained at 160^{o}C/10 min in aqueous citric acid media.

As shown in **Fig. 3.3B**, the content of hexA was enhanced as the temperature increased up to 200^{o}C, indicating that pectin was hydrolyzed at a higher extent. Consequently, there would be more hydroxyl groups exposed, leading to the higher scavenging activity of 90.6% at 160^{o}C.

Moreover, at the higher extent of hydrolysis, the molecular weight of pectin was also considerably reduced. Due to the low molecular weight, the pectic hydrolysates would have better water solubility, hence efficiently interacted with free radicals, leading to the increased scavenging activity (Guo et al., 2014; Zhi et al., 2017). In addition, the intramolecular forces of low MW pectin were mainly weak hydrogen bonds, hence, it provided more chance for the free hydroxyl groups to neutralize the $DPPH^-$ free radicals (Guo et al., 2014).

Beside citric acid, the catalytic effect of malic acid was also investigated in the scavenging activity of pectic hydrolysates, as shown in **Fig. 3.8C**. The hydrolysates with malic acid had a significantly higher activity than the ones obtained with citric acid, where the highest value was 92.6% obtained at 160^{o}C/10 min. Malic acid is a carboxylic acid containing two carboxyl groups, the catalytic effect of malic acid was reported in the study of Valdivieso-Ramirez et al. (2021b) who hydrolyzed pea hulls in the media modified by citric and malic acid. They noticed that aqueous malic acid exhibited a stronger catalytic effect than citric acid regarding the yield of gluco-oligosaccharides. In particular, the gluco-oligosaccharides yields were 11.7% and 9.7% in malic and citric acid modified media, respectively. Due to the higher content of oligosaccharides, the free hydroxyl groups are, therefore, increased. That explained the high scavenging activity of malic acid hydrolysates in this study.

In addition to $DPPH^-$ scavenging activity, the ferric reducing antioxidant power (FRAP) of pectic hydrolysates was also evaluated as shown in **Fig. 3.9**. The FRAP assay determines the ability of a substance to reduce ferrous ions based on the color intensity of blue the complex formed by ferrous ions and tripyridyltriazine (Benzie & Strain, 1996).

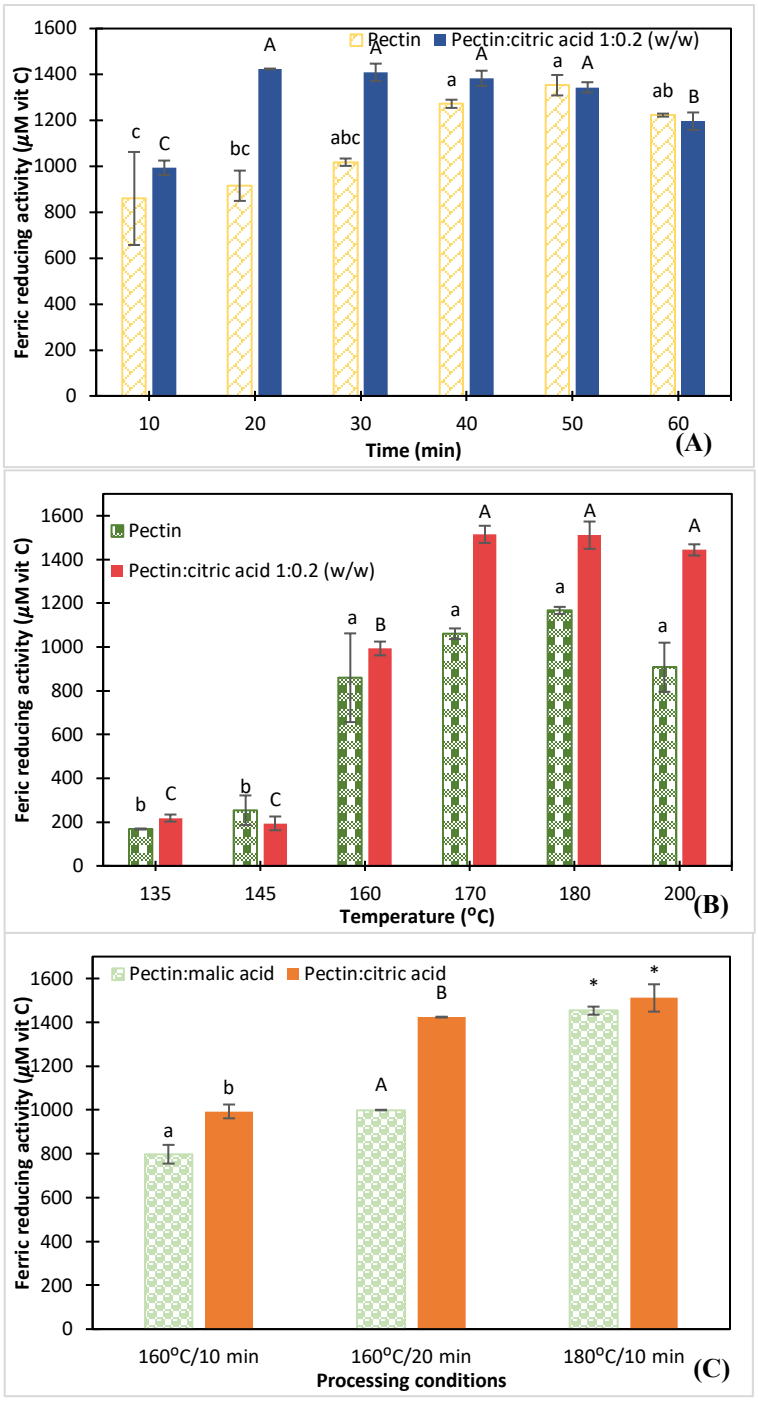


Fig. 3.9. Ferric reducing antioxidant power of pectic hydrolysates in sCW and sCW modified with citric acid as a function of reaction time at 160°C and 50 bar (A), temperature at 50 bar and 10 min (B), and types of solvent (0.2% (w/v) malic acid and 0.2 % (w/w) citric acid) (C). The averages that do not share the same letter or symbol are statistically different.

The effect of time was shown in **Fig. 3.9A,** the reducing power of the pectic hydrolysates was improved by increasing time and obtained the highest value of 1423.8 μM at 160°C/20 min in the aqueous citric acid media. After longer heating the value remained almost constant up to 60 min of hydrolysis. The control samples also showed a similar behavior however, the maximum value was 1353.2 μM obtained at longer time of 160°C/50 min. The effect of temperature was not noticeable at lower temperatures of 135 and 145°C (**Fig. 3.9B**), however, the reducing power drastically increased approx. 5 times at the elevated temperatures and reached the maximum value of 1515 μM at 170°C/10 min in the citric acid media. As shown in **Fig 3.3B**, the content of HexA significantly enhanced at temperature higher than 160°C, indicating that pectin was hydrolyzed at higher extent. Therefore, low molecular weight pectin could be produced which interacted with ferrous ions more effectively leading to the high ferric reducing activity at higher temperature (>170°C). Interestingly, the malic acid hydrolysates had significantly lower ferric reducing activity than the ones obtained with citric acid as shown in **Fig. 3.9C.** The differences among two different carboxylic acids hydrolysates could be contributed to their hydrolysis patterns. In particular, malic acid hydrolyzed the pectic polysaccharides in a random manner, meaning that the side chains and backbone structure of pectin were simultaneously cleaved. On the other hand, citric acid could break down pectin in a progressive pattern i.e., the side chains was first hydrolyzed then the backbone (Valdivieso-Ramirez et al., 2021b). Therefore, the composition of the hydrolysates with citric and malic acid varied leading to the differences in the ferric reducing power.

Although $DPPH^-$ and FRAP methods have different mechanisms: electron donating and electron receiving, respectively. The results demonstrated that the subcritical water hydrolysis was an effective, innovative, and green method to generate pectic hydrolysates with lower molecular weight of approx. 2.65 kDa and significantly enhanced antioxidant activity.

3.4. Conclusions

Subcritical water modified by carboxylic acids (malic and citric acid) was an ecofriendly technology to hydrolyze pectin, producing hexuronic acids and rhamnose. The content of hexuronic acid significantly improved as the temperature increased from 135 to 200°C with a maximum value of 0.7 g/g pectin at 200°C/50 bar/10 min. The hydrolysates obtained with malic had (0.9 g/g pectin) significantly higher content of HexA than the ones obtained with citric acid (0.6 g/g pectin). Rhamnose is the main monosaccharide in the hydrolysates which content was significantly influenced by the reaction temperature. The highest content of rhamnose was 0.08 g/g pectin obtained at 180°C/50 bar/10 min. The malic acid hydrolysates had higher content of rhamnose than the ones obtained with citric acid because the acid strength of malic acid was stronger than citric acid indicated by the pK_a values. Besides having rhamnose as the main monosaccharide (0.08 g/g pectin), arabinose was also detected in the hydrolysates with the highest content obtained at 160°C/50 bar/20 min (1.6 mg/g pectin). The dominant oligosaccharide in the hydrolysates were arabinooligosaccharide with the highest content obtained at 160°C/50 bar/20 min and its molecular weight was estimated at approx. 2.65 kDa, corresponding to DP17-18.

The antioxidant activity of the hydrolysates with carboxylic acids (malic and citric acids) were significantly higher than the control sample, due to the exposure of carbonyl and hydroxyl groups which were able to donate electrons/protons to scavenge the free radicals. The $DPPH^-$ scavenging activity of citric acid hydrolysates was lower than ones obtained with malic acid. Oppositely, the ferric reducing activity of the citric acid hydrolysates were significantly higher than the malic acid ones. Due to the hydrolysis pattern of malic and citric acids being random and stepwise, respectively, leading to the different composition in the hydrolysates hence difference in the antioxidant activities. For that reason, the type of catalyst can be specifically chosen to produce

the hydrolysates with the desired functionality. Ultimately, the pectin hydrolysates can be freeze dried or sprayed dried to produce the powder which can be used directly as fiber supplement or added into food products to increase their fiber content. In addition, rhamnose in the hydrolysates can be isolated to be used as the substrate for biofuel production (1,2-propanediol) via microbial fermentation. The hydrolysis of citrus pectin is graphically described in **Fig. 3.10.**

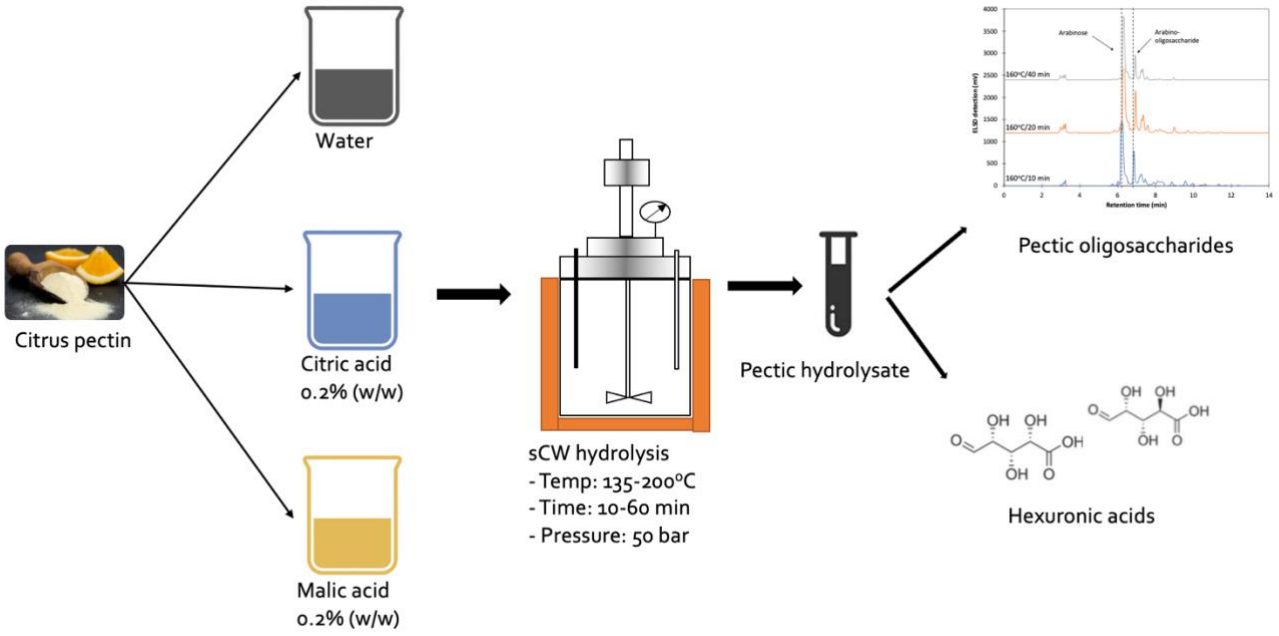


Fig. 3.10. Schematic description of citrus pectin hydrolysis in subcritical water with citric and malic acids as catalysts.

Chapter 4: Hydrolysis of pea protein concentrate in subcritical water media with addition of citrus pectin and citric acid[2]

4.1. Introduction

The protein consumption worldwide is witnessing a major shift from animal-based to plant-based sources. It is not only because plant protein is of high quality, affordable, and sustainable food source; but it also offers multiple potential health benefits, such as reduce the risk of cardiovascular diseases, hypertension, diabetes, LDL cholesterol level, and balance blood sugar level (Boukid et al., 2021; Klost et al., 2020). Pea, *Pisum sativum*, is one of the pulse crops that can provide a considerable amount of protein from pea seeds. Canada is the world largest pea producer, its production yield was 2.5 million tons in 2021, followed by Russia, China, and the USA (Agri-Food Canada, 2021). The protein content in pea seeds can range from 23.3% to 31.7%, depending on the genotypes, environment, and soil factors (Reinkensmeier et al., 2015). The commercial pea protein products are pea flour, pea protein concentrate, and pea protein isolate listed from the lowest to the highest protein content.

Pea protein is mainly consisted of globulin which accounts for 70-80%, and a minor portion of albumin which accounts for 10-20% of total protein (Acquah et al., 2020). Globulin is a salt soluble globular protein which is made up of three different fractions with different sedimentation coefficients and molecular masses (legumin, vicilin, and convicilin). Legumin (11S, ~360 kDa) is a hexameric protein which subunits consist of α-acidic chain and β-basic chain linked together by disulfide bonds. Vicilin (7S, ~180 kDa) is a trimer which is held together by hydrophobic interaction and lastly convicilin (7S, ~290±40 kDa) is trimeric fraction (Bogahawaththa et al.,

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2019; Klost & Drusch, 2019). Albumin (2S, 5-80 kDa) is a water-soluble protein, which contains more essential amino acids than globulin, such as tryptophan, lysine, threonine, and methionine (Boukid et al., 2021).

Recently, pea protein has drawn the attention of the scientific community due to its functional properties and its derived bioactive peptides. Several studies have demonstrated that pea protein hydrolysates possess antioxidant activities as well as health promoting properties. Li & Aluko. (2010) used alcalase enzyme to hydrolyze pea protein. The peptides fraction that had the most positive charge showed the strongest angiotensin converting enzyme (ACE) and renin inhibitory activities. In addition, the more hydrophilic the peptides, the stronger inhibitory activities against ACE and renin. There were three dipeptides identified to have moderate to strong ACE and renin inhibitory activities: IR (isoleucine-arginine), KF (lysine-phenylalanine), and EF (glutamic acid-phenylalanine). Even though, the authors were able to determine and characterize the health promoting effects of pea peptides, the degree of hydrolysis as well as molecular mass of the peptides were not determined. Hence, Barbana & Boye (2010) investigated the changes in molecular weight (MW), degree of hydrolysis (DH), and ACE inhibitory activities of pea protein hydrolysates generated by enzymatic digestion. The MW range of the native protein was between 30 and 670 kDa, the MW distribution was smaller after enzymatic treatment, 0.244 - 44 kDa, with more than 50% had MW below 4 kDa. Regarding the degree of hydrolysis, alcalase and flavourzyme (AF) combination had the highest DH (78.58%) compared to other hydrolysates generated by other enzymes: papain (31.18%) and gastrointestinal (31.08%). The antihypertensive effect of pea hydrolysate was tested in humans (Li et al., 2011), where the participants were orally administered pea protein hydrolysates daily for 3 weeks. The consumption of 3 g/day resulted in a maximum 10 mmHg reduction in systolic blood pressure compared to the placebo group.

On the other hand, the antioxidant activities of pea protein have also been improved by hydrolysis mainly due to the formation of peptides and amino acids. Pea protein hydrolysates were generated by thermolysin digestion (Pownall et al., 2010) and their scavenging activities against DPPH free radicals and metal ions were enhanced. After protein hydrolysis, the total content of hydrophobic amino acids (alanine, valine, isoleucine, leucine, etc.) increased, leading to the improvement of DPPH scavenging activity. However, the ability to neutralize superoxide ions, hydroxyl radicals, and hydrogen peroxide did not significantly increase after protein depolymerization. Ding et al. (2020) also observed that after hydrolyzing pea protein with alkaline protease, the DPPH radical scavenging rate of the hydrolysate fraction (M_w<1kDa) was 37.9%. Contrasting with the previous study, the scavenging ability of pea protein hydrolysate against hydroxyl radical was reported to be 27.8%. Ding et al. (2020) identified three main peptides contributing to the antiradical activities of the hydrolysates: YSSPIHIW (Tyr-Ser-Ser-Pro-Ile-His-Ile-Trp), ADLYNPR (Ala-Asp-Leu-Tyr-Asn-Pro-Arg), and HYDSEAILF (His-Tyr-Asp-Ser-Glu-Ala-Ile-Leu-Phe).

Besides the enzymatic hydrolysis, pea protein hydrolysate can also be generated by heat- and pressure-pretreatment (Chao et al., 2013; Girgih et al., 2015). Both studies used high pressure (2000-6000 bar/24 ^{o}C/5 min) or high temperature (100^{o}C/30 min) prior to the enzymatic treatment with alcalase (1-4%). With the pretreatment of high pressure or temperature, lower concentration of alcalase needed to generate low MW peptides. The improvement of antioxidant activities was observed after the high pressure pretreatment: superoxide and hydroxyl, DPPH, and oxygen radical scavenging. Probably under high pressure of 4000 bar, the peptide bonds were more exposed and more prone to alcalase hydrolysis, resulting in the formation of bioactive peptides which led to the enhancement of free radical scavenging.

Enzymatic digestion is a well-known approach to hydrolyze protein; however, the method bears several disadvantages such as high-cost enzymes, time consuming, complex protocol, numerous buffer systems, and strictly controlled parameters. In addition, acid or base hydrolysis is also commonly used to breakdown protein. Similarly, this method could generate environmentally harmful waste, toxic compounds (e.g., lysinoalanine), difficult to control peptides size, and destroy and racemize the amino acids (Espinoza et al., 2012; Marcet et al., 2016; Sereewatthanawut et al., 2008). Therefore, subcritical water (sCW) has been employed as a green technology to hydrolyze protein or protein-rich biomass. Not only because sCW is environmentally friendly but it also has unique characteristics that could favor depolymerization reaction such as low dielectric constant, high ionic species, and high hydronium and hydroxide ion concentrations (Kus, 2012; Zhao & Saldaña, 2019).

Even though sCW has been extensively used to hydrolyze protein from various sources, it has been known to have quite low specificity hydrolysis. Powell et al. (2016) concluded that cleavage could happen elsewhere in the protein molecules besides the breakdown of peptide bonds, possibly in the amino acids side chain. Previously, Rogalinski et al. (2005) reported the low selectivity of sCW in bovine serum albumin hydrolysis. However, they increased the selectivity by adding 90% CO_2 to the media, as a result, the amino acid yield improved by 4 times higher than the sCW alone. Valdivieso-Ramirez et al. (2021) observed a similar phenomenon however with pea hull fiber hydrolysis and found that citric and malic acid could enhance the selectivity of sCW towards cleavage of glycosidic bonds. Specifically, targeting gluco-oligosaccharides (2–6 DP) which was approximately 5-fold higher compared to the media with only water.

There were various types of protein that had been hydrolyzed or isolated using sCW technology (**Table 2.1**). However, to the best of our knowledge, there is no study investigating the

hydrolysis reaction of pea protein concentrate in sCW media. Therefore, the objectives of this study are to: (1) evaluate the effect of reaction parameters (temperatures, times, and protein/pectin ratios) on the degree of hydrolysis, (2) elucidate the effect of pectin and citric acid on pea protein hydrolysis in sCW media, and (3) determine the antioxidant activities of the hydrolysate towards DPPH free radical scavenging and ferric reducing activity.

4.2. Materials and methods

4.2.1. Materials

Pea protein concentrate (51% protein) was kindly provided by AGT Food and Ingredients Inc. (Saskatoon, SK, Canada). Citrus pectin (30% degree of esterification) was generously supplied by CP Kelco (Atlanta, GA, USA). Chemicals involved in the sCW hydrolysis were citric acid (> 99.5%, ACS grade) from Sigma Aldrich (Oakville, ON, Canada), potassium phosphate monobasic from ICN Biomedicals, Inc. (Aurora, OH, USA), sodium hydroxide (>95%) from Fisher Scientific (Ottawa, ON, Canada), water from the Milli-Q system (18.2 MΩ cm, Millipore, Billerica, MA, USA), and nitrogen gas (99.9% purity) from Praxair (Edmonton, AB, Canada).

For characterizations, all chemicals were of analytical grade. Sodium tetrahydroborate, sodium dodecyl sulfate, sodium carbonate, sodium acetate, sodium potassium tartrate, copper (II) sulfate, iron (III) chloride hexahydrate, β-mercaptoethanol, o-Phthaldialdehyde, Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), L-lysine and bovine serum albumin were obtained from Sigma Aldrich (Oakville, ON, Canada). Methanol (99.9%) was purchased from Fisher Scientific (Ottawa, ON, Canada). The calibration standards for peptide size exclusion chromatography were blue dextran (2000 kDa), bovine serum albumin (66.4 kDa), carbonic acid (29 kDa), cytochrome C (12.4 kDa), aprotin (6.5 kDa). L-serine, L-Histidine, L-Threonine, L-Arginine, L-Alanine, L-Tyrosine, L-Methionine, L-Valine, L-

Phenylalaline, L-Lysine, L-Isoleucine, L-Glycine, L-Leucine, L-Aspartic acid, and L-Glutamic were used as standards for amino acid HPLC analysis were purchased from Sigma Aldrich (Oakville, ON, Canada).

4.2.2. Subcritical water hydrolysis

The suspensions were prepared by mixing pea protein concentrate with pectin at different mass ratios of 1:0.1, 1:0.5, 1:1, 1:2, and 1:3 (w/w) (e.g., the pea protein and pectin ratio 1:1 (w/w) was prepared by mixing 1g of pea protein concentrate with 1g of pectin). The protein suspensions with citric acid were prepared by mixing 1g of pea protein concentrate with 0.2g of citric acid. The mixtures were then dissolved in 100mL of 0.2M phosphate buffer at pH 8 using a Heidolph homogenizer (Heidolph Instruments GmbH & Co., Germany) at 3,200 rpm for 1 min. After that, the suspensions were stirred overnight under constant speed at 4^{o}C. Then, subcritical water was employed to hydrolyze the suspensions according to the procedure described by Valdivieso Ramirez et al. (2021) with slight modifications.

The hydrolysis was conducted using a high pressure system which was described in detail in the previous Chapter 3 in section 3.2.2. Briefly, the system was equipped with a 600mL batch stirred reactor, a 780W heating mantle, a nitrogen tank, and a temperature controller. The stirring speed was estimated around 660 rpm and kept constant throughout the hydrolysis.

The suspensions were loaded into the reactor then purged with N_2 gas for 12 min under constant stirring. Due to the viscous solutions obtained from mixing pea protein with pectin, prolonged purging time was used to remove the dissolved air oxygen and prevent any undesirable oxidation reaction. After that, the reactor was pressurized with N_2 gas to a certain extent (e.g., use of 30 bar for 180^{o}C, use of 22 bar for 240^{o}C, etc.) so that after reaching the desired temperature, a pressure value of 50 bar could be reached. The reaction was performed at different temperatures

(160, 180, 200, 220, and 240°C) and times (10, 20, 30, 40, 50, and 60 min) under constant pressure of 50 bar. The reactor was immediately cooled down to 40°C in approximately 8-10 min (e.g., use of 8 min for 180°C, use of 10 min for 240°C, etc.) using a water bath. Then, the reactor was depressurized within 4 min. The resulting hydrolysates were centrifuged at 8,000g for 20 min to collect the supernatant which was then stored at -18°C until further characterizations. The hydrolysis of pea protein was conducted in duplicate, and the processing conditions were summarized in **Table 4.1**.

Table 4.1. Hydrolysis conditions of pea protein concentrate in subcritical water media.

<table>
<tr><th>Temperature (°C)</th><th>Time (min)</th><th>Solvent</th></tr>
<tr><td>160</td><td rowspan="5">10</td><td rowspan="12">Water</td></tr>
<tr><td>180</td></tr>
<tr><td>200</td></tr>
<tr><td>220</td></tr>
<tr><td>240</td></tr>
<tr><td rowspan="6">180</td><td>10</td></tr>
<tr><td>20</td></tr>
<tr><td>30</td></tr>
<tr><td>40</td></tr>
<tr><td>50</td></tr>
<tr><td>60</td></tr>
<tr><td>160</td><td rowspan="5">10</td><td rowspan="12">Citric acid
0.2% (w/w)</td></tr>
<tr><td>180</td></tr>
<tr><td>200</td></tr>
<tr><td>220</td></tr>
<tr><td>240</td></tr>
<tr><td rowspan="6">180</td><td>10</td></tr>
<tr><td>20</td></tr>
<tr><td>30</td></tr>
<tr><td>40</td></tr>
<tr><td>50</td></tr>
<tr><td>60</td></tr>
</table>

Table 4.1. *Continued*

160	10	Protein:pectin 1:1 (w/w)
180		
200		
220		
240		
180	10	
	20	
	30	
	40	
	50	
	60	

4.2.3. Characterizations of the protein hydrolysates

4.2.3.1. Degree of hydrolysis

The extent of protein hydrolysis was measured by spectrophotometry method based on the reaction of primary amine and OPA with the presence of β-mercaptoethanol to form an OPA adduct that could be detected at 340 nm (Church et al., 1983). The procedure was adopted from (Mirzaei et al., 2015) with slight modifications. Briefly, OPA reagent was prepared by mixing 40mg of OPA dissolved in 1mL of methanol, 25mL of 0.1M sodium tetrahydroborate, 2.5mL of 20% SDS (w/v), and 100 μL of β-mercaptoethanol were later added. The final volume was adjusted to 50 mL using mili-Q water. The OPA reagent was freshly prepared daily. To perform the assay, 0.1mL of the hydrolysate was mixed with 2mL of OPA reagent and incubated for exactly 2 min at room temperature. Finally, the absorbance was read at 340 nm using a UV-Vis spectrophotometer. The concentration of free amino group was estimated by a calibration curve with L-Lysine as the standard (0-2 mM) (See **Fig. A5** in Appendix A). The degree of hydrolysis (DH) was calculated using Eq. (4.1):

$$DH\ (\%) = \frac{L-Lo}{Lmax-Lo} \text{ x}100 \quad (4.1)$$

where, L is the concentration of free amino groups after hydrolysis, L_o is the concentration of free amino groups of the untreated sample, and L_{max} is the concentration of free amino groups obtained by conventional acid hydrolysis (6M HCl at 110°C/ 24h).

4.2.3.2. Protein/peptide/amino acid content determination

The content of protein/peptide/amino acid is reflected by the color intensity which was formed by two reactions: (i) reaction with alkaline copper to produce Cu^+ ions, and (ii) reaction with Folin-Ciocalteu reagent by Cu^+ ions. The procedure was adopted from Waterborg (2009) with minor modifications. First, 1mL of 4-fold-diluted hydrolysate was mixed with 1mL of 2N NaOH solution and incubated at 100°C/10 min in a water bath. The solution was then cooled down to room temperature and mixed with 10mL of complex-forming reagent (100mL of 2% (w/v) sodium carbonate + 1mL of 1% (w/v) copper (II) sulfate + 1mL of 2% (w/v) sodium potassium tartrate). After 10 min at room temperature, 1mL of Folin-Ciocalteu reagent was added to the solution. The mixture stood in the dark at room temperature for 35 min. The absorbance was read at 550 nm using the UV-Vis spectrophotometer. The protein/peptide/amino acid content was calculated by a calibration curve with bovine serum albumin as the standard (0-2 mg/mL) (See **Fig. A6** in Appendix A).

4.2.3.4. Total amino acid profile

The hydrolysate was treated with hydrochloric acid before determination of amino acids profile; by mixing 1mL of the hydrolysate with 6mL of 6M HCl at 110°C/24h. The mixture was then mixed with 0.2 mL of internal standard of B-amino-n-butyric acid and ethanolamine at 25 µmol/mL; followed by centrifugation at 2500 rpm for 15 min. The vials for HPLC injection included 50 µL of the supernatant, 50 µL of 4.29M NaOH, and 400 µL of mili-Q water. More

NaOH solution can be added to maintain pH of the solution at 9, which is crucial for derivatization. The mobile phase included two eluents: (A) 1600mL of 0.1M sodium acetate buffer pH 7.2, 180mL methanol, 10mL tetrahydrofolic acid, and 210mL of mili-Q water; (B) 100% methanol. The elution gradient was: 0-1 min, isocratic 100% A; 5-25 min, isocratic 85% A and 15% B; 38-39 min, linear from 55% to 35% A and 45% to 65% B; 40-42.5 min, isocratic 100% B, and 43-48 min, isocratic 100% A. The separation was performed using Supelcosil LC-18 column (Sigma Aldrich, Oakville, ON, Canada), 150mm x 4.6mm, 3µm with the flow rate of 1.1 mL/min. The fluorescence intensity of the samples was measured at the wavelength of 340 nm (excitation) and 450 nm (emission). The calibration curves were prepared by three different concentrations of L-configuration amino acids (150, 300, and 600 nM).

4.2.3.5. Peptide molecular weight distribution

Size exclusion chromatography was used to evaluate the size distribution of peptides. The procedure was adopted from Klost & Drusch (2019) with minor modifications. The diluted hydrolysates were filtered and injected through a Superdex 75 Increase 10/300 GL (GE healthcare GmbH, Solingen, Germany) column with 0.1M phosphate buffer at pH 7 as the mobile phase. The UV detector was used at 280 nm. The estimation of peptide molecular weight distribution was carried out via calibration standards (Sigma Aldrich, Oakville, ON, Canada): blue dextran (2000 kDa), bovine serum albumin (66.4 kDa), carbonic acid (29 kDa), cytochrome C (12.4 kDa), and aprotin (6.5 kDa) (See **Fig. A7** in Appendix A).

4.2.3.6. Intrinsic fluorescence spectroscopy

The intrinsic fluorescence intensity of protein is mainly contributed by the aromatic hydrophobic amino acids of tyrosine, phenylalanine, and tryptophan. The fluorescence intensity indicates the conformational changes of the protein molecules (Lakowicz, 2006). The procedure

for intrinsic fluorescence determination was previously described by Chao & Aluko (2018) with minor modifications. Briefly, the hydrolysates were diluted with phosphate buffer pH 8 or aqueous citric acid until the protein concentration reached 20 μg/mL. The solutions were then centrifuged at 10,000 g for 5 min to collect the supernatant. The fluorescence intensities of protein solutions were scanned with a spectrofluorometer (SpectraMax® M3, Molecular Devices, San Jose, CA, USA) with excitation wavelength of 295 nm and emission spectra from 350 nm to 450 nm with a 2 nm increment.

4.2.3.7. Antioxidant activity

The $DPPH^-$ scavenging ability of the hydrolysates was determined according to the method of Zhang et al. (2008). Briefly, 0.1 mM DPPH solution was prepared with absolute methanol. Then, 1mL of 2-fold-diluted hydrolysates was mixed with 3mL of DPPH solution then stood at room temperature for 35 min. After that, the solution was centrifuged at 7000g for 5 min to collect the supernatant. The absorbance of the supernatant was read at 514 nm using the UV-Vis spectrophotometer. The scavenging ability was expressed as the percentage of scavenging ability using Eq. (4.2):

$$\text{DPPH scavenging ability (\%)} = \frac{Ac-As}{Ac}\text{x}100 \quad (4.2)$$

where, A_c: absorbance of the DPPH solution, and A_s: absorbance of the samples.

The ferric reducing antioxidant power (FRAP) was measured using the method described by Ekaette & Saldaña (2021) with minor modifications. The TPTZ solution included three reagents at the ratio of 10:1:1 v/v/v: 0.3 M sodium acetate, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$. The hydrolysates were diluted by 4-fold using deionized water before the measurement. Briefly, 0.4mL of diluted hydrolysates was mixed with 3mL of TPTZ solution. The mixture was incubated in the water bath at 37°C/30 min then centrifuged at 7000g for 7 min to

collect the supernatant. The absorbance of the supernatant was measured at 593 nm using the UV-Vis spectroscopy. The reducing power was estimated using the calibration curve with vitamin C as the standard at the concentrations from 50 to 300 μM (See **Fig. A4** in Appendix A).

4.2.3.8. Statistical analysis

The hydrolysis of pea protein concentrate was conducted in duplicate. The results were reported as mean ± standard deviation. The statistical difference between means was determined using one-way ANOVA ($p<0.05$) with grouping information obtained from Tukey's test and t-test. The comparisons were performed using Minitab version 17.0 (Minitab Inc., State College, PA, USA).

4.3. Results and discussion

4.3.1. Degree of hydrolysis

The degree of hydrolysis (DH) is indicated by the amount of free amino groups in the hydrolysates. The higher DH demonstrates that the protein is cleaved into free amino groups at higher extent. With increasing time, the DH increased and reached the maximum value of 50.5% at 40 min (**Fig. 4.1A**). Afterwards, the DH remained unchanged even at longer times of 50 and 60 min. The phenomenon could be attributed to the dissociation constant (K_w) of water, which depends on temperature and pressure of the media (Martinez-Monteagudo & Saldaña, 2014) i.e., the concentration of H_3O^+ and OH^- ions do not change under constant pressure and temperature conditions. For that reason, the DH could not further increase after the protein was hydrolyzed by the available ionic species in the sCW medium. Regarding the reaction time from 10 to 40 min, the DH was significantly enhanced due to better interaction between ionic species and protein. Similar result was observed in the study of Sereewatthanawut et al. (2008) after hydrolyzing rice bran using subcritical water at 100-220°C/30 min. They reported that the protein as well as amino acid yields did not increase with the increase of the reaction time. In fact, the yields remained

constant after 5 min until the end of the experiment at 30 min. Espinoza et al. (2012) reported that the reaction time had no significant effect on the DH of whey protein hydrolysates. After 10 min of hydrolysis at 250°C, the DH values remained unchanged up to 60 min.

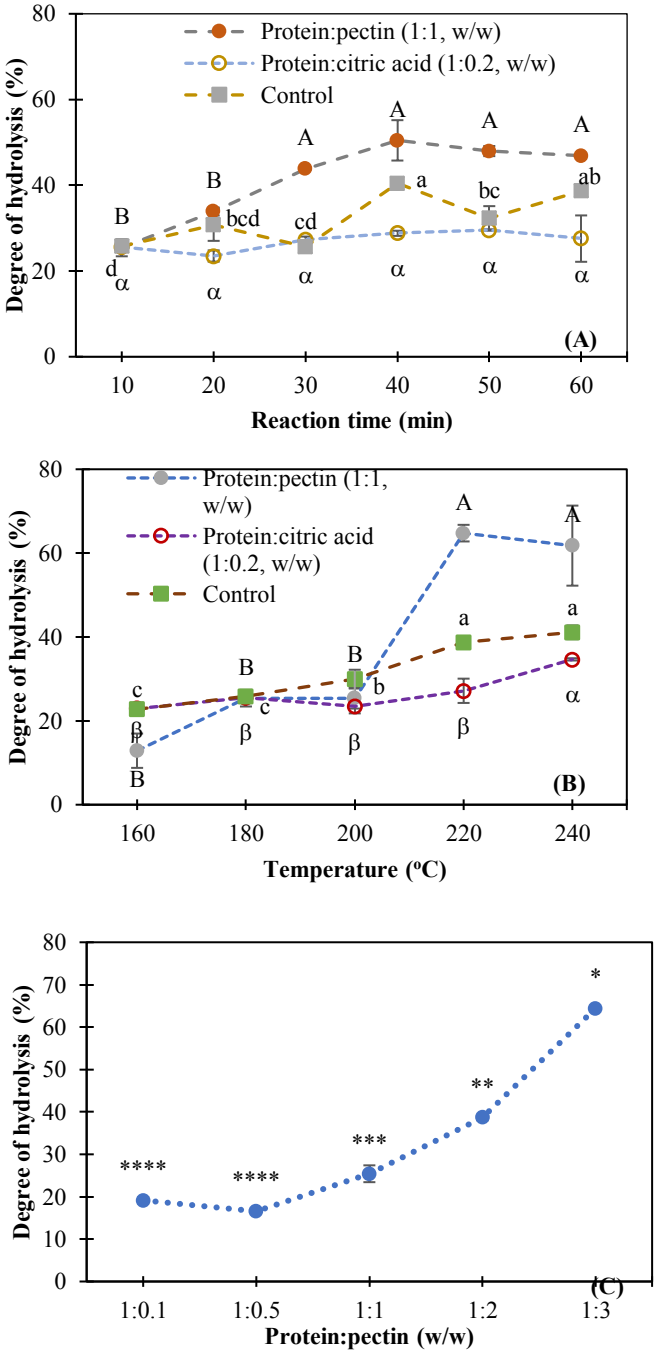


Fig. 4.1. The effect of reaction times (A) at 180°C, temperatures (B) for 10 min, and mass ratios (C) at 180°C/10 min on the degree of hydrolysis of pea protein:pectin and pea protein:citric acid suspensions.

Fig. 4.1B shows the effect of temperature on the protein DH. When the temperature increased from 160°C to 200°C, the DH only slightly increased. However, the DH was considerably improved and peaked at 220°C with the highest DH value of 64.8%. As mentioned earlier, the K_w value of water increases with temperature, K_w value at 220°C is approximately 700 times higher than the one at ambient conditions (Sereewatthanawut et al., 2008). Consequently, the protein underwent a more severe hydrolysis due to higher concentration of ionic species which could potentially break the peptide bonds in the protein; eventually increasing the amount of free amino groups in the hydrolysates. Ramachandraiah et al. (2017) reported hydrolysis reaction of soybean powder using sCW where they observed that when the temperature increased from 150°C to 190°C, the content of free amino groups appreciably increased from 28 to 89 mg/L. The yield of amino acids in the study conducted by Pińkowska & Oliveros (2014) had similar changes and considerably increased at 220°C then remained constant up to 260°C and eventually decreased upon higher temperatures (260°C to 300°C). The possible mechanism of peptide bond cleavage by H_3O^+ is shown in **Fig. 2.4** and **Fig. 4.2**.

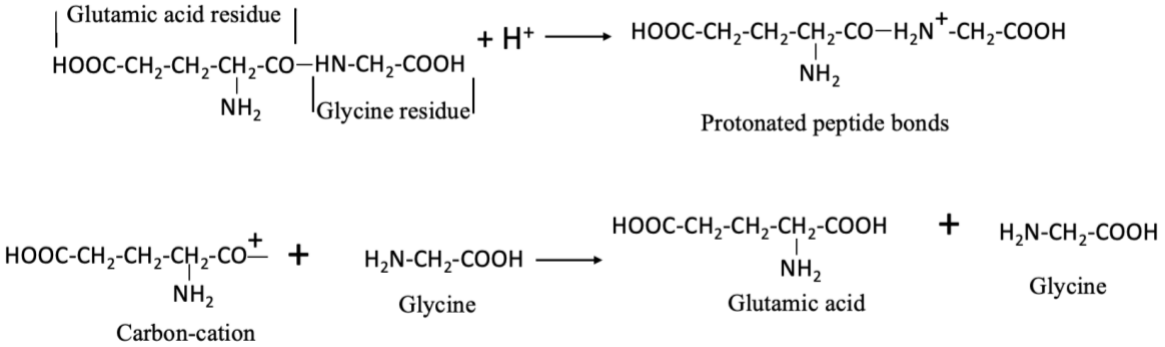


Fig 4.2. Schematic presentation of peptide bond cleavage between glutamic acid and glycine residues.

Interestingly, the mixing ratio of pea protein and pectin had a noticeable effect on the DH. Particularly, with the increasing concentration of pectin, the DH was significantly enhanced. The maximum DH value was 64.4% at a ratio pea protein:pectin of 1:3 (w/w) (**Fig. 4.1C**). Pectin is a heterogeneous polysaccharide with D-galacturonic acid (GalA) as the predominant monomer (60%) which are bonded together by glycosidic linkages (Valdivieso-Ramirez, Sanchez Gallego, et al., 2021). Rogalinski et al. (2008) suggested that the stability of peptide bonds is considerably higher than glycosidic bonds. Therefore, pectin could be broken down into its monomers, D-galacturonic acids and other hexuronic acids (**Fig 3.2**) before the protein was hydrolyzed. The released acidic sugars from pectin could then act as a catalyst for the hydrolysis reaction of protein. Earlier, Valdivieso Ramirez et al. (2021) reported that with the addition of 0.2% citric acid to the sCW, the yield of gluco-oligosaccharides (2–6 DP) was 5 times higher than the one with sCW only. Rogalinski et al. (2005) used sCW media with 90% CO_2 and reported that the yield of amino acids from bovine serum albumin hydrolysis increased by 4 times compared to the one without CO_2 addition. For that reason, at higher concentrations of pectin, there would be more acidic sugars released into the reaction media which further hydrolyzed the protein, resulting in a higher degree of hydrolysis.

Pea protein hydrolysis reaction was also conducted in sCW media modified by citric acid. The reaction time did not exert a significant effect on the DH of the hydrolysates (**Fig. 4.1A**), which ranged from 23.5% to 27.6%. On the other hand, the processing temperatures had a more pronounced effect on the DH (**Fig. 4.1B**), specifically, at 240^{o}C, the DH value reached the maximum of 34.7% meanwhile, the DH was only 22.9% at 160^{o}C. Compared to the media using pectin, the aqueous citric acid media had a considerably less proteolytic effect, resulting in a significantly lower DH. The pH of the suspensions could be the primary reason attributed to the

cleavage of pea protein. In the media with pectin, the pH value was maintained at 8 which is far above the isoelectric point of pea protein (~4.3). The protein molecules were then negatively charged due to the alkaline environment used. Therefore, the possibility to combine with hydrogen ions is appreciably increased compared to the positively charged ones (Steinhardt & Fugitt, 1942). Eventually, the proton donated by pectic acidic sugars could protonate and break down the peptide bonds, leading to the escalation of free amino groups in the samples with pectin added. On the contrary, the pH of aqueous citric acid solution was 2.6 which had the protein positively charged, reducing the hydrolysis potential of hydronium ions in the sCW media. Moreover, the proteolytic potential of the basic solution is proven to be more effective than acidic one (Espinoza & Morawicki, 2012), probably due to the recombination of peptides under acidic pH.

4.3.2. Protein/peptide/amino acid content of hydrolysates

The content of protein/peptide/amino acid was determined by Lowry's method, which detects peptide bonds from its reaction with copper ions and Folin reagent. The resulting complex has a strong blue color which is detectable at 550 nm (Waterborg, 2009). The content steadily improved when the reaction time increased from 10 to 30 min; then reached the maximum value of 903.1 mg/g protein at 40 min. With prolonged heating periods from 40 to 60 min, the protein/peptide/amino acid content was considerably reduced (**Fig. 4.3A**). The results agreed with the protein degree of hydrolysis where the DH value significantly increased from 10 to 40 min of reaction time. Under high temperature and pressure conditions, the structure of globular proteins could be unfolded (Chao et al., 2013; Lu et al., 2016). Therefore, more peptide bonds, which used to be buried inside, were exposed; hence, provided better interactions of the peptide bonds with the assay's reagents. Consequently, the protein/peptide/amino acid content increased.

Lu et al. (2016) reported a similar behavior of soy protein hydrolysis reaction, under the effect of extended heating time (>30 min), a significant decrease in protein yield was observed. Additionally, Sunphorka et al. (2012) noticed a similar behavior in rice bran protein hydrolysis, where the protein content increased up to 60 min of processing time. However, within the first period of the reaction (5 min), the proteins aggregated then assembled into solid precipitates. Afterwards, with the increasing reaction time, the particles disaggregated and released the unfolded structure of protein, smaller soluble peptides, and amino acids. As a result, the protein content of the hydrolysates was improved. The results also agreed with the findings in the study of Sereewatthanawut et al. (2008) who reported that the protein content of soy protein hydrolysates gradually increased at longer holding times from 10 to 30 min.

On the other hand, the protein suspensions supplemented with citric acid had a quite limited increase in the content of protein/peptide/amino acid, specifically the highest value was 543.9 mg/g protein obtained at 40 min, which was only 1.23 times higher than the lowest value at 10 min. The main reason, as mentioned above, could be due to the acidic pH of the reaction medium. As citric acid was supplemented, the pH value dropped to 2.6 which was below the isoelectric point of pea protein, hence, the protein molecules obtained the positive charges; eventually, reduced the hydrolytic ability of H_3O^+ ions.

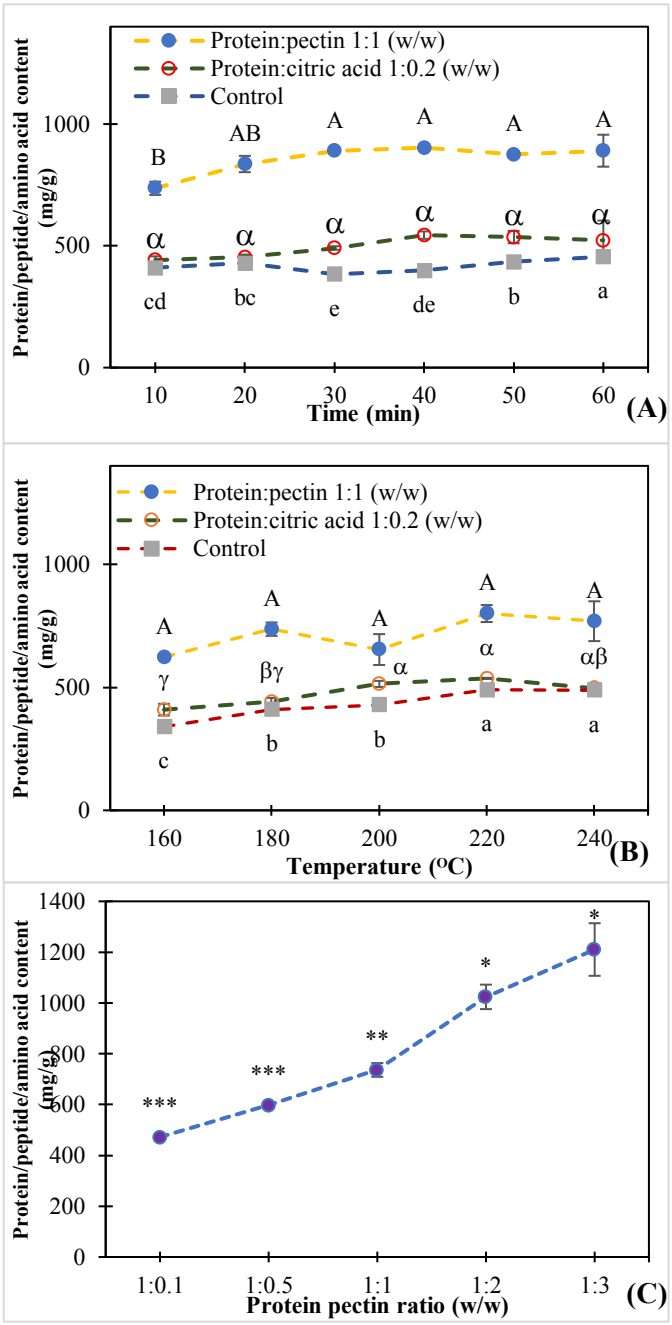


Fig. 4.3. The protein/peptide/amino acid content of the hydrolysates under the effects of (A) reaction time at 180°C, (B) reaction temperature during 10 min, and (C) mass ratio of protein and pectin at 180°C/10 min.

Fig. 4.3B shows the effect of temperature on the content protein/peptide/amino acid in the hydrolysates. With the increasing temperatures from 160°C to 220°C, the content was progressively increasing and peaked at 220°C (799.9 mg/g protein). Afterwards, it remained almost constant at 240°C with the value of 768.7 mg/g protein. The results agreed with findings in the study of Ndlela et al. (2012), who reported that the protein contents of soybean protein hydrolysates were enhanced from 68% to 73% as temperatures increased from 150°C to 234°C. Sunphorka et al. (2012) noticed a similar phenomenon for rice bran protein hydrolysis with the increasing temperature, where the protein content significantly increased. The dissociation of aggregated protein particles and unfolding of the protein structure could be likely leading to the increase in peptide bonds of the hydrolysates as explained previously. The hydrolysates with citric acid added had considerably lower protein/peptide/amino acid content, the highest content was obtained at 220°C with the value of 536.3 mg/g protein and decreased upon further heating at a higher temperature of 240°C.

The protein/peptide/amino acid content of the hydrolysates are consistent with the degree of hydrolysis in **Fig. 4.1B**, where the DH value also peaked at 220°C. However, there was no sudden increase observed in the content of protein/peptide/amino acid when the temperatures increased from 200°C to 220°C. The employed assays could be the major reason attributed to the variation between the two sets of data. The degree of hydrolysis was quantified by the OPA assay which detected the free amino groups in the hydrolysates. On the other hand, the reagents in Lowry's method would react with peptide bonds of the protein molecules. Therefore, it is obvious that the peptide bonds in the hydrolysates did not increase but instead broken down after the treatment at 220°C. Increasing the amount of free amino groups in the hydrolysates was evident as

shown in **Fig. 4.1B** to confirm the cleavage of peptide bonds. Then, peptide size distribution was estimated using high performance size exclusion chromatography.

The mixing ratio of protein and pectin significantly influenced the protein/peptide/amino acid content of the hydrolysates (**Fig. 4.3C**). With increasing concentrations of pectin, there was more protein/peptide/amino acid in the hydrolysates. Its content significantly increased, approximately 2.6 times from 471.9 mg/g protein to 1210.3 mg/g protein when the ratio of protein and pectin increased from 1:0.1 to 1:3 (w/w). As discussed earlier, pectin could be broken down into hexuronic acids under sCW conditions; the more pectin, the higher the content of hexuronic acids in the hydrolysate (**Fig. 3.2**). Therefore, pectic acids together with H_3O^+ ions in the media could hydrolyze the protein at a higher extent i.e., they could disrupt the inter- and intramolecular linkages: H-bonds, electrostatic and hydrophobic interactions, and disulfide bonds, leading to the unfolding of its structure, eventually increasing the protein/peptide/amino acid content of the hydrolysates (Zhang et al., 2018). Another reason that could contribute to the increase in protein/peptide/amino acid content was the pH of the media which also influenced the conformational changes of protein molecules. At pH 8, which is far above the isoelectric point (~4.3) of pea protein, the electrostatic repulsion and ion hydration were enhanced, therefore, the unfolding of protein was induced (Wen et al., 2019).

4.3.3. Intrinsic fluorescence spectroscopy

Fluorescence spectroscopy is a sensitive method to monitor the conformational changes of protein tertiary structure. The fluorescence intensity of protein is largely attributed to the aromatic amino acid residues. **Fig. 4.4** shows that sCW treatment modified the conformation of pea protein concentrate mainly for phenylalaline, tyrosine, and tryptophan, which was indicated by the intensity of the emission spectra and the maximum emission wavelength (λ_{max}).

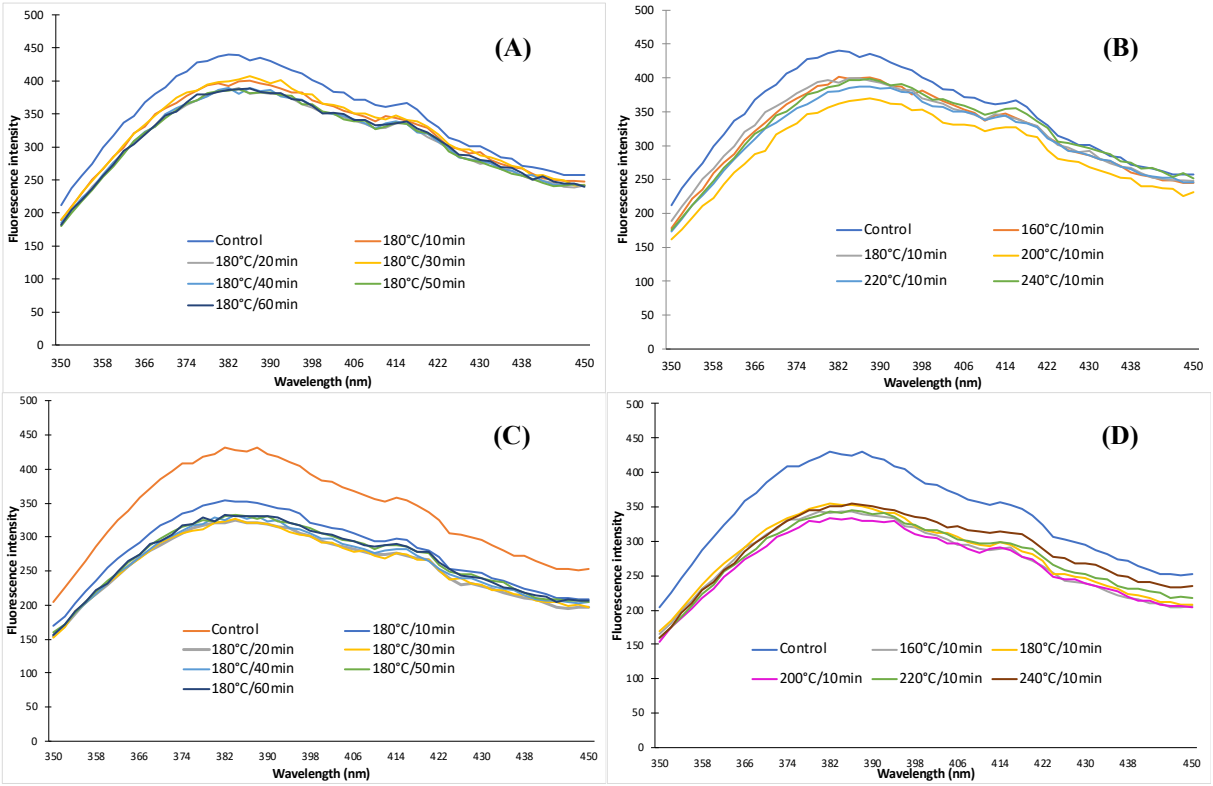


Fig. 4.4. Emission fluorescence spectra of pea protein concentrate hydrolysates prepared by sCW hydrolysis with (A, B) pectin and (C, D) citric acid.

Compared to the untreated sample, all sCW-hydrolyzed protein with pectin had appreciably lower fluorescence intensity (**Fig. 4.4A-B**). The decrease in the fluorescence intensity indicated the unfolding of the protein occurred, leading to the exposure of the interior hydrophobic clusters to the surface of the protein. Lu et al. (2016) observed similar behavior of enhancing surface hydrophobicity by hydrolyzing soy protein using sCW. Chao et al. (2018) reported the reduction in fluorescence intensity of pea protein after 6000 bar treatment. They suggested that the protein could be severely denatured and unfolded to expose the structures that were previously buried in the interior. The maximum emission wavelength is another indicator of protein

denaturation level (Schmid, 1989). Compared to the untreated sample which had λ_{max} at 382 nm, the sCW hydrolyzed proteins experienced a slight red-shifting in λ_{max} ranging from 384 nm to 388 nm. Due to the exposure of tryptophan residues to the aqueous environment. The interactions of aromatic amino acids with aqueous media could enhance the fluorescence quenching of water therefore resulting in the lower fluorescence intensity and the red shift of λ_{max}. A similar observation was reported in the study of Chao et al. (2013) where the λ_{max} of pea protein slightly shifted from 372 nm to 375-378 nm after the high pressure pretreatment (2000-6000 bar). The change of the λ_{max} from short to long wavelengths is an indication of the denaturation of protein and exposure of interior hydrophobic regions.

For the hydrolysis of pea protein concentrate with citric acid, the λ_{max} values of the hydrolyzed proteins were in the range of 382-386 nm and had slightly red-shifted compared to the untreated protein (λ_{max}=382 nm) and the fluorescence intensity was also reduced (**Fig. 4.4C-D**). Chao et al. (2018) observed that the intensity of fluorescence signal was considerably lower pH 3 than the samples at pH 5 and 7. They suggested that at the protein was excessively denatured and exposed the tryptophan residues that were previously buried in the interior at pH 3. The extensive interactions of tryptophan residues with the aqueous environment increased the fluorescence quenching of water, as a result, the fluorescence intensity was reduced. In this study, the pH of the citric acid and protein suspensions were 2.6, therefore a similar phenomenon could have occurred for the pea protein. Another possible reason is that the protein-protein interactions increased at pH 2.6 due to the isoelectric point of pea protein being 4.3. The lack of repulsive forces and the associated steric hindrance could have resulted in the reduced fluorescence intensity (Chao & Aluko, 2018).

The temperature and time treatment could reduce the fluorescence intensity and shift the λ_{max} of protein. However, there was no linear relationship observed between the fluorescence intensity and sCW treatments. Chang et al. (2022) hydrolyzed egg white protein using sCW and reported that temperatures did not correlate to the fluorescence intensity of the protein and λ_{max} of the hydrolysates remained unchanged after sCW treatment.

4.3.4. Peptide size distribution

The changes in the molecular weight of peptide chains were monitored by size exclusion chromatography shown in **Figs. 4.5-4.6**. After 40 min hydrolysis, the intensity of 4.3 kDa peak considerably increased. Also, the peak areas of 4.3 kDa at 20 min and 40 min were 1.8 and 5.5 times higher than the one obtained at 10 min, respectively. This observation agreed with the degree of hydrolysis (**Fig. 4.1A**) where the highest DH was obtained at 40 min, which is likely due to the high content of peptides in the hydrolysate. Compared to the reaction time, temperature had the most significant effect in reducing the peptides molecular mass. With increasing temperature, the better hydrolysis and the smaller peptides were produced. The peptides found at 180^{o}C had molecular masses of 4.5-4.9 kDa with relatively low intensities from 53-55 mAu. However, at a higher temperature of 220^{o}C, the peaks eluted at 30 and 31 min disappeared, instead, there was a prominent peak detected at 32 min with a considerably higher intensity of 153 mAu (**Fig. 4.5B**). The molecular mass of the identified peptides was 4.1 kDa with the estimated degree of polymerization of 30-31. As shown in the chromatograms, the intensity of peptides at 220^{o}C was approximately 3 times higher than the one obtained at 180^{o}C. The area of 4.1 kDa peptides at 220^{o}C were 6.5 times higher than the one at 180^{o}C, suggesting that the proteolytic activity of the media was enhanced at a higher temperature. The result well-explained the increment of the degree of hydrolysis at 220^{o}C in **Fig. 4.1B** which was due to the higher content of peptides in the media.

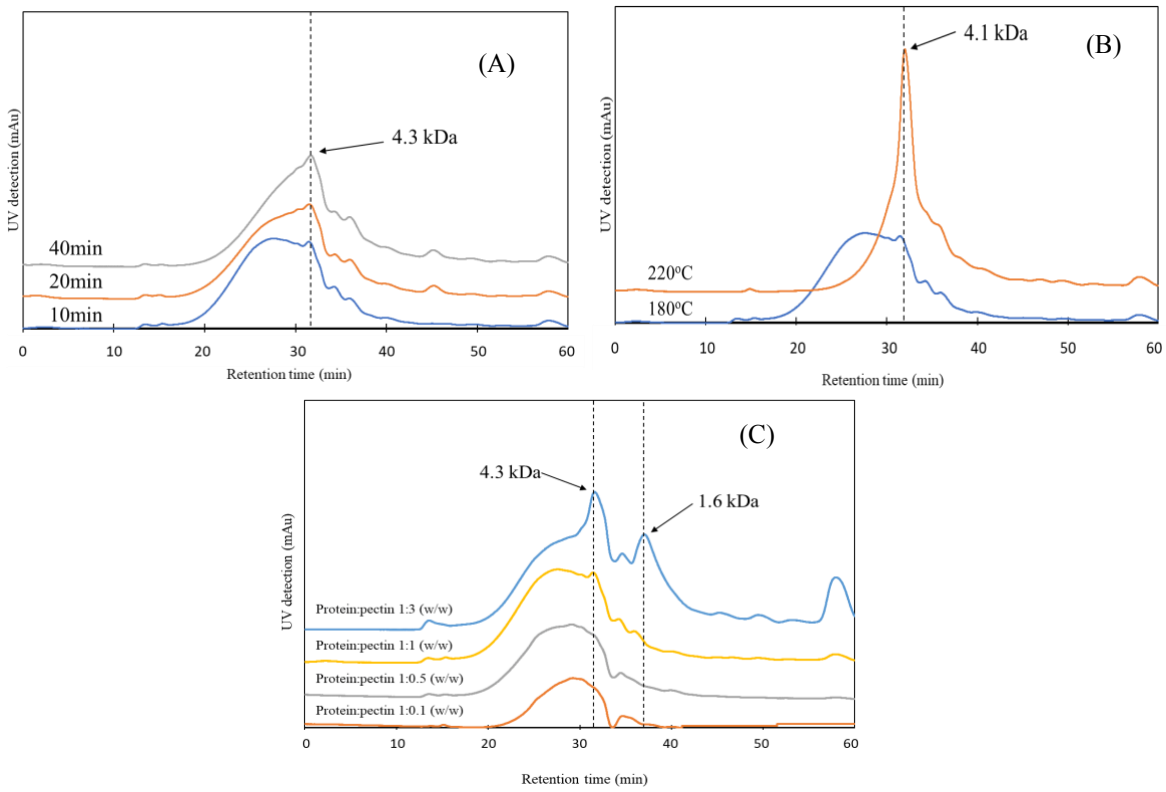


Fig. 4.5. Distribution of peptide size in the hydrolysates of protein and pectin at (A) protein:pectin 1:1 (w/w)/180°C, (B) protein:pectin 1:1 (w/w)/10 min, and (C) 180°C/10 min.

The mixing ratios of pectin and protein also showed a noticeable effect on the hydrolysis of protein. At the ratios of 1:0.1 and 1:0.5 protein:pectin (w/w), there were no considerable changes in the peptides profile of the hydrolysates, the major peak was eluted at approximately 29 min corresponding to the MW of 6.3-6.5 kDa (**Fig. A8**). However, at the ratios of 1:1 and 1:3 protein:pectin (w/w), the smaller peptides gradually appeared with the retention times of 30-37 min which correlated to the MW of 4.3-1.6 kDa, respectively (**Fig 4.5C**). Wang et al. (2019) observed a similar result after hydrolyzing soy protein with sCW. At 120°C, the MW of the peptides decreased to less than 75 kDa and the percentage of aggregated protein particles was reduced. They suggested that the disulfide bonds were broken under sCW treatment, limiting the formation of aggregated particles.

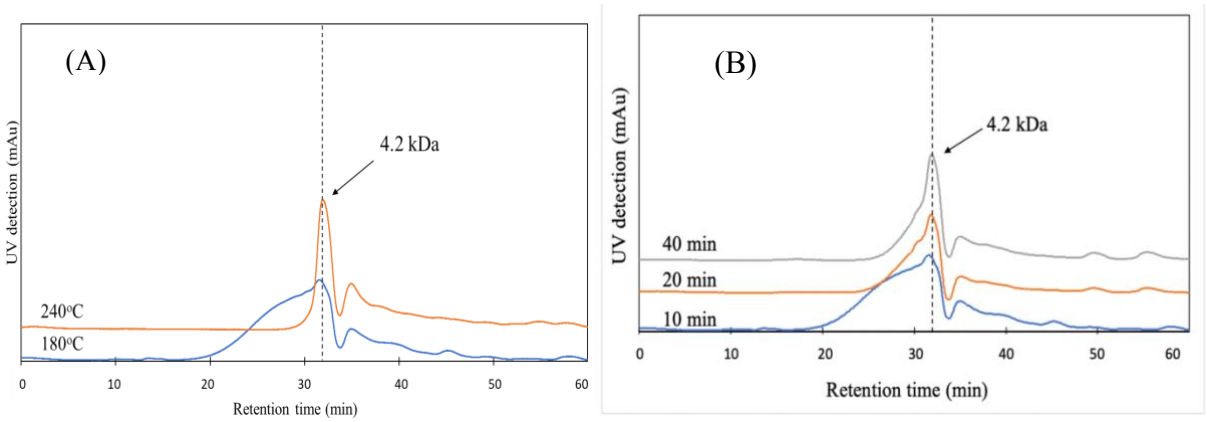


Fig. 4.6. Size exclusion chromatograms of pea protein hydrolysates with citric acid at (A) 10 min and (B) 180°C.

Regarding the hydrolysis of pea protein with citric acid (**Fig. 4.6**), the molecular weight distributions had a relatively similar elution pattern. The prominent peak eluted at ~32 min which had a MW of 4.2 kDa. The peak's intensity gradually increased and reached a maximum of 72 mAu at 240°C/10 min (**Fig 4.6A**). The reaction time did not greatly affect the intensity of the ~32 min peak, there was only a slight increase in its intensity from 49 to 56 mAu at the reaction time of 20 and 40 min, respectively.

4.3.5. Amino acid profile

The amino acid profiles of pea protein and pectin hydrolysates are reported in **Table 4.2.** The dominant amino acid was glutamic acid (43.12g/100g protein at 240°C). This agreed with the data reported by Gorissen et al. (2018) that glutamic acid is the most abundant amino acid (12.9g/100g) in pea flour. Most of the essential amino acids were degraded at temperatures higher than 180°C, especially threonine, arginine, and serine were undetectable at 240°C. Hao et al. (2019) reported that threonine could not be found in the hydrolysates of abalone viscera extract at 230°C. On the other hand, the hydrophobic amino acids had increasing trends at higher sCW temperatures, particularly, hydrophobic amino acids contents were 33.08, 35.93, and 42.08 g/100g at 160°C, 180°C, and 220°C, respectively. Among which Met/Val, Phe, and Ala had the highest contents at

240^{o}C (5.36, 8.85, and 12.74 g/100g protein, respectively). Similarly, Domenico-Ziero et al. (2022) that the content of hydrophobic amino acids (Ile, Phe, Trp) were higher at elevated temperatures. It suggested that the hydrophobic characteristic of the hydrolysates was enhanced by the high temperatures of sCW which related to the exposure of hydrophobic amino acids in the interior of protein molecules to the surface as it was previously proposed by the fluorescence intensity in **Fig. 4.4A-B**. The dielectric constant of sCW decreases at increasing temperature which favors the dissolution of non-polar molecules. Hydrophobic amino acids normally possess non-polar side chains which solubility is considerably enhanced at high temperature (Domenico-Ziero et al., 2022).

Table 4.2. Total amino acid composition of the protein and pectin hydrolysates.

Amino acids (g/100g protein)	180°C/10min			Protein:pectin 1:1 (w/w), 10 min				Protein:pectin 1:1 (w/w), 180°C		
	Protein:pectin 1:0.1 (w/w)	Protein:pectin 1:1 (w/w)	Protein:pectin 1:3 (w/w)	160°C	180°C	220°C	240°C	10min	40min	60min
Asp	15.67	15.7	15.05	15.43	15.7	5.27	1.7	15.7	14.03	12.73
Glu	24.65	26.24	24.36	23.39	26.24	31.62	43.12	26.24	26.95	27.72
Ser	2.33	2.56	4.94	5	2.56	0.44	0	2.56	1.93	2
His	3.09	3.38	3.03	2.9	3.38	3.15	2.5	3.38	3.25	3.08
Gly	7.79	7.5	6.83	6.37	7.5	9.73	9.26	7.5	7.38	8.46
Thr	1.03	1.34	3.84	3.55	1.34	0	0	1.34	1.18	1.07
Arg	3.7	1.32	1.06	2.37	1.32	0	0	1.32	0.99	0.69
Ala	6.91	7.79	6.93	6.41	7.79	10.39	12.74	7.79	8.14	8.33
Tyr	4.11	4.37	5.1	5.56	4.37	5.14	5.37	4.37	4.94	4.37
Met/Val	4.2	4	4.17	3.93	4	4.88	5.36	4	4.53	4.3
Phe	6.82	7.09	6.58	6.47	7.09	7.9	8.85	7.09	7.03	7.12
Ile	6.25	6.54	6.32	6.16	6.54	6.33	5.29	6.54	6.63	6.78
Leu	10.48	10.51	10.33	10.11	10.51	12.58	2.75	10.51	11.08	11.1
Lys	2.97	1.65	1.46	2.36	1.65	2.55	3.07	1.65	1.95	2.24
Basic[b]	9.76	6.35	5.55	7.63	6.35	5.7	5.57	6.35	6.19	6.01
Uncharged polar[c]	15.26	15.77	20.71	20.48	15.77	15.31	14.63	15.77	15.43	15.9
Hydrophobic[d]	34.66	35.93	34.33	33.08	35.93	42.08	34.99	35.93	37.41	37.63
Hydrophilic[e]	50.08	48.29	44.96	46.45	48.29	42.59	50.39	48.29	47.17	46.46

[a] Asp: aspartic acid, Glu: glutamic acid. [b] His: histidine, Arg: arginine, Lys: lysine. [c] Ser: serine, Gly: glycine, Thr: threonine, Tyr: tyrosine. [d] Ala: alanine, Met: methionine/Val: valine, Phe: phenylalaline, Ile: isoleucine, Leu: leucine. [e] Asp, Glu, His, Arg, Lys.

The contents of hydrophilic amino acids were the highest compared to other amino acid categories. In general, hydrophilic amino acids increased at increasing temperatures and reached the maximum value of 50.39 g/100g protein at 240^{o}C. The exposure of hydrophilic amino acids suggested the potential to chelate the ferrous ions of pea protein hydrolysates (Phongthai et al., 2018). The reaction times again did not considerably affect the changes in amino acids profiles of pea protein hydrolysates. There was a slight increase in hydrophobic amino acids content from 35.93 to 37.63g/100g protein at 10 and 60 min of reaction times, respectively. However, Sunphorka et al. (2012) contrastingly reported the amino acid contents of rice bran protein hydrolysates significantly increased with longer time from 10 to 60 min, the possible reason was due to the differences in temperature. The authors employed sCW at 250^{o}C which was much higher than this study, 180^{o}C. Hence, under higher temperature, the amino acids content was very likely to increase.

Table 4.3 shows the amino acid profiles of pea protein and citric acid hydrolysates. Similarly, Glu was the most prominent amino acid in the hydrolysate with the maximum content of 40.05g/100g protein at 240^{o}C. Most of the amino acids were unstable at high temperature of 240^{o}C, except for the hydrophobic ones which content improved with increasing temperature and peaked at 240^{o}C with 39.52 g/100g. That explained the unfolding of protein at high temperature, which was previously indicated by the intrinsic fluorescence intensity in **Fig. 4.4C-D**. Most of the amino acid contents were not significantly influenced by the reaction times.

Table 4.3. Total amino acid composition of the protein and citric acid hydrolysates.

Amino acid (g/100g protein)	Protein:citric acid ratio of 1:0.2 (w/w), 10 min			Protein:citric acid ratio of 1:0.2 (w/w), 180°C		
	160°C	180°C	240°C	10 min	20 min	40 min
Asp	13.97	11.95	1.56	11.95	10.3	7.55
Glu	21.56	23.77	40.05	23.77	23.59	26.84
Ser	5.66	5.63	1.29	5.63	5.59	5.46
His	3.09	3.38	3.06	3.38	2.91	3.29
Gly	5.61	6.08	8.21	6.08	5.91	6.47
Thr	4.35	4.19	0.98	4.19	4	4.21
Arg	9.88	7.98	1.95	7.98	7.97	6.39
Ala	5.47	5.97	10.58	5.97	6.14	6.86
Tyr	3.01	4.03	0	4.03	5.22	2.94
Met/Val	3.11	3.35	4.72	3.35	3.73	4.06
Phe	5.65	5.88	6.65	5.88	6.12	6.43
Ile	4.92	5.28	6.08	5.28	5.64	6.18
Leu	8.63	9.09	11.49	9.09	9.45	10.07
Lys	5.07	3.42	3.38	3.42	3.44	3.26
Acidic[a]	35.53	35.72	41.61	35.72	33.89	34.39
Basic[b]	18.04	14.78	8.39	14.78	14.32	12.94
Uncharged polar[c]	18.63	19.93	10.48	19.93	20.72	19.08
Hydrophobic[d]	27.78	29.57	39.52	29.57	31.08	33.6
Hydrophilic[e]	53.57	50.50	50.00	50.50	48.21	47.33

[a] Asp: aspartic acid, Glu: glutamic acid. [b] His: histidine, Arg: arginine, Lys: lysine. [c] Ser: serine, Gly: glycine, Thr: threonine, Tyr: tyrosine. [d] Ala: alanine, Met: methionine/Val: valine, Phe: phenylalaline, Ile: isoleucine, Leu: leucine. [e] Asp, Glu, His, Arg, Lys.

4.3.6. Antioxidant activity of pea protein hydrolysates

The antioxidant activity of pea protein hydrolysates was estimated by its ability to neutralize $DPPH^-$ free radicals. Once $DPPH^-$ encounters proton-donating species, its absorbance reduces thus reflect its scavenging ability of that species. The reaction time did not significantly influence the antioxidant activity of pea protein hydrolysates with citric acid (**Fig. 4.7A**), however, all the hydrolysates possessed relatively high antioxidant activity. At least 88% of $DPPH^-$ free radicals were scavenged. On the other hand, the $DPPH^-$ scavenging activity of hydrolysates with pectin were significantly affected by the reaction time, the scavenging activity was 72.27% at 10 min, decreasing upon longer heating.

Regarding the effect of temperature, the antioxidant activity of hydrolysates obtained from protein with both citric acid and pectin gradually increased and reached the maximum values of 90.88%, and 76.95% respectively at 200^{o}C. However, higher temperatures of 220 and 240^{o}C resulted in the reduction of the scavenging activity. This could be related to the small peptide size (4.1 kDa) and the peptides composition in the hydrolysates. At lower temperatures of 160-200^{o}C, there were different peptides with various MW detected (**Figs. 4.5-4.6**). It was likely that these peptides could have encountered $DPPH^-$ radicals then transformed them to more stable products; hence, contributed to the antioxidant activity of the hydrolysates. At higher temperatures of 220 and 240^{o}C, the degradation of high MW peptides was clearly observed (**Fig. 4.5**) where there was only one dominant peptide with MW of ~4.1 kDa detected which probably had lower antioxidant activity than the ones at 200^{o}C. Consequently, the antioxidant activity of the hydrolysates at 220 and 240^{o}C was reduced. Another plausible reason is related to the content of aromatic amino acids (Tyr, Phe, Trp) that can donate hydrogen atoms from the hydroxyl groups to neutralize the free radicals (Phongthai et al., 2018). As indicated in **Table 4.2**, the contents of aromatic amino acids

(Tyr and Phe) considerably decreased from 9.91 to 6.65 g/100g protein at 180 and 240^{o}C, respectively; therefore, that may affect the $DPPH^{-}$ scavenging activity of the hydrolysates. Compared to citric acid hydrolysates, the protein and pectin hydrolysates showed a lower scavenging activity against $DPPH^{-}$ indicated in **Fig. 4.7A-B.** This potentially demonstrated that pectin and citric acid catalyzed pea protein hydrolysis reaction in different patterns, thus different peptides were formed, leading to the differences in $DPPH^{-}$ scavenging activity. It is noteworthy to mention that the antioxidant activity seems to be inversely proportional to the degree of hydrolysis of pea protein. The higher DH (**Fig 4.1B**), the lower antioxidant activity (**Fig 4.7B**). A similar observation was reported in the study of Klompong et al. (2007) where with increasing the DH of yellow stripe trevally animal protein from 5 to 25%, the DPPH scavenging activity was reduced from approximately 97% to 78%, respectively.

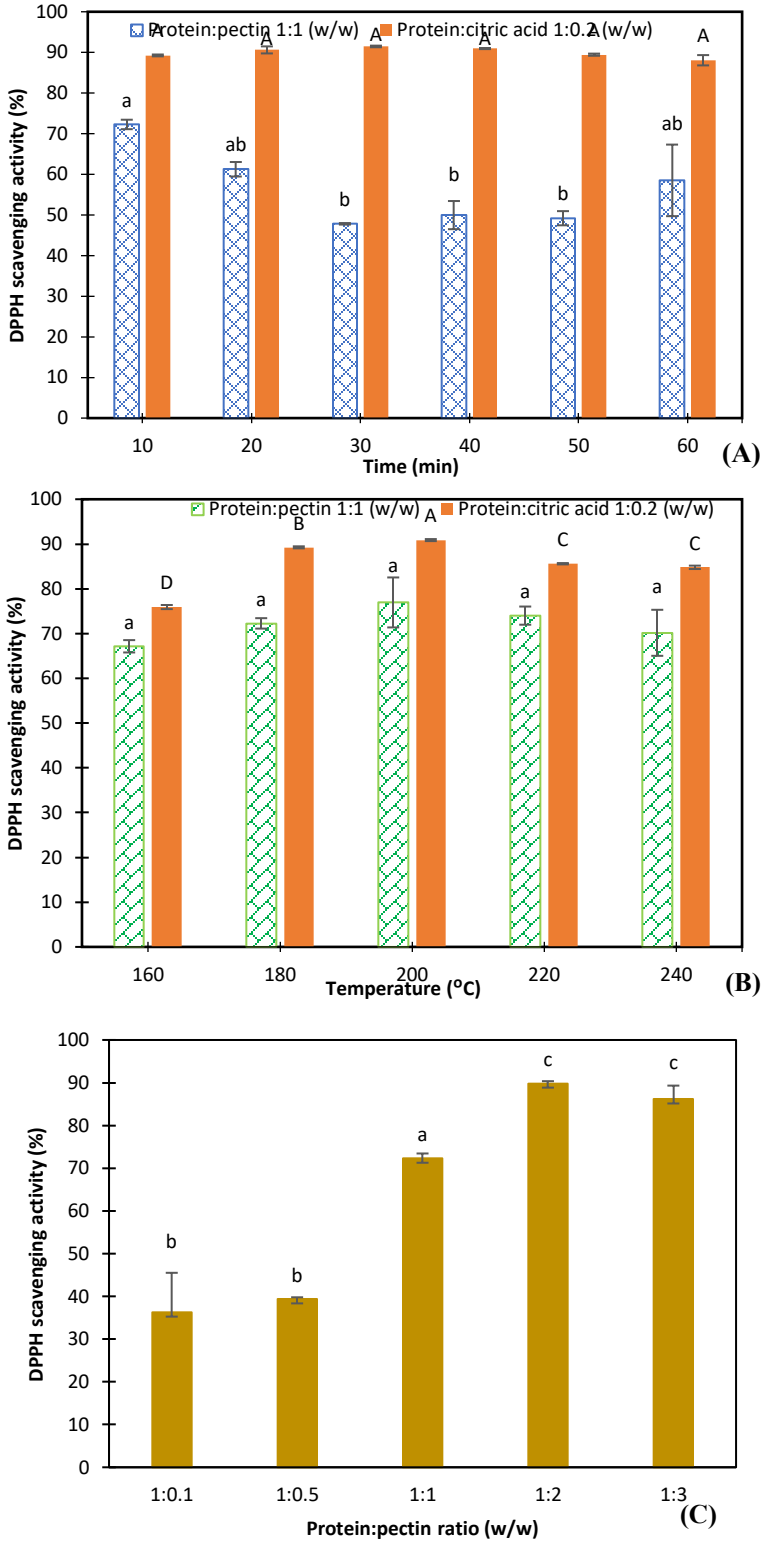


Fig. 4.7. DPPH$^{\cdot}$ radicals scavenging activity of pea protein hydrolysates with pectin 1:1 (w/w) and citric acid 1:0.2 (w/w) at: (A) 10 min, (B) 180ºC and (C) 180ºC/10 min. The averages that do not share the same letter are statistically different.

Gomes & Kurozawa (2020) investigated the antioxidant capacity of rice protein hydrolyzed by alcalase and flavourzyme. They reported that with increasing degree of hydrolysis, the DPPH scavenging activity increased as the smaller peptides are more effective to engage with $DPPH^{\cdot}$ radicals, consequently increased the antioxidant activity of the protein hydrolysates. However, the data reported in this study did not agree with those findings. The degree of hydrolysis at 200°C and 220°C were found to be 25.1% and 64.8% respectively; however, the antioxidant activity at 220°C was significantly lower than the one at 200°C. Gomes & Kurozawa (2020) only evaluated the hydrolysates at the DH from 1 to 10% of rice protein. Meanwhile, in this study, we obtained hydrolysates with appreciably higher degree of hydrolysis from 13% to 65%. Therefore, the antioxidant activity is not proportionally related to the degree of hydrolysis but likely depends on the peptide size and its composition.

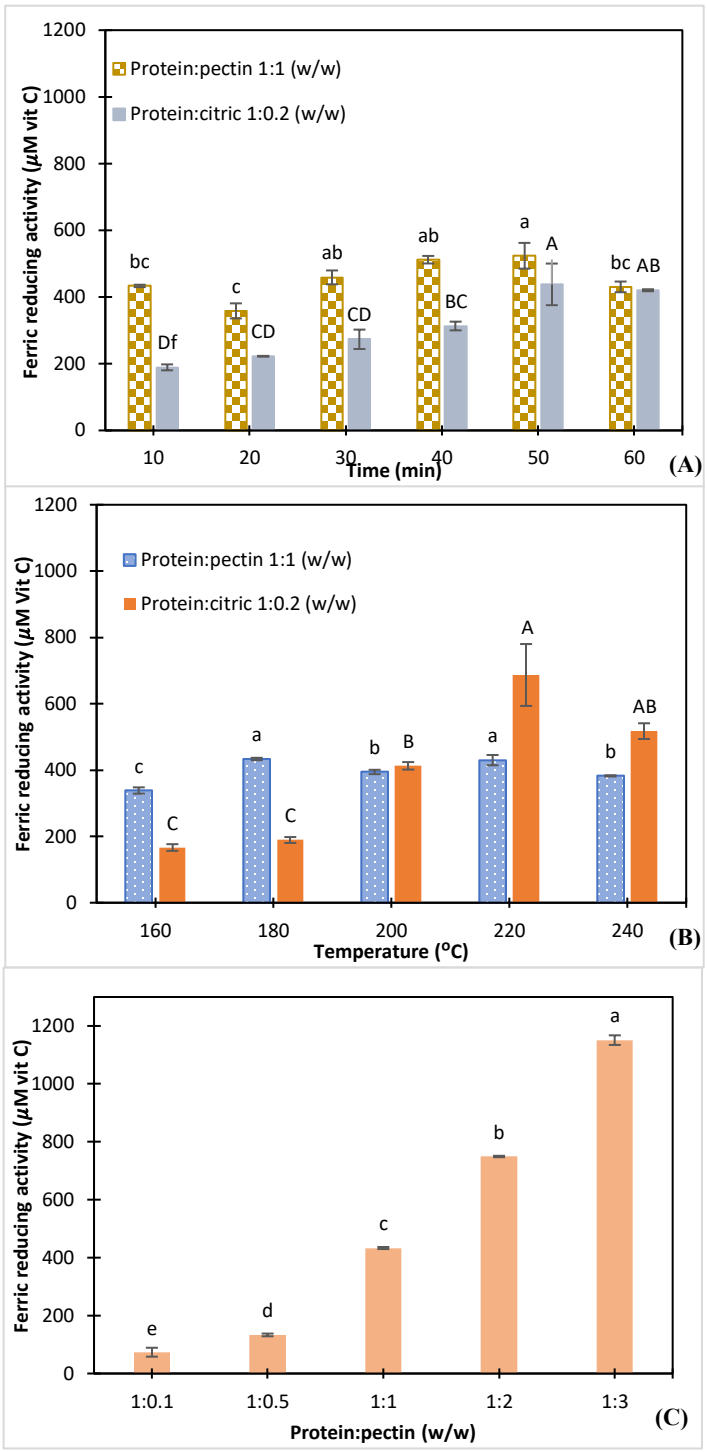


Fig. 4.8. Ferric reducing antioxidant power of the protein:pectin (1:1, w/w) hydrolysates and protein:citric acid (1:0.2, w/w) at: (A) 10 min, (B) 180ºC, and (C) 180ºC/10 min. The samples that do not share the same letter are statistically different.

The ferric reducing activity measures the ability of a substance to donate the electrons which reduce the ferrous ions in TPTZ solution. Similar to the $DPPH^-$ scavenging activity, the reaction time did not have significant effect on ferric reducing activity of pea protein hydrolysates with both citric acid and pectin, as shown in **Fig 4.8A.** However, it clearly showed that the hydrolysates with pectin had significantly stronger ferric reducing activity than the ones obtained with citric acid. Interestingly, the degree of hydrolysis of the hydrolysates with pectin were, as well, significantly higher than the ones obtained with citric acid (**Fig 4.1A**). Therefore, it was very likely that, at higher DH, the smaller MW peptides readily interacted with the ferrous ions leading to the higher ferric reducing activity. Moreover, due to the cleavage of peptide bonds, the small MW peptides had additional source of protons and electrons in the specific side chain which helped to effectively reduce the ferrous ions (Centenaro et al., 2011). A similar observation was reported in the study of Gomes & Kurozawa (2020) who mentioned that at higher degree of hydrolysis, the ferric reducing activity of rice protein hydrolysates were significantly increased. The highest activity was 957 μmol TE obtained at DH 10% using alcalase enzyme for the hydrolysis.

The effect of temperature on the ferric reducing activity was shown in **Fig 4.8B.** At the temperatures lower than 200^{o}C, the protein hydrolysates with pectin had significantly higher ferric reducing activity than the ones with citric acid. However, at 220^{o}C, there was a significantly increase in the ferric reducing activity of hydrolysates with citric acid and the highest value was 686.7 μM obtained at 220^{o}C/ 10 min. Upon further heating up to 240^{o}C, the ferric reducing activity was significantly reduced to 517.3 μM. The mixing ratio of protein and pectin had significant influence on the activity of the hydrolysates, as shown in **Fig 4.8C.** With increasing concentration of pectin, the activity significantly increased and reached the highest value of 1151.1 μM at protein:pectin 1:3 (w/w). The degree of hydrolysis, as shown in **Fig 4.1C,** significantly improved

with increasing pectin concentration. As mentioned earlier, at higher DH, smaller MW peptides was released which could easily align with the ferrous ions and donate the electrons to reduce them, leading to the improvement in the ferric reducing activity.

4.4. Conclusions

For the first time, pea protein concentrate was hydrolyzed by sCW with citrus pectin and citric acid as the additives. The highest DH was found to be 64.8% at 200^{o}C/50 bar/10 min, and the protein:pectin ratio of 1:1 (w/w). The hydrolysates with citric acid had significantly lower DH, the highest value was 34.66% at 240^{o}C/50 bar/10 min and protein:citric acid ratio of 1:0.2 (w/w). The effect of temperature was more pronounced than the reaction time in terms of influencing the degree of hydrolysis. With increasing DH, the protein content of the hydrolysates increased due to the breakdown of protein molecules into smaller peptides with MW of 6.5-1.6 kDa. Among them, the most prominent peptide was 4.1 kDa (with the estimated degree of polymerization 30-31) that eluted at ~32 min. All the protein hydrolysates experienced the unfolding and the exposure of hydrophobic clusters as indicated by the fluorescence intensity and the λ_{max} values. The amino acid profiles showed that hydrophilic amino acids were dominant in the hydrolysates, which accounted for at least 50% of total amino acids. The $DPPH^{-}$ scavenging activity of hydrolysates with citric acid were appreciably higher than the ones obtained with pectin. This indicated that pectin and citric acid could assist the hydrolysis of pea protein concentrate in different mechanisms. Overall, sCW is a green technology that has great potential to produce small MW peptides from pea protein concentrate, moreover, the selectivity of sCW can be adjusted to generate hydrolysate that has high DH with high ferric reducing activity or moderate DH with high $DPPH^{-}$ scavenging activity by adding either citrus pectin or citric acid, respectively to the media. The experimental scheme of pea protein hydrolysis is shown in **Fig. 4.9.**

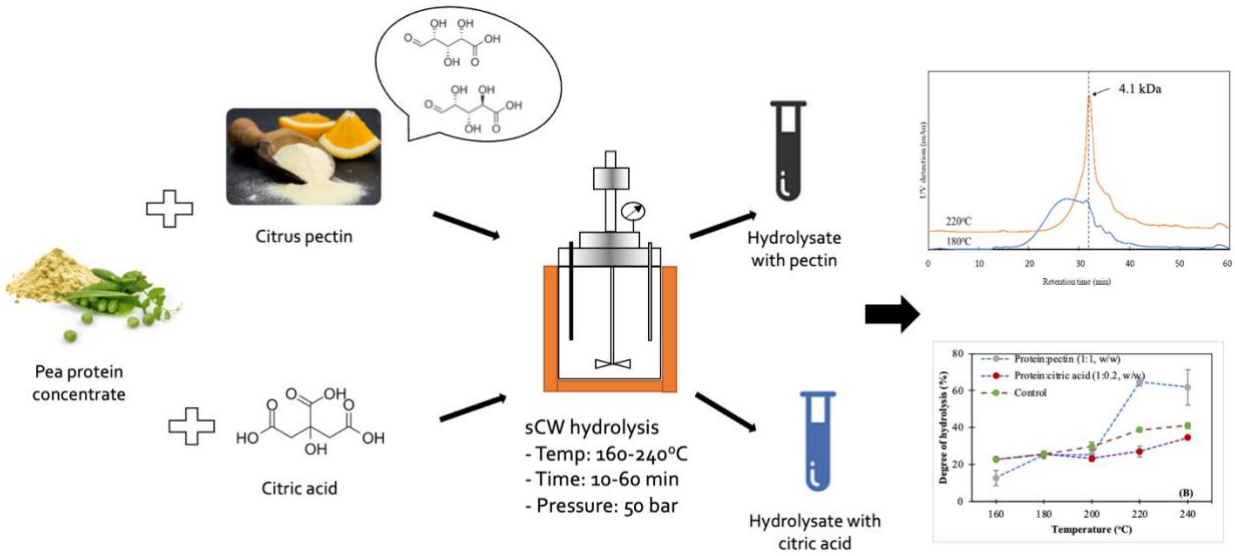


Fig 4.9. Schematic representation of pea protein concentrate hydrolysis with citrus pectin and citric acid as catalysts.

Chapter 5: Conclusions and Recommendations

5.1. Conclusions

In this thesis, bioactive oligosaccharides and bioactive peptides were produced from the hydrolysis of citrus pectin and pea protein concentrate, respectively, using subcritical water technology.

In the first study, citrus pectin was hydrolyzed in sCW media modified by either 0.2% (w/w) malic acid or 0.2% (w/w) citric acid. The hexuronic acids content of the pectic hydrolysates was significantly enhanced by increasing temperature. At temperatures above 145ºC, the concentration of ionic species (H_3O^+ and OH^-) improved which catalyzed the hydrolysis at a higher extent, leading to the increase in hexuronic acid content up to 0.712g HexA/g pectin at 200ºC/50 bar/10 min. The hexuronic acid content obtained in this study was significantly higher than the reported values of the hydrolysates obtained by subcritical water hydrolysis of apple pectin (0.079g/g pectin), apple pomace (0.12 g/g pectin), and passion fruit pectin (0.11 g/g pectin). Besides hexuronic acids, the content of rhamnose also improved at increasing temperatures, which had the highest value of 0.075g/g pectin at 180ºC/50 bar/10 min in the aqueous citric acid media. The rhamnose content in this study was significantly higher than the content obtained by subcritical water hydrolysis of lemon peel pectin (0.058g/g pectin) but lower than the hydrolysate of *Enteromorphan prolifera* macroalgae (0.42 g/g algae) and soybean rhamnogalacturonan (0.113g/g RG). The malic acid hydrolysates had a significantly higher rhamnose content (0.082g/g pectin) than the ones obtained with citric acid (0.075g/g pectin) at 180ºC/50 bar/10 min. The stronger catalytic effect of malic acid could be due to the pK_a values of 3.4 and 5.1 which were lower than pK_a values of citric acid (3.1, 4.7, and 6.4). The lower pK_a, the more readily to donate hydrogen atom, hence, malic acid could catalyze the hydrolysis reaction at a higher extent. Rhamnose is one of the constituents of the backbone of rhamnogalacturonan (RG) regions. The more rhamnose in

the hydrolysate, the more RG was hydrolyzed, releasing the oligosaccharides attached to the RG's backbone. Therefore, oligosaccharides were determined using the hydrophilic interaction liquid chromatography. The dominant oligosaccharide was arabinooligosaccharide eluted at 6.8 min with the highest content at 160°C/50 bar/20 min and a molecular weight of the oligosaccharide was estimated at approx. 2.65 kDa with DP17-DP18. In addition, arabinose was also detected in the hydrolysate which had the highest content of 1.55mg/g pectin at 160°C/50 bar/20 min. The functionality of the pectic hydrolysates was determined based on the $DPPH^-$ scavenging activity and ferric reducing antioxidant power (FRAP) methods. The $DPPH^-$ scavenging activity improved with increasing temperature which had the highest activity of 90.6% at 160°C/50 bar/10 min. Similarly, the ferric reducing power also increased at elevated temperatures and reached the maximum value of 1515 μM at 170°C/50 bar/10 min. The $DPPH^-$ scavenging activity of malic acid hydrolysates (92.6%) was significantly higher than the ones obtained with citric acid (90.6%) at 160°C/50 bar/10 min. But the citric acid hydrolysates (993.5 μM) had higher ferric reducing power than the ones obtained with malic acid (798.1 μM) at 160°C/50 bar/10 min. This behavior might be because malic acid had random catalytic pattern, implying that the side chain and the backbone could be hydrolyzed simultaneously. On the other hand, citric acid catalyzed the pectin hydrolysis in a stepwise pattern, meaning that the side chain was broken down prior to the backbone. Due to the different catalytic patterns, the compositions of hydrolysates obtained in malic and citric acid media were different, leading to variations in their antioxidant activities.

In the second study, due to the release of hexuronic acids from pectin hydrolysis, pectin was used as the catalyst for pea protein hydrolysis to obtain bioactive peptides. Pea protein concentrate was hydrolyzed using subcritical water with citrus pectin and citric acid as catalysts. Regardless of the catalysts used, the conformation of pea protein after subcritical water processing unfolded as

indicated by the reduction of the fluorescence intensity and the red shift of λ_{max} from 382 to 388 nm. The unfolded protein was severely denatured as determined by the protein content. The total protein/peptide/amino acid content of the hydrolysates increased up to 799.9 mg/g protein at 220°C/50 bar/10 min with a protein:pectin ratio of 1:1 (w/w). The reaction time also played a significant effect on the protein content which was enhanced as the time increased from 10 to 40 min, reaching the highest value of 903.1 mg/g protein at 180°C/50 bar/40 min. After the subcritical water hydrolysis, the structure of globular protein unfolded, leading to the exposure of peptide bonds which were buried in the molecule interior. Therefore, the higher amount of peptide bond in the hydrolysates, the higher protein content. The use of citric acid hydrolysis, on the other hand, had a significantly lower protein content of 543.9 mg/g protein obtained at 180°C/50 bar/40 min. The protein degree of hydrolysis (DH) agreed with the protein content, that also increased at high temperatures. The hydrolysates with pectin had significantly higher DH than the ones obtained with citric acid. At 220°C/50 bar/10 min, the highest DH was 64.7% obtained with a protein:pectin ratio of 1:1 (w/w), while the DH of citric acid hydrolysate was only 27.2%. The high DH (64.8%) indicated the presence of small MW peptides in the hydrolysates. The peptides were detected in the range of 6.5-1.6 kDa, among them, a peptide with MW of 4.1 kDa was the most prominent obtained at 220°C/50 bar/10 min with a protein:pectin ratio of 1:1 (w/w). The hydrophilic amino acids were the dominant type in the hydrolysates, with the highest contents of 50.39 and 53.57g/100g obtained at 240°C/50 bar/10 min and 160°C/50 bar/10 min in the pectin and citric acid hydrolysates, respectively. Interestingly, the content of hydrophobic amino acids significantly increased from 33.08 to 42.08 g/100g as the temperature increased from 160 to 220°C. As known, the dielectric constant of water reduces at higher temperature, which favors the dissolution of non-polar side chains of the hydrophobic amino acids, leading to the higher content of hydrophobic

amino acids at high temperatures. The functionality of pea protein hydrolysates was determined based on the ability of scavenge $DPPH^-$ free radicals and reduce ferric ions. The citric acid hydrolysates had significantly higher $DPPH^-$ scavenging activity than the ones obtained with pectin. Meanwhile, the pectin hydrolysates had higher ferric reducing activity than the citric acid ones, indicating that the catalytic patterns of pectin and citric acid were different, leading to the differences in the composition of the hydrolysates, hence, difference in the antioxidant activity. Therefore, depending on the application, the type of catalyst can tailor to either obtain hydrolysates with high degree of hydrolysis and high proton donating activity or moderate degree of hydrolysis with high electron donating activity.

5.2. Recommendations and challenges

5.2.1. Recommendations

To further advance this research, there are some suggestions for future work.

For the first study:

- Ultrafiltration of pectic hydrolysates should be conducted with the molecular weight membrane cut-off of 3 kDa as the hydrolysates have 2.65 kDa oligosaccharides to obtain the oligosaccharides in the retentate and rhamnose in the permeate.
- The retentate stream can then be freeze-dried or spray-dried to obtain oligosaccharide powder that can be directly used as fiber supplements or as fortifying agent to improve fiber content in food products.
- The prebiotic effect of the obtained oligosaccharides should be tested *in vitro* using the gastrointestinal microorganisms such as *Bifidobacterium bifidum, Lactobacillus spp., Streptococcus spp.,* and *Escherichia spp. In vivo* evaluation can be eventually conducted with rats and human subjects.

- In this study, the effect of dicarboxylic (malic acid) and tricarboxylic acid (citric acid) on pectin hydrolysis was evaluated. Further study can employ mono- or tetra-carboxylic acids such as acetic acid and pyromellitic acid, respectively to understand their catalytic patterns in the hydrolysis reaction. The pKa values of pyromellitic acid are 1.92, 2.87, 4.49, and 5.63 which, while the pKa value of acetic acid is 4.76. Due to having lower pKa values, pyromellitic acid is expected to have higher hydrolytic potential than acetic acid.

For the second study:

- Ultrafiltration should be performed with a molecular weight membrane cut-off of 5 kDa due to the presence of 4.1 kDa peptides in the hydrolysates to isolate the bioactive peptides in the protein hydrolysate.
- The isolated peptides can then be freeze-dried to produce powder which can be used as protein supplements. Moreover, the angiotensin converting enzymes inhibitory effect of the isolated peptides powder should be determined and eventually tested in rats and human subjects.
- Identification of peptide sequence in the hydrolysate by tandem mass spectrometry method (MS/MS) and online database (https://web.expasy.org/peptide_cutter/) should be performed to clarify the peptide sequences that have bioactivities.
- The unfolded protein has amphiphilic property due to the exposure of the hydrophobic core to the surface, therefore, the evaluation of the techno-functionalities of the protein hydrolysate such as emulsifying ability, foaming capacity, and encapsulating ability should be conducted.

- This study evaluated the effect of hexuronic acids on protein hydrolysis. Therefore, the catalytic effect of $NaHCO_3$ and CO_2 towards protein hydrolysis reaction should also be determined due to their potential of forming H_2CO_3 in subcritical water media.
- The crosslinking of bovine serum albumin in subcritical water media was reported in the literature. Therefore, crosslinking of pea protein to form gels in subcritical water media can be possible and should be evaluated. Gels obtained from pea protein can later be used as delivery systems such as hydrogel or aerogel using the supercritical CO_2 drying process.
- Subcritical water might be able to remove the beany flavor volatiles (e.g., alcohols, aldehydes, ketones) of the pea protein powder, therefore the sensorial evaluation of pea protein powder should be performed. Moreover, different techniques for beany flavor removal such as supercritical CO_2 extraction and deep eutectic solvents extraction should be conducted for comparison purposes.

5.2.2. Challenges

Throughout my research, there were some challenges that I have successfully solved.

For the process:

- The process involved high temperatures and high pressures; therefore, a high level of attention was needed to safely operate the high pressure-temperature system and accurately duplicate the experiments.
- The system was pressurized using the N_2 tank pressure, therefore, it was necessary to frequently check the tank's pressure to avoid any insufficient supply of N_2.

For the analyses:

- Spectrophotometry method was employed throughout the research, therefore, calibrating before each measurement was required to obtain accurate values.

- High performance liquid chromatography (HPLC) analysis also used for quantification of compounds of interest (e.g., rhamnose, oligosaccharides, amino acids, etc.), therefore, additional time was needed to learn the use of HPLC.
- During the pandemic, the chemical orders were delayed, hence, postponed the progress of some analysis.

References

Abdelmoez, W., & Abdelfatah, R. (2017). Therapeutic Compounds From Plants Using Subcritical Water Technology. In *Water Extraction of Bioactive Compounds: From Plants to Drug Development* (pp. 51–68). Elsevier. https://doi.org/10.1016/B978-0-12-809380-1.00002-4

Abdelmoez, W., & Yoshida, H. (2013). Production of amino and organic acids from protein using sub-critical water technology. *International Journal of Chemical Reactor Engineering*, *11*(1), 369–384. https://doi.org/10.1515/IJCRE-2013-0017/MACHINEREADABLECITATION/RIS

Acquah, C., Zhang, Y., Dubé, M. A., & Udenigwe, C. C. (2020). Formation and characterization of protein-based films from yellow pea (Pisum sativum) protein isolate and concentrate for edible applications. *Current Research in Food Science*, *2*, 61–69. https://doi.org/10.1016/J.CRFS.2019.11.008

Ahmed, R., & Chun, B. S. (2018). Subcritical water hydrolysis for the production of bioactive peptides from tuna skin collagen. *The Journal of Supercritical Fluids*, *141*, 88–96. https://doi.org/10.1016/J.SUPFLU.2018.03.006

Alenezi, R., Baig, M., Wang, J., Santos, R., & Leeke, G. A. (2009). Continuous Flow Hydrolysis of Sunflower Oil for Biodiesel. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects* , *32*(5), 460-468. https://doi.org/10.1080/15567030802612341

Amnuaycheewa, P., Hengaroonprasan, R., Rattanaporn, K., Kirdponpattara, S., Cheenkachorn, K., & Sriariyanun, M. (2016). Enhancing enzymatic hydrolysis and biogas production from rice straw by pretreatment with organic acids. *Industrial Crops and Products*, *87*, 247–254. https://doi.org/10.1016/J.INDCROP.2016.04.069

Arnaut, L., Formosinho, S., & Burrows, H. (2007). Elementary Reactions in Solution. In *Chemical Kinetics* (pp. 223–250). Elsevier. https://doi.org/10.1016/B978-044452186-6/50009-2

Awosika, T., & Aluko, R. E. (2019). Enzymatic pea protein hydrolysates are active trypsin and chymotrypsin inhibitors. *Foods*, *8*(6), 200. https://doi.org/10.3390/foods8060200

Awosika, T. O., & Aluko, R. E. (2019). Inhibition of the *in vitro* activities of α-amylase, α-glucosidase and pancreatic lipase by yellow field pea (*Pisum sativum* L.) protein hydrolysates. *International Journal of Food Science & Technology*, *54*(6), 2021–2034. https://doi.org/10.1111/ijfs.14087

Babbar, N., Baldassarre, S., Maesen, M., Prandi, B., Dejonghe, W., Sforza, S., & Elst, K. (2016). Enzymatic production of pectic oligosaccharides from onion skins. *Carbohydrate Polymers*, *146*, 245–252. https://doi.org/10.1016/j.carbpol.2016.03.011

Babbar, N., Dejonghe, W., Gatti, M., Sforza, S., & Elst, K. (2015). Pectic oligosaccharides from agricultural by-products: production, characterization and health benefits. *Critical Reviews in Biotechnology*, *36*(4), 594-606. https://doi.org/10.3109/07388551.2014.996732

Barba, F. J., Zhu, Z., Koubaa, M., Sant'Ana, A. S., & Orlien, V. (2016). Green alternative methods for the extraction of antioxidant bioactive compounds from winery wastes and by-products: A review. *Trends in Food Science & Technology*, *49*, 96–109. https://doi.org/10.1016/J.TIFS.2016.01.006

Barbana, C., & Boye, J. I. (2010). Angiotensin I-converting enzyme inhibitory activity of chickpea and pea protein hydrolysates. *Food Research International*, *43*(6), 1642–1649. https://doi.org/10.1016/j.foodres.2010.05.003

Basak, S., & Annapure, U. S. (2022). Trends in "green" and novel methods of pectin modification - A review. *Carbohydrate Polymers*, *278*, 118967. https://doi.org/10.1016/J.CARBPOL.2021.118967

Batista, G., Souza, R. B. A., Pratto, B., dos Santos-Rocha, M. S. R., & Cruz, A. J. G. (2019). Effect of severity factor on the hydrothermal pretreatment of sugarcane straw. *Bioresource Technology*, *275*, 321–327. https://doi.org/10.1016/J.BIORTECH.2018.12.073

Bélafi-Bakó, K., Eszterle, M., Kiss, K., Nemestóthy, N., & Gubicza, L. (2007). Hydrolysis of pectin by Aspergillus niger polygalacturonase in a membrane bioreactor. *Journal of Food Engineering*, *78*(2), 438–442. https://doi.org/10.1016/j.jfoodeng.2005.10.012

Benjamin, M., Stéphane, R., Gérard, V., & Pierre-Yves, P. (2017). Pressurized water extraction of isoflavones by experimental design from soybean flour and soybean protein isolate. *Food Chemistry*, *214*, 9–15. https://doi.org/10.1016/j.foodchem.2016.07.053

Benzie, I. F. F., & Strain, J. J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "'Antioxidant Power'": The FRAP Assay. *Analytical Biochemistry*, *239*(1), 70–76.

Bogahawaththa, D., Bao Chau, N. H., Trivedi, J., Dissanayake, M., & Vasiljevic, T. (2019). Impact of selected process parameters on solubility and heat stability of pea protein isolate. *LWT-Food Science and Technology*, *102*, 246–253. https://doi.org/10.1016/J.LWT.2018.12.034

Boukid, F., Rosell, C. M., & Castellari, M. (2021). Pea protein ingredients: A mainstream ingredient to (re)formulate innovative foods and beverages. *Trends in Food Science & Technology*, *110*, 729–742. https://doi.org/10.1016/J.TIFS.2021.02.040

Boye, J. I., Aksay, S., Roufik, S., Ribéreau, S., Mondor, M., Farnworth, E., & Rajamohamed, S. H. (2010). Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation techniques. *Food Research International*, *43*(2), 537–546. https://doi.org/10.1016/J.FOODRES.2009.07.021

Boye, J., Zare, F., & Pletch, A. (2010). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International*, *43*(2), 414–431. https://doi.org/10.1016/J.FOODRES.2009.09.003

Brethauer, S., & Wyman, C. E. (2010). Review: Continuous hydrolysis and fermentation for cellulosic ethanol production. *Bioresource Technology*, *101*(13), 4862–4874. https://doi.org/10.1016/J.BIORTECH.2009.11.009

Brunner, G. (2009). Near critical and supercritical water. Part I. Hydrolytic and hydrothermal processes. *The Journal of Supercritical Fluids*, *47*(3), 373–381. https://doi.org/10.1016/J.SUPFLU.2008.09.002

Centenaro, G. S., Mellado, M. S., & Prentice-Hernández, C. (2011). Antioxidant activity of protein hydrolysates of fish and chicken bones. *Advance Journal of Food Science and Technology*, *3*(4), 280–288.

Chakraborty, S., Shaik, L., & Gokhale, J. S. (2021). Subcritical Water: An Innovative Processing Technology. In *Innovative Food Processing Technologies: A Comprehensive Review* (pp. 552–566). Elsevier. https://doi.org/10.1016/B978-0-08-100596-5.22966-1

Chalamaiah, M., Yu, W., & Wu, J. (2018). Immunomodulatory and anticancer protein hydrolysates (peptides) from food proteins: A review. *Food Chemistry*, *245*, 205–222. https://doi.org/10.1016/J.FOODCHEM.2017.10.087

Chandler, K., Liotta, C. L., Eckert, C. A., & Schiraldi, D. (1998). Tuning alkylation reactions with temperature in near-critical water. *AIChE Journal*, *44*(9), 2080–2087. https://doi.org/10.1002/AIC.690440915

Chang, K., Jiang, W., & Liu, J. (2022). Effect of subcritical water treatment on the structure and foaming properties of egg white protein. *Food Hydrocolloids*, *124*. https://doi.org/10.1016/j.foodhyd.2021.107241

Chao, D., & Aluko, R. E. (2018). Modification of the structural, emulsifying, and foaming properties of an isolated pea protein by thermal pretreatment. *CYTA - Journal of Food*, *16*(1), 357–366. https://doi.org/10.1080/19476337.2017.1406536

Chao, D., He, R., Jung, S., & Aluko, R. E. (2013). Effect of pressure or temperature pretreatment of isolated pea protein on properties of the enzymatic hydrolysates. *Food Research International*, *54*(2), 1528–1534. https://doi.org/10.1016/j.foodres.2013.09.020

Chao, D., Jung, S., & Aluko, R. E. (2018). Physicochemical and functional properties of high pressure-treated isolated pea protein. *Innovative Food Science and Emerging Technologies*, *45*, 179–185. https://doi.org/10.1016/j.ifset.2017.10.014

Chen, J., Wu, S. S., Liang, R. H., Liu, W., Liu, C. M., Shuai, X. X., & Wang, Z. J. (2014). The effect of high speed shearing on disaggregation and degradation of pectin from creeping fig seeds. *Food Chemistry*, *165*, 1–8. https://doi.org/10.1016/J.FOODCHEM.2014.05.096

Choi, J. W., Synytsya, A., Capek, P., Bleha, R., Pohl, R., & Park, Y. il. (2016). Structural analysis and anti-obesity effect of a pectic polysaccharide isolated from Korean mulberry fruit Oddi (Morus alba L.). *Carbohydrate Polymers*, *146*, 187–196. https://doi.org/10.1016/j.carbpol.2016.03.043

Chum, H. L., Johnson, D. K., Black, S. K., & Overend, R. P. (1990). Pretreatment-Catalyst Effects and the Combined Severity Parameter. *Applied Biochemistry and Biotechnology*, *24*(1), 1–14.

Chung, W. S. F., Meijerink, M., Zeuner, B., Holck, J., Louis, P., Meyer, A. S., Wells, J. M., Flint, H. J., & Duncan, S. H. (2017). Prebiotic potential of pectin and pectic oligosaccharides to promote anti-inflammatory commensal bacteria in the human colon. *FEMS Microbiology Ecology*, *93*(11), 127. https://doi.org/10.1093/FEMSEC/FIX127

Church, F. C., Swaisgood, H. E., Porter, D. H., & Catignani, G. L. (1983). Spectrophotometric Assay Using o-Phthaldialdehyde for Determination of Proteolysis in Milk and Isolated Milk Proteins. *Journal of Dairy Science*, *66*(6), 1219–1227. https://doi.org/10.3168/jds.S0022-0302(83)81926-2

Ciftci, D., & Saldaña, M. D. A. (2015). Hydrolysis of sweet blue lupin hull using subcritical water technology. *Bioresource Technology*, *194*, 75–82. https://doi.org/10.1016/J.BIORTECH.2015.06.146

Ciftci, D., Ubeyitogullari, A., Huerta, R. R., Ciftci, O. N., Flores, R. A., & Saldaña, M. D. A. (2017). Lupin hull cellulose nanofiber aerogel preparation by supercritical CO2 and freeze drying. *The Journal of Supercritical Fluids*, *127*, 137–145. https://doi.org/10.1016/J.SUPFLU.2017.04.002

Ciriminna, R., Fidalgo, A., Scurria, A., Ilharco, L. M., & Pagliaro, M. (2022). Pectin: New science and forthcoming applications of the most valued hydrocolloid. *Food Hydrocolloids*, *127*, 107483. https://doi.org/10.1016/J.FOODHYD.2022.107483

Combo, A. M. M., Aguedo, M., Goffin, D., Wathelet, B., & Paquot, M. (2012). Enzymatic production of pectic oligosaccharides from polygalacturonic acid with commercial pectinase preparations. *Food and Bioproducts Processing*, *90*(3), 588–596. https://doi.org/10.1016/J.FBP.2011.09.003

Cui, J., Zhao, C., Feng, L., Han, Y., Du, H., Xiao, H., & Zheng, J. (2021). Pectins from fruits: Relationships between extraction methods, structural characteristics, and functional properties. *Trends in Food Science & Technology*, *110*, 39–54. https://doi.org/10.1016/J.TIFS.2021.01.077

Das, I., & Arora, A. (2021). Kinetics and mechanistic models of solid-liquid extraction of pectin using advance green techniques- a review. *Food Hydrocolloids*, *120*, 106931. https://doi.org/10.1016/J.FOODHYD.2021.106931

de Oliveira, M. B., dos Santos, M. S. N., Pogorzelski, E. S., de Souza, V. L., Pfeifenberg, R., Vieira, J., da Silva, J. F., Draszewski, C. P., Mayer, F. D., Castilhos, F., Abaide, E. R., Zabot, G. L., & Tres, M. v. (2022). Potential of canola feedstocks for fermentable sugars production by subcritical water hydrolysis. *Biomass and Bioenergy*, *162*, 106505. https://doi.org/10.1016/j.biombioe.2022.106505

Diaz, J. v., Anthon, G. E., & Barrett, D. M. (2007). Nonenzymatic degradation of citrus pectin and pectate during prolonged heating: Effects of pH, temperature, and degree of methyl esterification. *Journal of Agricultural and Food Chemistry*, *55*(13), 5131–5136. https://doi.org/10.1021/jf0701483

Ding, J., Liang, R., Yang, Y., Sun, N., & Lin, S. (2020). Optimization of pea protein hydrolysate preparation and purification of antioxidant peptides based on an in silico analytical approach. *LWT-Food Science and Technology*, *123*, 109126. https://doi.org/10.1016/j.lwt.2020.109126

Domenico-Ziero, H., Ampese, L. C., Sganzerla, W. G., Torres-Mayanga, P. C., Timko, M. T., Mussatto, S. I., & Forster-Carneiro, T. (2022). Subcritical water hydrolysis of poultry feathers for amino acids production. *The Journal of Supercritical Fluids*, *181*, 105492. https://doi.org/10.1016/J.SUPFLU.2021.105492

Donati, I., Paoletti, S., Donati, I., & Paoletti, S. (2009). Material Properties of Alginates. In *Alginates: Biology and Applications* (Vol. 13, pp. 1–53). Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-540-92679-5_1

Dranca, F., & Oroian, M. (2018). Extraction, purification and characterization of pectin from alternative sources with potential technological applications. *Food Research International*, *113*, 327–350. https://doi.org/10.1016/J.FOODRES.2018.06.065

Eblaghi, M., Bronlund, J. E., Yedro, F. M., & Archer, R. H. (2021). Kinetics of Pectin Reactions in Apple Pomace During Hydrothermal Treatment. *Food and Bioprocess Technology*, *14*(4), 739–750. https://doi.org/10.1007/s11947-021-02603-4/Published

Ekaette, I., & Saldaña, M. D. A. (2021). Ultrasound processing of rutin in food-grade solvents: Derivative compounds, antioxidant activities and optical rotation. *Food Chemistry*, *344*, 128629. https://doi.org/10.1016/J.FOODCHEM.2020.128629

Esfahan, Z. M., Izhar, S., Ismail, M. H. S., & Yoshida, H. (2021). Subcritical water treatment of bovine serum albumin pathway to produce superabsorbent biomaterial as green technology. *Materials Today Sustainability*, *16*, 100087. https://doi.org/10.1016/j.mtsust.2021.100087

Espinoza, A. D., & Morawicki, R. O. (2012). Effect of additives on subcritical water hydrolysis of whey protein isolate. *Journal of Agricultural and Food Chemistry*, *60*(20), 5250–5256. https://doi.org/10.1021/jf300581r

Espinoza, A. D., Morawicki, R. O., & Hager, T. (2012). Hydrolysis of whey protein isolate using subcritical water. *Journal of Food Science*, *77*(1), C20–C26. https://doi.org/10.1111/J.1750-3841.2011.02462.X

Fraeye, I., Duvetter, T., Doungla, E., van Loey, A., & Hendrickx, M. (2010). Fine-tuning the properties of pectin–calcium gels by control of pectin fine structure, gel composition and environmental conditions. *Trends in Food Science & Technology*, *21*(5), 219–228. https://doi.org/10.1016/J.TIFS.2010.02.001

Galkin, A. A., & Lunin, V. v. (2005). Subcritical and supercritical water: a universal medium for chemical reactions. *Russian Chemical Reviews*, *74*(1), 21–35. https://doi.org/10.1070/RC2005v074n01ABEH001167

García-Arteaga, V., Apéstegui Guardia, M., Muranyi, I., Eisner, P., & Schweiggert-Weisz, U. (2020). Effect of enzymatic hydrolysis on molecular weight distribution, techno-functional properties and sensory perception of pea protein isolates. *Innovative Food Science & Emerging Technologies*, *65*, 102449. https://doi.org/10.1016/J.IFSET.2020.102449

Garcia-Moscoso, J. L., Teymouri, A., & Kumar, S. (2015). Kinetics of Peptides and Arginine Production from Microalgae (*Scenedesmus sp.*) by Flash Hydrolysis. *Industrial and Engineering Chemistry Research*, *54*(7), 2048–2058. https://doi.org/10.1021/IE5047279

Garna, H., Mabon, N., Nott, K., Wathelet, B., & Paquot, M. (2006). Kinetic of the hydrolysis of pectin galacturonic acid chains and quantification by ionic chromatography. *Food Chemistry*, *96*(3), 477–484. https://doi.org/10.1016/j.foodchem.2005.03.002

Girgih, A. T., Chao, D., Lin, L., He, R., Jung, S., & Aluko, R. E. (2015). Enzymatic protein hydrolysates from high pressure-pretreated isolated pea proteins have better antioxidant properties than similar hydrolysates produced from heat pretreatment. *Food Chemistry*, *188*, 510–516. https://doi.org/10.1016/j.foodchem.2015.05.024

Gomes, M. G., Gurgel, L. V. A., Baffi, M. A., & Pasquini, D. (2020). Pretreatment of sugarcane bagasse using citric acid and its use in enzymatic hydrolysis. *Renewable Energy*, *157*, 332–341. https://doi.org/10.1016/J.RENENE.2020.05.002

Gomes, M. H. G., & Kurozawa, L. E. (2020). Improvement of the functional and antioxidant properties of rice protein by enzymatic hydrolysis for the microencapsulation of linseed oil. *Journal of Food Engineering*, *267*. https://doi.org/10.1016/j.jfoodeng.2019.109761

Gómez, B., Gullón, B., Remoroza, C., Schols, H. A., Parajó, J. C., & Alonso, J. L. (2014). Purification, characterization, and prebiotic properties of pectic oligosaccharides from orange peel wastes. *Journal of Agricultural and Food Chemistry*, *62*(40), 9769–9782. https://doi.org/10.1021/JF503475B/ASSET/IMAGES/MEDIUM/JF-2014-03475B_0009.GIF

Gómez, B., Gullón, B., Yáñez, R., Parajó, J. C., & Alonso, J. L. (2013). Pectic oligosacharides from lemon peel wastes: Production, purification, and chemical characterization. *Journal of Agricultural and Food Chemistry*, *61*(42), 10043–10053. https://doi.org/10.1021/jf402559p

Gómez, B., Gullón, B., Yáñez, R., Schols, H., & Alonso, J. L. (2016). Prebiotic potential of pectins and pectic oligosaccharides derived from lemon peel wastes and sugar beet pulp: A comparative evaluation. *Journal of Functional Foods*, *20*, 108–121. https://doi.org/10.1016/j.jff.2015.10.029

González-Pérez, S., & Arellano, J. B. (2009). Vegetable protein isolates. In *Handbook of Hydrocolloids: Second Edition* (pp. 383–419). Woodhead Publishing. https://doi.org/10.1533/9781845695873.383

Gorissen, S. H. M., Crombag, J. J. R., Senden, J. M. G., Waterval, W. A. H., Bierau, J., Verdijk, L. B., & van Loon, L. J. C. (2018). Protein content and amino acid composition of commercially available plant-based protein isolates. *Amino Acids*, *50*(12), 1685–1695. https://doi.org/10.1007/s00726-018-2640-5

Gullón, B., Gómez, B., Martínez-Sabajanes, M., Yáñez, R., Parajó, J. C., & Alonso, J. L. (2013). Pectic oligosaccharides: Manufacture and functional properties. In *Trends in Food Science and Technology* (Vol. 30, Issue 2, pp. 153–161). https://doi.org/10.1016/j.tifs.2013.01.006

Guo, X., Ye, X., Sun, Y., Wu, D., Wu, N., Hu, Y., & Chen, S. (2014). Ultrasound effects on the degradation kinetics, structure, and antioxidant activity of sea cucumber fucoidan. *Journal of Agricultural and Food Chemistry*, *62*(5), 1088–1095. https://doi.org/10.1021/JF404717Y/SUPPL_FILE/JF404717Y_SI_001.PDF

Hall, F., & Liceaga, A. (2020). Effect of microwave-assisted enzymatic hydrolysis of cricket (*Gryllodes sigillatus*) protein on ACE and DPP-IV inhibition and tropomyosin-IgG binding. *Journal of Functional Foods*, *64*, 103634. https://doi.org/10.1016/J.JFF.2019.103634

Hao, G., Cao, W., Li, T., Chen, J., Zhang, J., Weng, W., Osako, K., & Ren, H. (2019). Effect of temperature on chemical properties and antioxidant activities of abalone viscera subcritical water extract. *Journal of Supercritical Fluids*, *147*, 17–23. https://doi.org/10.1016/j.supflu.2019.02.007

Ho, C. H. L., Cacace, J. E., & Mazza, G. (2007). Extraction of lignans, proteins and carbohydrates from flaxseed meal with pressurized low polarity water. *LWT - Food Science and Technology*, *40*(9), 1637–1647. https://doi.org/10.1016/j.lwt.2006.12.003

Huerta, R. R., & Saldaña, M. D. A. (2018). Pressurized fluid treatment of barley and canola straws to obtain carbohydrates and phenolics. *The Journal of Supercritical Fluids*, *141*, 12–20. https://doi.org/10.1016/J.SUPFLU.2017.11.029

Husain, A., Khan, S. A., Iram, F., Iqbal, M. A., & Asif, M. (2019). Insights into the chemistry and therapeutic potential of furanones: A versatile pharmacophore. *European Journal of Medicinal Chemistry*, *171*, 66–92. https://doi.org/10.1016/J.EJMECH.2019.03.021

Illmann, S., Davidek, T., Gouézec, E., Gouézec, G., Rytz, A., Schuchmann, H. P., & Blank, I. (2009). Generation of 4-Hydroxy-2,5-Dimethyl-3(2H)-Furanone from Rhamnose as Affected by Reaction Parameters: Experimental Design Approach. *Journal of Agricultural and Food Chemistry*, *57*(7), 2889-2895. https://doi.org/10.1021/jf803776k

Jakubczyk, A., Karaś, M., Baraniak, B., & Pietrzak, M. (2013). The impact of fermentation and in vitro digestion on formation angiotensin converting enzyme (ACE) inhibitory peptides from pea proteins. *Food Chemistry*, *141*(4), 3774–3780. https://doi.org/10.1016/J.FOODCHEM.2013.06.095

Kalinichev, A. G., & Churakov, S. v. (1999). Size and topology of molecular clusters in supercritical water: a molecular dynamics simulation. *Chemical Physics Letters*, *302*(5–6), 411–417. https://doi.org/10.1016/S0009-2614(99)00174-8

Kapoor, S., & Dharmesh, S. M. (2017). Pectic oligosaccharide from tomato exhibiting anticancer potential on a gastric cancer cell line: Structure-function relationship. *Carbohydrate Polymers*, *160*, 52–61. https://doi.org/10.1016/j.carbpol.2016.12.046

Kedare, S. B., & Singh, R. P. (2011). Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science and Technology*, *48*(4), 412-422. https://doi.org/10.1007/s13197-011-0251-1

Kellock, M., Maaheimo, H., Marjamaa, K., Rahikainen, J., Zhang, H., Holopainen-Mantila, U., Ralph, J., Tamminen, T., Felby, C., & Kruus, K. (2019). Effect of hydrothermal pretreatment severity on lignin inhibition in enzymatic hydrolysis. *Bioresource Technology*, *280*, 303–312. https://doi.org/10.1016/J.BIORTECH.2019.02.051

Khodaei, N., & Karboune, S. (2014). Enzymatic extraction of galactan-rich rhamnogalacturonan I from potato cell wall by-product. *LWT - Food Science and Technology*, *57*(1), 207–216. https://doi.org/10.1016/J.LWT.2013.12.034

Khodaei, N., & Karboune, S. (2016). Enzymatic generation of galactose-rich oligosaccharides/oligomers from potato rhamnogalacturonan I pectic polysaccharides. *Food Chemistry*, *197*, 406–414. https://doi.org/10.1016/J.FOODCHEM.2015.10.122

Khodaei, N., Karboune, S., & Orsat, V. (2016). Microwave-assisted alkaline extraction of galactan-rich rhamnogalacturonan I from potato cell wall by-product. *Food Chemistry*, *190*, 495–505. https://doi.org/10.1016/J.FOODCHEM.2015.05.082

Khuwijitjaru, P., Pokpong, A., Klinchongkon, K., & Adachi, S. (2014). Production of oligosaccharides from coconut meal by subcritical water treatment. *International Journal of Food Science and Technology*, *49*(8), 1946–1952. https://doi.org/10.1111/ijfs.12524

Klinchongkon, K., Khuwijitjaru, P., & Adachi, S. (2017). Degradation kinetics of passion fruit pectin in subcritical water. *Bioscience, Biotechnology, and Biochemistry*, *81*(4), 712–717. https://doi.org/10.1080/09168451.2016.1277941

Klinchongkon, K., Khuwijitjaru, P., & Adachi, S. (2018). Properties of subcritical water-hydrolyzed passion fruit (*Passiflora edulis*) pectin. *Food Hydrocolloids*, *74*, 72–77. https://doi.org/10.1016/j.foodhyd.2017.07.034

Klompong, V., Benjakul, S., Kantachote, D., & Shahidi, F. (2007). Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. *Food Chemistry*, *102*(4), 1317–1327. https://doi.org/10.1016/J.FOODCHEM.2006.07.016

Klost, M., & Drusch, S. (2019). Functionalisation of pea protein by tryptic hydrolysis – Characterisation of interfacial and functional properties. *Food Hydrocolloids*, *86*, 134–140. https://doi.org/10.1016/j.foodhyd.2018.03.013

Klost, M., Giménez-Ribes, G., & Drusch, S. (2020). Enzymatic hydrolysis of pea protein: Interactions and protein fractions involved in fermentation induced gels and their influence on rheological properties. *Food Hydrocolloids*, *105*, 105793. https://doi.org/10.1016/J.FOODHYD.2020.105793

Kohn, R., & Kovác, P. (1978). Dissociation constants of D-galacturonic and D-glucuronic acid and their O-methyl derivatives. *Chem. Zvesti*, *32*(4), 478–485.

Kornet, C., Venema, P., Nijsse, J., van der Linden, E., van der Goot, A. J., & Meinders, M. (2020). Yellow pea aqueous fractionation increases the specific volume fraction and viscosity of its dispersions. *Food Hydrocolloids*, *99*, 105332. https://doi.org/10.1016/J.FOODHYD.2019.105332

Kroll, J., Rawel, H., & Kröck, R. (1998). Microwave digestion of proteins. *Z Lebensm Unters Forsch A*, *207*, 202–206. www.dife.de

Kruse, A., & Dinjus, E. (2007). Hot compressed water as reaction medium and reactant: Properties and synthesis reactions. *The Journal of Supercritical Fluids*, *39*(3), 362–380. https://doi.org/10.1016/J.SUPFLU.2006.03.016

Kühnel, S., Schols, H. A., & Gruppen, H. (2011). Aiming for the complete utilization of sugar-beet pulp: Examination of the effects of mild acid and hydrothermal pretreatment followed by enzymatic digestion. *Biotechnology for Biofuels*, *4*(1), 1–14. https://doi.org/10.1186/1754-6834-4-14/FIGURES/7

Kus, N. S. (2012). Organic reactions in subcritical and supercritical water. *Tetrahedron*, *68*(4), 949–958. https://doi.org/10.1016/J.TET.2011.10.070

Lam, A. C. Y., Can Karaca, A., Tyler, R. T., & Nickerson, M. T. (2016). Pea protein isolates: Structure, extraction, and functionality. *Food Reviews International*, *34*(2), 126–147. https://doi.org/10.1080/87559129.2016.1242135

Larsen, L. R., van der Weem, J., Caspers-Weiffenbach, R., Schieber, A., & Weber, F. (2021). Effects of ultrasound on the enzymatic degradation of pectin. *Ultrasonics Sonochemistry*, *72*, 105465. https://doi.org/10.1016/J.ULTSONCH.2021.105465

Li, F., Liu, L., An, Y., He, W., Themelis, N. J., & Li, G. (2016). Hydrothermal liquefaction of three kinds of starches into reducing sugars. *Journal of Cleaner Production*, *112*, 1049–1054. https://doi.org/10.1016/J.JCLEPRO.2015.08.008

Li, H., & Aluko, R. E. (2010). Identification and inhibitory properties of multifunctional peptides from pea protein hydrolysate. *Journal of Agricultural and Food Chemistry*, *58*(21), 11471–11476. https://doi.org/10.1021/jf102538g

Li, H., Prairie, N., Udenigwe, C. C., Adebiyi, A. P., Tappia, P. S., Aukema, H. M., Jones, P. J. H., & Aluko, R. E. (2011). Blood Pressure Lowering Effect of a Pea Protein Hydrolysate in Hypertensive Rats and Humans. *Journal of Agricultural and Food Chemistry*, *59*(18), 9854–9860. https://doi.org/10.1021/jf201911p

Li, J., Xu, Y., Zhang, M., & Wang, D. (2017). Determination of Furfural and 5-Hydroxymethylfurfural in Biomass Hydrolysate by High-Performance Liquid Chromatography. *Energy & Fuels*, *31*(12), 13769–13774. https://doi.org/10.1021/acs.energyfuels.7b02827

Li, M., Xia, S., Zhang, Y., & Li, X. (2018). Optimization of ACE inhibitory peptides from black soybean by microwave-assisted enzymatic method and study on its stability. *LWT-Food Science and Technology*, *98*, 358–365. https://doi.org/10.1016/J.LWT.2018.08.045

Liao, W., Fan, H., Liu, P., & Wu, J. (2019). Identification of angiotensin converting enzyme 2 (ACE2) up-regulating peptides from pea protein hydrolysate. *Journal of Functional Foods*, *60*, 103395. https://doi.org/10.1016/J.JFF.2019.05.051

Lin, D., Ma, Y., Qin, W., Loy, D. A., Chen, H., & Zhang, Q. (2022). The structure, properties and potential probiotic properties of starch-pectin blend: A review. *Food Hydrocolloids*, *129*, 107644. https://doi.org/10.1016/J.FOODHYD.2022.107644

Lin, Y., An, F., He, H., Geng, F., Song, H., & Huang, Q. (2021). Structural and rheological characterization of pectin from passion fruit (*Passiflora edulis f. flavicarpa*) peel extracted by high-speed shearing. *Food Hydrocolloids*, *114*, 106555. https://doi.org/10.1016/J.FOODHYD.2020.106555

Lu, W., Chen, X. W., Wang, J. M., Yang, X. Q., & Qi, J. R. (2016). Enzyme-assisted subcritical water extraction and characterization of soy protein from heat-denatured meal. *Journal of Food Engineering*, *169*, 250–258. https://doi.org/10.1016/j.jfoodeng.2015.09.006

Ma, X., Zhang, L., Wang, W., Zou, M., Ding, T., Ye, X., & Liu, D. (2016). Synergistic Effect and Mechanisms of Combining Ultrasound and Pectinase on Pectin Hydrolysis. *Food and Bioprocess Technology*, *9*(7), 1249–1257. https://doi.org/10.1007/S11947-016-1689-Y/FIGURES/4

Machmudah, S., Wahyudiono, Kanda, H., & Goto, M. (2017). Hydrolysis of Biopolymers in Near-Critical and Subcritical Water. In *Water Extraction of Bioactive Compounds: From Plants to Drug Development* (pp. 69–107). Elsevier. https://doi.org/10.1016/B978-0-12-809380-1.00003-6

Malmberg, C. G., & Maryott, A. A. (1956). Dielectric Constant of Water from 0° to 100°C. *Journal of Research of the National Bureau of Standards*, *56*(I), 1–8.

Manderson, K., Pinart, M., Tuohy, K. M., Grace, W. E., Hotchkiss, A. T., Widmer, W., Yadhav, M. P., Gibson, G. R., & Rastall, R. A. (2005). In vitro determination of prebiotic properties of oligosaccharides derived from an orange juice manufacturing by-product stream. *Applied*

and Environmental Microbiology, *71*(12), 8383–8389. https://doi.org/10.1128/AEM.71.12.8383-8389.2005/ASSET/BD120AA4-10EF-4619-9D5F-1319F2558F00/ASSETS/GRAPHIC/ZAM0120561930001.JPEG

Mao, G., Wu, D., Wei, C., Tao, W., Ye, X., Linhardt, R. J., Orfila, C., & Chen, S. (2019). Reconsidering conventional and innovative methods for pectin extraction from fruit and vegetable waste: Targeting rhamnogalacturonan I. *Trends in Food Science & Technology*, *94*, 65–78. https://doi.org/10.1016/J.TIFS.2019.11.001

Marcet, I., Álvarez, C., Paredes, B., & Díaz, M. (2016). The use of sub-critical water hydrolysis for the recovery of peptides and free amino acids from food processing wastes. Review of sources and main parameters. *Waste Management*, *49*, 364–371. https://doi.org/10.1016/J.WASMAN.2016.01.009

Marić, M., Grassino, A. N., Zhu, Z., Barba, F. J., Brnčić, M., & Rimac Brnčić, S. (2018). An overview of the traditional and innovative approaches for pectin extraction from plant food wastes and by-products: Ultrasound-, microwaves-, and enzyme-assisted extraction. *Trends in Food Science & Technology*, *76*, 28–37. https://doi.org/10.1016/J.TIFS.2018.03.022

Marshall, W. L., & Franck, E. U. (1981). Ion product of water substance, 0-1000 °C, 1-10,000 bars New International Formulation and its background. *Journal of Physical and Chemical Reference Data*, *10*(2), 295–304. https://doi.org/10.1063/1.555643

Martinez-Monteagudo, S. I., & Saldaña, M. D. A. (2014). Chemical Reactions in Food Systems at High Hydrostatic Pressure. *Food Engineering Reviews*, *6*(4), 105–127. https://doi.org/10.1007/S12393-014-9087-6/FIGURES/2

Matsumoto, T., Moriya, M., Sakurai, M. H., Kiyohara, H., Tabuchi, Y., & Yamada, H. (2008). Stimulatory effect of a pectic polysaccharide from a medicinal herb, the roots of *Bupleurum falcatum L.*, on G-CSF secretion from intestinal epithelial cells. *International Immunopharmacology*, *8*(4), 581–588. https://doi.org/10.1016/J.INTIMP.2008.01.006

Message, J. L., Assifaoui, A., Cayot, P., & Saurel, R. (2012). Effect of pea proteins extraction and vicilin/legumin fractionation on the phase behavior in admixture with alginate. *Food Hydrocolloids*, *29*(2), 335–346. https://doi.org/10.1016/J.FOODHYD.2012.03.003

Mirzaei, M., Mirdamadi, S., Ehsani, M. R., Aminlari, M., & Hosseini, E. (2015). Purification and identification of antioxidant and ACE-inhibitory peptide from *Saccharomyces cerevisiae* protein hydrolysate. *Journal of Functional Foods*, *19*, 259–268. https://doi.org/10.1016/J.JFF.2015.09.031

Mohd Thani, N., Mustapa Kamal, S. M., Taip, F. S., Sulaiman, A., & Omar, R. (2019). Effect of sub-critical water hydrolysis on sugar recovery from bakery leftovers. *Food and Bioproducts Processing*, *117*, 105–112. https://doi.org/10.1016/J.FBP.2019.07.002

Moldoveanu, S. C., & David, V. (2013). Mobile Phases and Their Properties. In *Essentials in Modern HPLC Separations* (pp. 363–447). Elsevier. https://doi.org/10.1016/B978-0-12-385013-3.00007-0

Möller, M., Nilges, P., Harnisch, F., & Schröder, U. (2011). Subcritical Water as Reaction Environment: Fundamentals of Hydrothermal Biomass Transformation. *Chemistry-Sustainability-Energy-Materials*, *4*(5), 566–579. https://doi.org/10.1002/CSSC.201000341

Moreno, J., & Peinado, R. (2012a). Carboxylic Acids: Structure and Properties. In *Enological Chemistry* (pp. 109–120). Academic Press. https://doi.org/10.1016/B978-0-12-388438-1.00008-X

Moreno, J., & Peinado, R. (2012b). Sugars in Must. In *Enological Chemistry* (pp. 95–107). Academic Press. https://doi.org/10.1016/B978-0-12-388438-1.00007-8

Muñoz-Almagro, N., Montilla, A., Moreno, F. J., & Villamiel, M. (2017). Modification of citrus and apple pectin by power ultrasound: Effects of acid and enzymatic treatment. *Ultrasonics Sonochemistry*, *38*, 807–819. https://doi.org/10.1016/J.ULTSONCH.2016.11.039

Nagamori, M., & Funazukuri, T. (2004). Glucose production by hydrolysis of starch under hydrothermal conditions. *Journal of Chemical Technology & Biotechnology*, *79*(3), 229–233. https://doi.org/10.1002/JCTB.976

Ndiaye, F., Vuong, T., Duarte, J., Aluko, R. E., & Matar, C. (2012). Anti-oxidant, anti-inflammatory and immunomodulating properties of an enzymatic protein hydrolysate from yellow field pea seeds. *European Journal of Nutrition*, *51*(1), 29–37. https://doi.org/10.1007/S00394-011-0186-3/FIGURES/7

Ndlela, S. C., de Moura, J. M. L. N., Olson, N. K., & Johnson, L. A. (2012). Aqueous Extraction of Oil and Protein from Soybeans with Subcritical Water. *Journal of the American Oil Chemists' Society*, *89*(6), 1145–1153. https://doi.org/10.1007/S11746-011-1993-7

Nikolić, M. v., & Mojovic, L. (2007). Hydrolysis of apple pectin by the coordinated activity of pectic enzymes. *Food Chemistry*, *101*(1), 1–9. https://doi.org/10.1016/J.FOODCHEM.2005.12.053

Nkurunziza, D., Ho, T. C., Protzman, R. A., Cho, Y. J., Getachew, A. T., Lee, H. J., & Chun, B. S. (2021). Pressurized hot water crosslinking of gelatin-alginate for the enhancement of spent coffee oil emulsion stability. *The Journal of Supercritical Fluids*, *169*, 105120. https://doi.org/10.1016/J.SUPFLU.2020.105120

Novo, L. P., Bras, J., García, A., Belgacem, N., & Curvelo, A. A. S. (2015). Subcritical Water: A Method for Green Production of Cellulose Nanocrystals. *ACS Sustainable Chemistry & Engineering*, *3*(11), 2839-2846. https://doi.org/10.1021/acssuschemeng.5b00762

Ogutu, F. O., & Mu, T. H. (2017). Ultrasonic degradation of sweet potato pectin and its antioxidant activity. *Ultrasonics Sonochemistry*, *38*, 726–734. https://doi.org/10.1016/J.ULTSONCH.2016.08.014

Onumpai, C., Kolida, S., Bonnin, E., & Rastall, R. A. (2011). Microbial Utilization and Selectivity of Pectin Fractions with Various Structures. *Applied and Environmental Microbiology*, *77*(16), 5747–5754. https://doi.org/10.1128/AEM.00179-11

Overend, R. P., & Chornet, E. (1987). Fractionation of Lignocellulosics by Steam-Aqueous Pretreatments. *Philosophical Transactions of the Royal Society of London. Series A, Mathematical and Physical Sciences*, *321*, 523–536. https://about.jstor.org/terms

Pageon, H., Azouaoui, A., Zucchi, H., Ricois, S., Tran, C., & Asselineau, D. (2019). Potentially beneficial effects of rhamnose on skin ageing: an in vitro and in vivo study. *International Journal of Cosmetic Science*, *41*(3), 213–220. https://doi.org/10.1111/ICS.12523

Papagianni, M. (2007). Advances in citric acid fermentation by Aspergillus niger: Biochemical aspects, membrane transport and modeling. *Biotechnology Advances*, *25*(3), 244–263. https://doi.org/10.1016/J.BIOTECHADV.2007.01.002

Park, J. S., Jeong, Y. R., & Chun, B. S. (2019). Physiological activities and bioactive compound from laver (*Pyropia yezoensis*) hydrolysates by using subcritical water hydrolysis. *The Journal of Supercritical Fluids*, *148*, 130–136. https://doi.org/10.1016/J.SUPFLU.2019.03.004

Phongthai, S., D'Amico, S., Schoenlechner, R., Homthawornchoo, W., & Rawdkuen, S. (2018). Fractionation and antioxidant properties of rice bran protein hydrolysates stimulated by in vitro gastrointestinal digestion. *Food Chemistry*, *240*, 156–164. https://doi.org/10.1016/J.FOODCHEM.2017.07.080

Picot-Allain, M. C. N., Ramasawmy, B., & Emmambux, M. N. (2020). Extraction, Characterisation, and Application of Pectin from Tropical and Sub-Tropical Fruits: A Review. *Food Reviews International*, *38*(3), 282–312. https://doi.org/10.1080/87559129.2020.1733008

Pińkowska, H., Krzywonos, M., Wolak, P., & Złocińska, A. (2019). Production of uronic acids by hydrothermolysis of pectin as a model substance for plant biomass waste. *Green Processing and Synthesis*, *8*(1), 683–690. https://doi.org/10.1515/gps-2019-0039

Pińkowska, H., & Oliveros, E. (2014). Application of the doehlert matrix for the determination of the optimal conditions of hydrolysis of soybean protein in subcritical water. *Industrial and Engineering Chemistry Research*, *53*(4), 1320–1326. https://doi.org/10.1021/ie403451b

Pińkowska, H., Wolak, P., Krzywonos, M., & Złocińska, A. (2021). Comprehensive Study of Hydrothermal Extraction of Pectin From Sugar Beet Pulp. *Waste and Biomass Valorization*, *12*(8), 4587–4598. https://doi.org/10.1007/S12649-020-01317-6/TABLES/3

Pollet, P., Davey, E. A., Ureña-Benavides, E. E., Eckert, C. A., & Liotta, C. L. (2014). Solvents for sustainable chemical processes. *Green Chemistry*, *16*(3), 1034–1055. https://doi.org/10.1039/C3GC42302F

Pourali, O., Asghari, F. S., & Yoshida, H. (2009). Sub-critical water treatment of rice bran to produce valuable materials. *Food Chemistry*, *115*(1), 1–7. https://doi.org/10.1016/j.foodchem.2008.11.099

Powell, T., Bowra, S., & Cooper, H. J. (2016). Subcritical Water Processing of Proteins: An Alternative to Enzymatic Digestion? *Analytical Chemistry*, *88*(12), 6425–6432. https://doi.org/10.1021/acs.analchem.6b01013

Pownall, T. L., Udenigwe, C. C., & Aluko, R. E. (2010). Amino Acid Composition and Antioxidant Properties of Pea Seed (*Pisum sativum L.*) Enzymatic Protein Hydrolysate Fractions. *Journal of Agricultural and Food Chemistry*, *58*, 4712–4718. https://doi.org/10.1021/jf904456r

Prado, J. M., Forster-Carneiro, T., Rostagno, M. A., Follegatti-Romero, L. A., Maugeri Filho, F., & Meireles, M. A. A. (2014). Obtaining sugars from coconut husk, defatted grape seed, and pressed palm fiber by hydrolysis with subcritical water. *The Journal of Supercritical Fluids*, *89*, 89–98. https://doi.org/10.1016/J.SUPFLU.2014.02.017

Purkayastha, S., Saha, S., & Ghosh, A. K. (2022). One-step poly vinyl alcohol-based hydrogel preparation using subcritical water/CO2 process. *Journal of Materials Science*, *57*(2), 1314–1327. https://doi.org/10.1007/s10853-021-06593-8

Qin, F., Johansen, A. Z., & Mussatto, S. I. (2018). Evaluation of different pretreatment strategies for protein extraction from brewer's spent grains. *Industrial Crops and Products*, *125*, 443–453. https://doi.org/10.1016/J.INDCROP.2018.09.017

Raji, M., Zari, N., el Kacem Qaiss, A., & Bouhfid, R. (2019). Chemical Preparation and Functionalization Techniques of Graphene and Graphene Oxide. In *Functionalized Graphene Nanocomposites and Their Derivatives: Synthesis, Processing and Applications* (pp. 1–20). Elsevier. https://doi.org/10.1016/B978-0-12-814548-7.00001-5

Ramachandraiah, K., Koh, B. B., Davaatseren, M., & Hong, G. P. (2017). Characterization of soy protein hydrolysates produced by varying subcritical water processing temperature. *Innovative Food Science and Emerging Technologies*, *43*, 201–206. https://doi.org/10.1016/j.ifset.2017.08.011

Reddy, H. K., Muppaneni, T., Sun, Y., Li, Y., Ponnusamy, S., Patil, P. D., Dailey, P., Schaub, T., Holguin, F. O., Dungan, B., Cooke, P., Lammers, P., Voorhies, W., Lu, X., & Deng, S. (2014).

Subcritical water extraction of lipids from wet algae for biodiesel production. *Fuel*, *133*, 73–81. https://doi.org/10.1016/J.FUEL.2014.04.081

Reinkensmeier, A., Bußler, S., Schlüter, O., Rohn, S., & Rawel, H. M. (2015). Characterization of individual proteins in pea protein isolates and air classified samples. *Food Research International*, *76*(P1), 160–167. https://doi.org/10.1016/J.FOODRES.2015.05.009

Robledo, V. R., & Vázquez, L. I. C. (2019). Pectin - Extraction, Purification, Characterization and Applications. In *Pectins - Extraction, Purification, Characterization and Applications* (pp. 1–19). IntechOpen. https://doi.org/10.5772/INTECHOPEN.85588

Rogalinski, T., Herrmann, S., & Brunner, G. (2005). Production of amino acids from bovine serum albumin by continuous sub-critical water hydrolysis. *The Journal of Supercritical Fluids*, *36*(1), 49–58. https://doi.org/10.1016/J.SUPFLU.2005.03.001

Rogalinski, T., Liu, K., Albrecht, T., & Brunner, G. (2008). Hydrolysis kinetics of biopolymers in subcritical water. *The Journal of Supercritical Fluids*, *46*(3), 335–341. https://doi.org/10.1016/J.SUPFLU.2007.09.037

Rudolph, S., Lunow, D., Kaiser, S., & Henle, T. (2017). Identification and quantification of ACE-inhibiting peptides in enzymatic hydrolysates of plant proteins. *Food Chemistry*, *224*, 19–25. https://doi.org/10.1016/J.FOODCHEM.2016.12.039

Sabater, C., Ferreira-Lazarte, A., Montilla, A., & Corzo, N. (2019). Enzymatic Production and Characterization of Pectic Oligosaccharides Derived from Citrus and Apple Pectins: A GC-MS Study Using Random Forests and Association Rule Learning. *Journal of Agricultural and Food Chemistry*, *67*(26), 7435–7447. https://doi.org/10.1021/acs.jafc.9b00930

Salapa, I., Topakas, E., & Sidiras, D. (2018). Simulation and optimization of barley straw organosolv pretreatment. *Industrial Crops and Products*, *113*, 80–88. https://doi.org/10.1016/J.INDCROP.2018.01.018

Saldaña, M. D. A., Silva, E. K., Cornejo, J. E. O., & Lopez, C. L. O. (2021). Green Processes in Foodomics: Biorefineries in the Food Industry. In *Comprehensive Foodomics*. Elsevier. https://doi.org/10.1016/B978-0-08-100596-5.22821-7

Saldaña, M. D. A., & Valdivieso-Ramírez, C. S. (2015). Pressurized fluid systems: Phytochemical production from biomass. *The Journal of Supercritical Fluids*, *96*, 228–244. https://doi.org/10.1016/J.SUPFLU.2014.09.037

Santos, D. I., Saraiva, J. M. A., Vicente, A. A., & Moldão-Martins, M. (2019). Methods for determining bioavailability and bioaccessibility of bioactive compounds and nutrients. In *Innovative Thermal and Non-Thermal Processing, Bioaccessibility and Bioavailability of Nutrients and Bioactive Compounds*. Woodhead Publishing. https://doi.org/10.1016/B978-0-12-814174-8.00002-0

Sato, T., Sekiguchi, G., Adschiri, T., & Arai, K. (2003). Alkylation of Phenol with Carbonyl Compounds in Supercritical Water. *Journal of Chemical Engineering of Japan*, *36*(3), 339–342. https://doi.org/10.1252/JCEJ.36.339

Sereewatthanawut, I., Prapintip, S., Watchiraruji, K., Goto, M., Sasaki, M., & Shotipruk, A. (2008). Extraction of protein and amino acids from deoiled rice bran by subcritical water hydrolysis. *Bioresource Technology*, *99*(3), 555–561. https://doi.org/10.1016/j.biortech.2006.12.030

Shao, Y., Shao, H., Hua, X., & Kohlus, R. (2022). Kinetics study of acid hydrolysis of citrus pectin in hydrothermal process. *Food Hydrocolloids*, *124*, 107300. https://doi.org/10.1016/J.FOODHYD.2021.107300

Shewry, P. R., Napier, J. A., & Tatham, A. S. (1995). Seed storage proteins: structures and biosynthesis. *The Plant Cell*, *7*(7), 945. https://doi.org/10.1105/TPC.7.7.945

Sikorski, Z. E. (2001). Functional properties of proteins in food systems. In *Chemical and Functional Properties of Food Proteins* (pp. 113–135). CRC Press. https://doi.org/10.1201/9781482279047-11

Silva, J. D. C., de França, P. R. L., Converti, A., & Porto, T. S. (2019). Pectin hydrolysis in cashew apple juice by Aspergillus aculeatus URM4953 polygalacturonase covalently-immobilized on calcium alginate beads: A kinetic and thermodynamic study. *International Journal of Biological Macromolecules*, *126*, 820–827. https://doi.org/10.1016/J.IJBIOMAC.2018.12.236

Singh, R. P., Prakash, S., Bhatia, R., Negi, M., Singh, J., Bishnoi, M., & Kondepudi, K. K. (2020). Generation of structurally diverse pectin oligosaccharides having prebiotic attributes. *Food Hydrocolloids*, *108*, 105988. https://doi.org/10.1016/J.FOODHYD.2020.105988

Singh, R. P., & Tingirikari, J. M. R. (2021). Agro waste derived pectin poly and oligosaccharides: Synthesis and functional characterization. *Biocatalysis and Agricultural Biotechnology*, *31*, 101910. https://doi.org/10.1016/J.BCAB.2021.101910

Steinhardt, J., & Fugitt, C. H. (1942). Catalyzed hydrolysis of amide and peptide bonds in proteins. *Journal of Research of the National Bureau of Standards*, *29*, 316–327.

Sunphorka, S., Chavasiri, W., Oshima, Y., & Ngamprasertsith, S. (2012). Kinetic studies on rice bran protein hydrolysis in subcritical water. *Journal of Supercritical Fluids*, *65*, 54–60. https://doi.org/10.1016/j.supflu.2012.02.017

Tamm, F., Herbst, S., Brodkorb, A., & Drusch, S. (2016). Functional properties of pea protein hydrolysates in emulsions and spray-dried microcapsules. *Food Hydrocolloids*, *58*, 204–214. https://doi.org/10.1016/j.foodhyd.2016.02.032

Toor, S. S., Rosendahl, L., & Rudolf, A. (2011). Hydrothermal liquefaction of biomass: A review of subcritical water technologies. *Energy*, *36*(5), 2328–2342. https://doi.org/10.1016/J.ENERGY.2011.03.013

Ueno, S., Ichinoi, H., Zhao, J., & Fujii, T. (2015). Degradation of fish gelatin using hot-compressed water and the properties of the degradation products. *High Pressure Research*, *35*(2), 203–213. https://doi.org/10.1080/08957959.2015.1023197

Ulug, S. K., Jahandideh, F., & Wu, J. (2021). Novel technologies for the production of bioactive peptides. *Trends in Food Science & Technology*, *108*, 27–39. https://doi.org/10.1016/J.TIFS.2020.12.002

Urbizo-Reyes, U., San Martin-González, M. F., Garcia-Bravo, J., López Malo Vigil, A., & Liceaga, A. M. (2019). Physicochemical characteristics of chia seed (*Salvia hispanica*) protein hydrolysates produced using ultrasonication followed by microwave-assisted hydrolysis. *Food Hydrocolloids*, *97*, 105187. https://doi.org/10.1016/J.FOODHYD.2019.105187

Uslu, H., & Kirbaşlar, Ş. I. (2010). Extraction of aqueous of malic acid by trioctylamine extractant in various diluents. *Fluid Phase Equilibria*, *287*(2), 134–140. https://doi.org/10.1016/j.fluid.2009.09.022

Valdivieso-Ramirez, C. S., Sanchez Gallego, J. E., Gänzle, M., Temelli, F., & Saldaña, M. D. A. (2021). Carboxylic acid-catalysed hydrolysis of polygalacturonic acid in subcritical water media. *The Journal of Supercritical Fluids*, *169*, 105103. https://doi.org/10.1016/J.SUPFLU.2020.105103

Valdivieso-Ramirez, C. S., Temelli, F., & Saldaña, M. D. A. (2021a). Carboxylic acid-catalyzed hydrolysis of rhamnogalacturonan in subcritical water media. *The Journal of Supercritical Fluids*, *175*, 105268. https://doi.org/10.1016/J.SUPFLU.2021.105268

Valdivieso-Ramirez, C. S., Temelli, F., & Saldaña, M. D. A. (2021b). Production of pea hull soluble fiber-derived oligosaccharides using subcritical water with carboxylic acids. *The Journal of Supercritical Fluids*, *178*, 105349. https://doi.org/10.1016/J.SUPFLU.2021.105349

Valladares-Diestra, K. K., Porto de Souza Vandenberghe, L., Zevallos Torres, L. A., Zandoná Filho, A., Lorenci Woiciechowski, A., & Ricardo Soccol, C. (2022). Citric acid assisted hydrothermal pretreatment for the extraction of pectin and xylooligosaccharides production from cocoa pod husks. *Bioresource Technology*, *343*, 126074. https://doi.org/10.1016/J.BIORTECH.2021.126074

Valladares-Diestra, K., Porto de Souza Vandenberghe, L., & Ricardo Soccol, C. (2022). A biorefinery approach for pectin extraction and second-generation bioethanol production from cocoa pod husk. *Bioresource Technology*, *346*, 126635. https://doi.org/10.1016/J.BIORTECH.2021.126635

van der Wal, H., Sperber, B. L. H. M., Houweling-Tan, B., Bakker, R. R. C., Brandenburg, W., & López-Contreras, A. M. (2013). Production of acetone, butanol, and ethanol from biomass of the green seaweed Ulva lactuca. *Bioresource Technology*, *128*, 431–437. https://doi.org/10.1016/J.BIORTECH.2012.10.094

Vedovatto, F., Ugalde, G., Bonatto, C., Bazoti, S. F., Treichel, H., Mazutti, M. A., Zabot, G. L., & Tres, M. v. (2021). Subcritical water hydrolysis of soybean residues for obtaining fermentable sugars. *The Journal of Supercritical Fluids*, *167*, 105043. https://doi.org/10.1016/J.SUPFLU.2020.105043

Wang, M. P., Chen, X. W., Guo, J., Yang, J., Wang, J. M., & Yang, X. Q. (2019). Stabilization of foam and emulsion by subcritical water-treated soy protein: Effect of aggregation state. *Food Hydrocolloids*, *87*, 619–628. https://doi.org/10.1016/J.FOODHYD.2018.08.047

Wang, T., Jiang, M., Tang, S., Lai, C., Huang, C., Fan, Y., & Yong, Q. (2021). Preparation of di- and tri- galacturonic acid by coupling hydrothermal pretreatment and enzymatic hydrolysis. *Process Biochemistry*, *102*, 180–185. https://doi.org/10.1016/J.PROCBIO.2021.01.002

Wang, W., Chen, W., Zou, M., Lv, R., Wang, D., Hou, F., Feng, H., Ma, X., Zhong, J., Ding, T., Ye, X., & Liu, D. (2018). Applications of power ultrasound in oriented modification and degradation of pectin: A review. *Journal of Food Engineering*, *234*, 98–107. https://doi.org/10.1016/J.JFOODENG.2018.04.016

Watchararuji, K., Goto, M., Sasaki, M., & Shotipruk, A. (2008). Value-added subcritical water hydrolysate from rice bran and soybean meal. *Bioresource Technology*, *99*(14), 6207–6213. https://doi.org/10.1016/j.biortech.2007.12.021

Waterborg, J. H. (2009). The Lowry Method for Protein Quantitation. In *The Protein Protocols Handbook* (pp. 7–10). Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-59745-198-7_2

Wen, C., Zhang, J., Yao, H., Zhou, J., Duan, Y., Zhang, H., & Ma, H. (2019). Advances in renewable plant-derived protein source: The structure, physicochemical properties affected by ultrasonication. *Ultrasonics Sonochemistry*, *53*, 83–98. https://doi.org/10.1016/J.ULTSONCH.2018.12.036

Wiboonsirikul, J., Mori, M., Khuwijitjaru, P., & Adachi, S. (2013). Properties of extract from Okara by Its Subcritical Water Treatment. *International Journal of Food Properties*, *16*(5), 974–982. https://doi.org/10.1080/10942912.2011.573119

Wikiera, A., Mika, M., Starzyńska-Janiszewska, A., & Stodolak, B. (2015). Development of complete hydrolysis of pectins from apple pomace. *Food Chemistry*, *172*, 675–680. https://doi.org/10.1016/J.FOODCHEM.2014.09.132

Xu, Y., Wang, P., Xue, S., Kong, F., Ren, H., & Zhai, H. (2020). Green biorefinery — the ultra-high hydrolysis rate and behavior of Populus tomentosa hemicellulose autohydrolysis under moderate subcritical water conditions. *RSC Advances*, *10*(32), 18908–18917. https://doi.org/10.1039/D0RA02350G

Yapo, B. M. (2011). Pectic substances: From simple pectic polysaccharides to complex pectins—A new hypothetical model. *Carbohydrate Polymers*, *86*(2), 373–385. https://doi.org/10.1016/J.CARBPOL.2011.05.065

Yedro, F. M., García-Serna, J., Cantero, D. A., Sobrón, F., & Cocero, M. J. (2014). Hydrothermal hydrolysis of grape seeds to produce bio-oil. *RSC Advances*, *4*(57), 30332–30339. https://doi.org/10.1039/C4RA00429A

Yeung, Y. K., Kang, Y. R., So, B. R., Jung, S. K., & Chang, Y. H. (2021). Structural, antioxidant, prebiotic and anti-inflammatory properties of pectic oligosaccharides hydrolyzed from okra pectin by Fenton reaction. *Food Hydrocolloids*, *118*. https://doi.org/10.1016/j.foodhyd.2021.106779

Zhang, J., Wen, C., Zhang, H., Zandile, M., Luo, X., Duan, Y., & Ma, H. (2018). Structure of the zein protein as treated with subcritical water. *International Journal of Food Properties*, *21*(1), 128–138. https://doi.org/10.1080/10942912.2017.1414839

Zhang, R., Chen, Y., Zhou, Y., Tong, D., & Hu, C. (2019). Selective Conversion of Hemicellulose in Macroalgae Enteromorpha prolifera to Rhamnose. *ACS Omega*, *4*(4), 7023–7028. https://doi.org/10.1021/acsomega.8b03600

Zhang, S. B., Wang, Z., & Xu, S. Y. (2008). Antioxidant and antithrombotic activities of rapeseed peptides. *JAOCS, Journal of the American Oil Chemists' Society*, *85*(6), 521–527. https://doi.org/10.1007/s11746-008-1217-y

Zhao, Y., & Saldaña, M. D. A. (2019). Hydrolysis of cassava starch, chitosan and their mixtures in pressurized hot water media. *The Journal of Supercritical Fluids*, *147*, 293–301. https://doi.org/10.1016/J.SUPFLU.2018.11.013

Zhao, Y., Teixeira, J. S., Gänzle, M. M., & Saldaña, M. D. A. (2018). Development of antimicrobial films based on cassava starch, chitosan and gallic acid using subcritical water technology. *The Journal of Supercritical Fluids*, *137*, 101–110. https://doi.org/10.1016/J.SUPFLU.2018.03.010

Zhi, Z., Chen, J., Li, S., Wang, W., Huang, R., Liu, D., DIng, T., Linhardt, R. J., Chen, S., & Ye, X. (2017). Fast preparation of RG-I enriched ultra-low molecular weight pectin by an ultrasound accelerated Fenton process. *Scientific Reports*, *7*(1), 1–11. https://doi.org/10.1038/s41598-017-00572-3

Zhu, G., Zhu, X., Fan, Q., & Wan, X. (2011). Kinetics of amino acid production from bean dregs by hydrolysis in sub-critical water. *Amino Acids*, *40*(4), 1107–1113. https://doi.org/10.1007/S00726-010-0734-9/TABLES/4

APPENDIX

Appendix A. Calibration curves

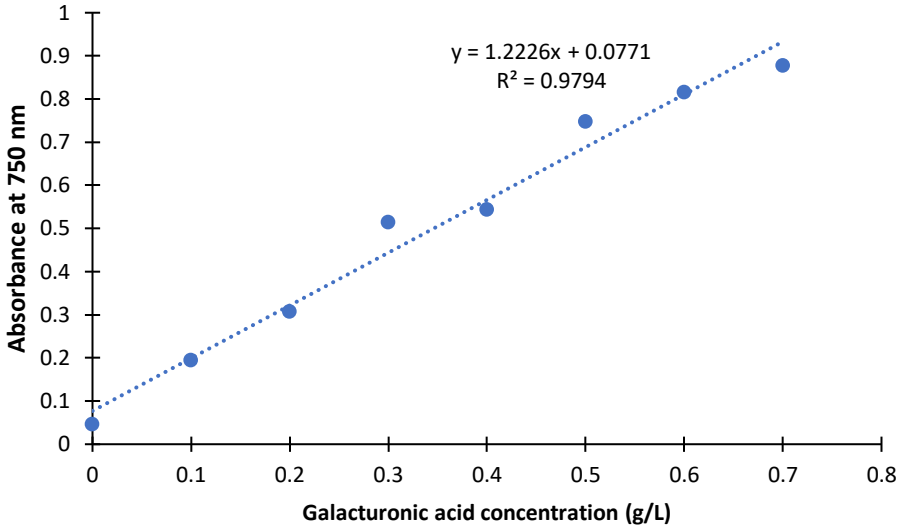


Fig. A1. Calibration curve of hexuronic acid determination based on galacturonic acid equivalent by spectrophotometry method.

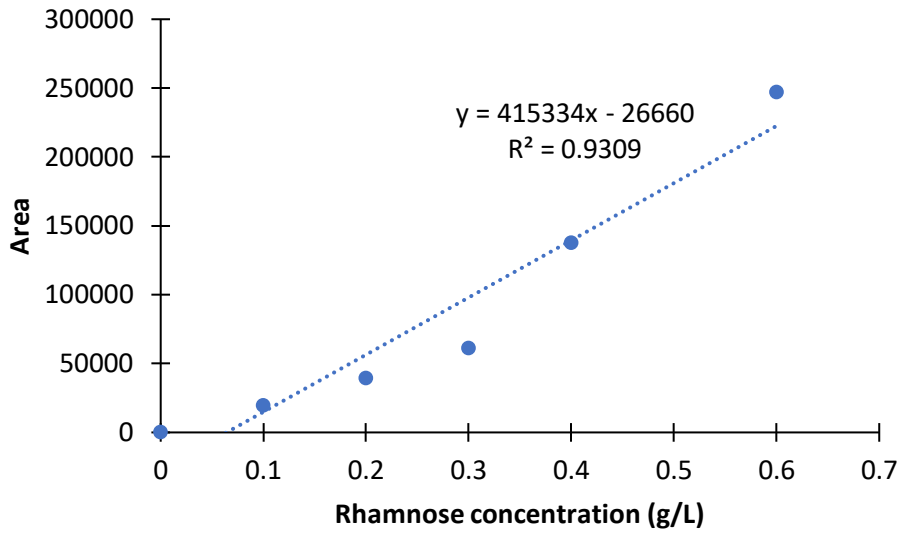


Fig. A2. Calibration curve for rhamnose determination by HPLC analysis.

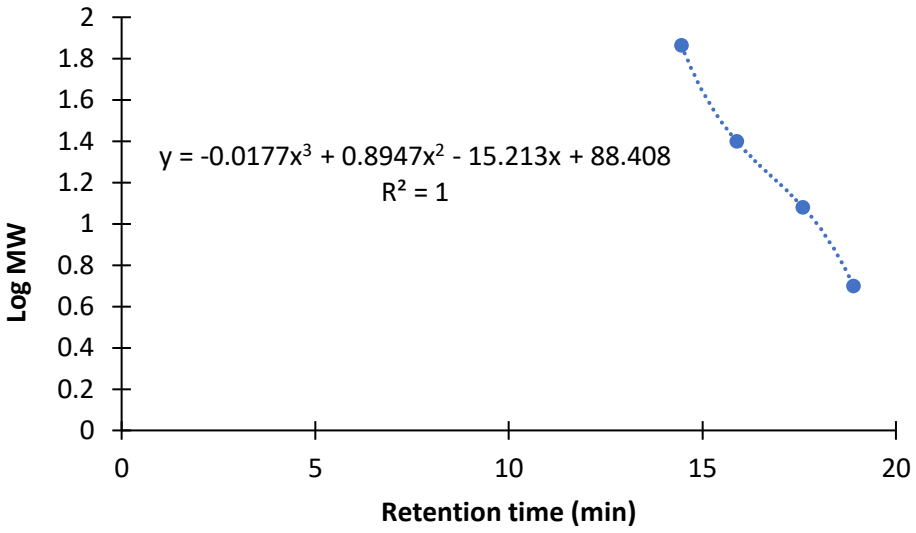


Fig. A3. Calibration curve for molecular weight (MW) determination based on dextran as the standard by HP-SEC.

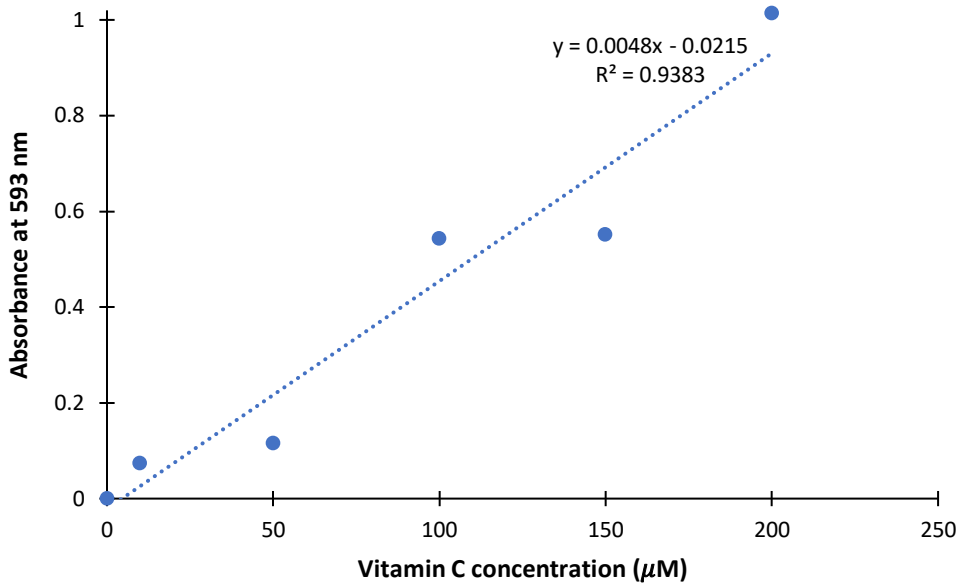


Fig. A4. Calibration curve of ferric reducing antioxidant power (FRAP) determination based on vitamin C equivalent by spectrophotometry method.

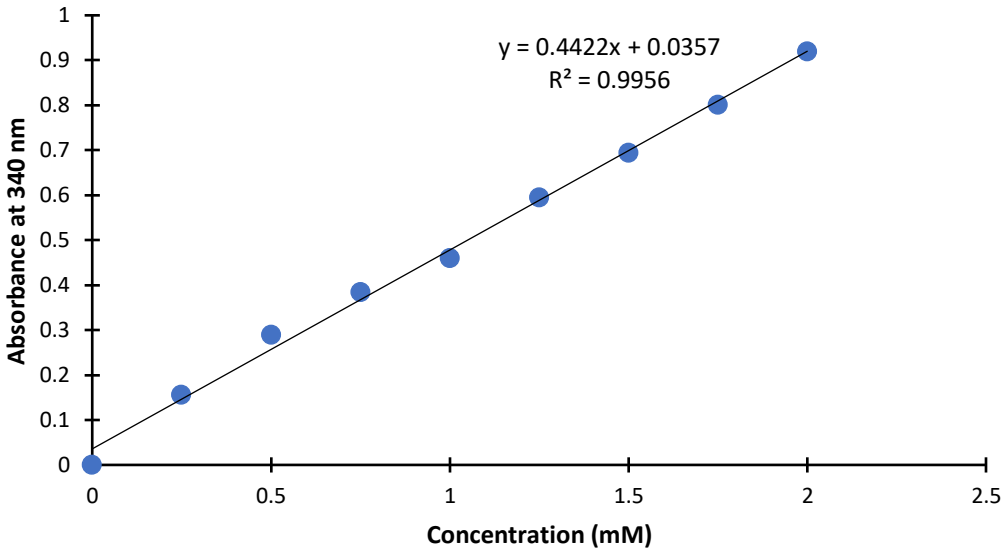


Fig. A5. Calibration curve for degree of hydrolysis determination using L-Lysine as the standard by spectrophotometry method.

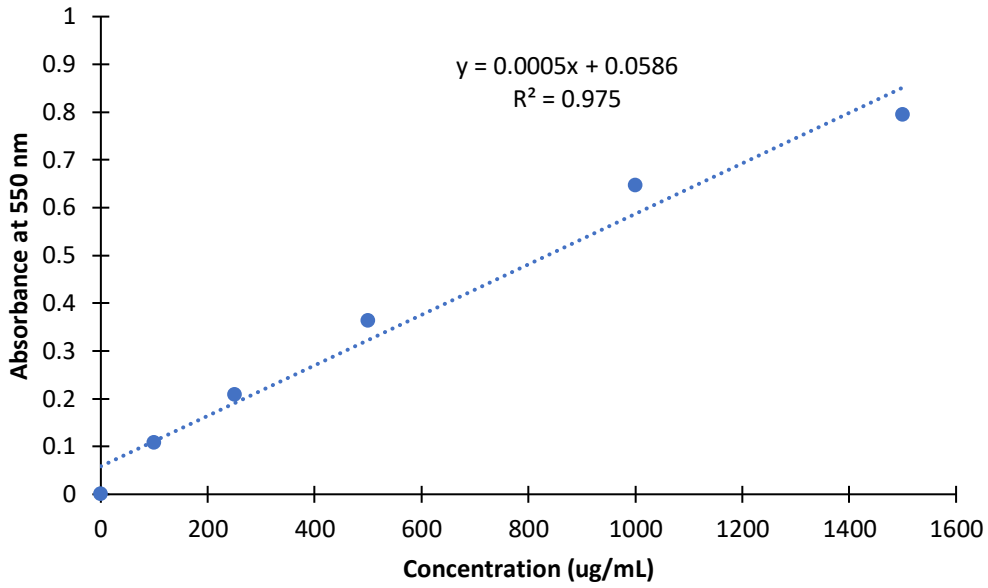
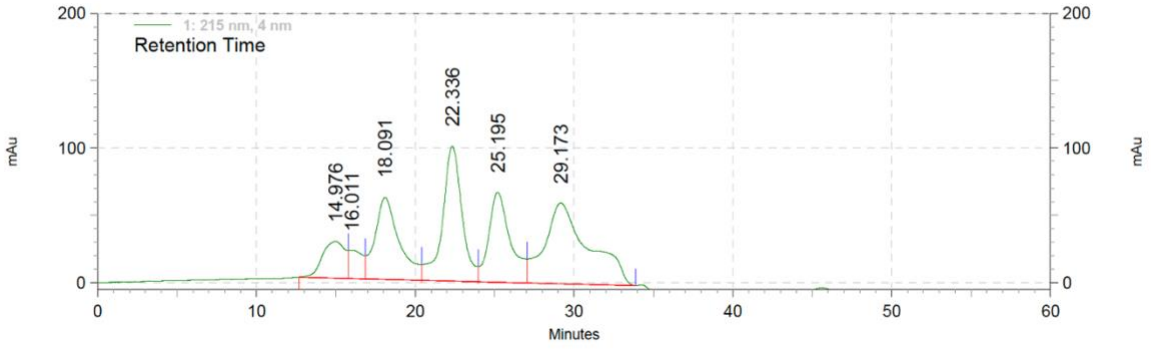


Fig. A6. Calibration curve for protein/peptide/amino acid content determination using bovine serum albumin as the standard by spectrophotometry method



Retention time (min)	Area	Name
14.98	2725805	Bluedex
16.01	1232056	
18.09	6456273	BSA
22.34	8581032	Carbonic acid
25.19	6357621	Cytochrome C
29.17	11519000	Aprotin

Fig. A7. Chromatogram of HPSEC standards used for peptides size determination.

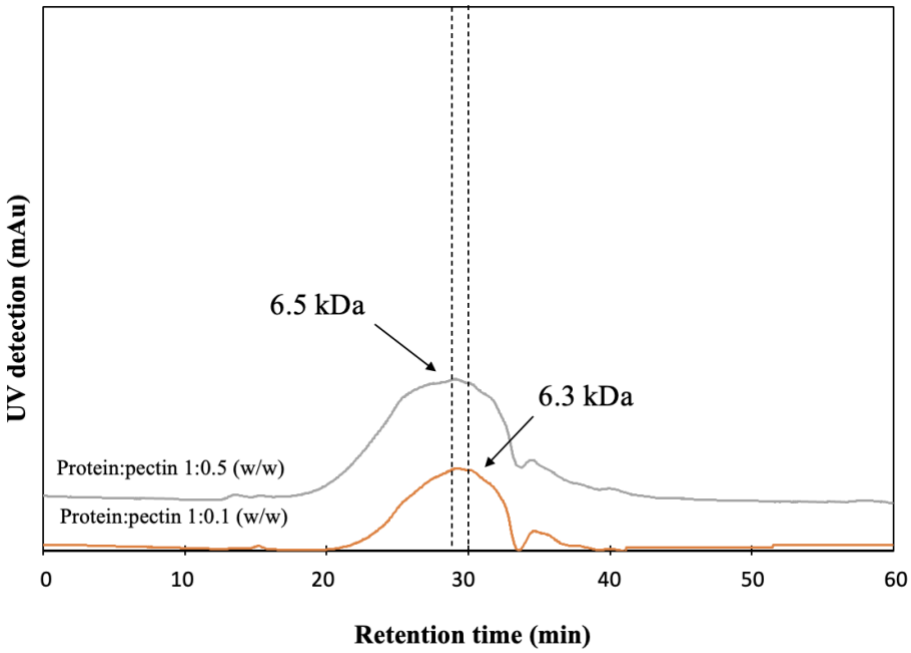


Fig. A8. High performance size exclusion chromatograms (HPSEC) of pea protein and pectin hydrolysates.

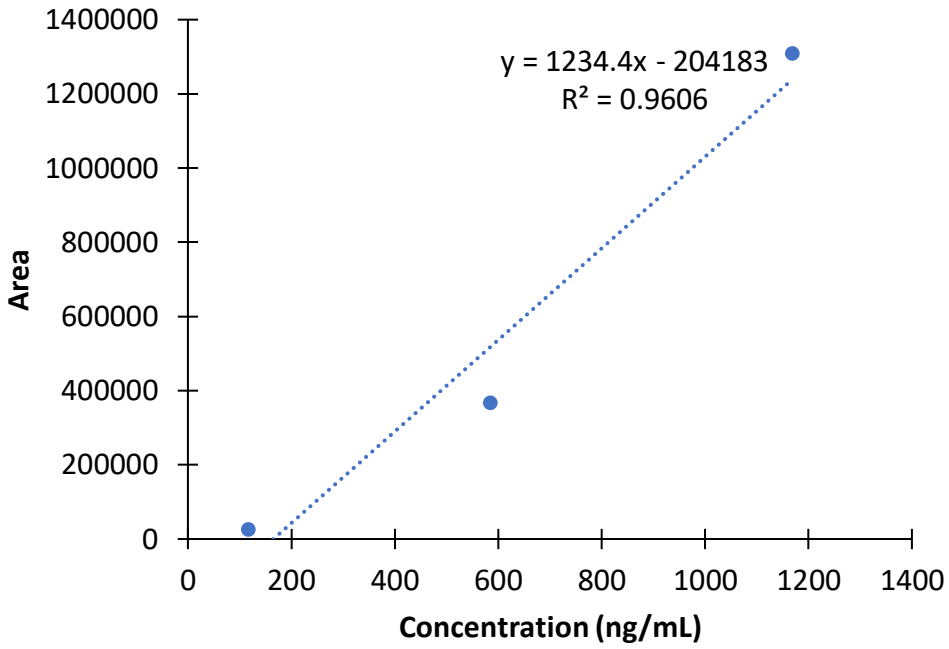


Fig. A9. Calibration curve for arabinose determination by HPLC analysis.

Appendix B. Hydrolysis of citrus pectin by subcritical water modified with carboxylic acids.

Table B1. Hexuronic acids content of pectic hydrolysates.

Time (min)	Temperature (°C)	Solvent	HexA concentration (g/g pectin)		Average concentration (g/g pectin)
			Rep1	Rep2	
10	160	Water	0.50	0.59	0.54±0.06[a]
20			0.56	0.54	0.55±0.01[a]
30			0.60	0.54	0.57±0.04[a]
40			0.52	0.58	0.55±0.04[a]
50			0.48	0.5	0.49±0.02[a]
60			0.46	0.48	0.47±0.01[a]
10	135	Water	0.09	0.03	0.06±0.04[c]
	145		0.12	0.13	0.13±0.00[c]
	160		0.50	0.59	0.54±0.06[b]
	170		0.48	0.41	0.45±0.05[b]
	180		0.52	0.53	0.52±0.01[b]
	200		0.71	0.71	0.71±0.00[a]
10	160	Citric acid 0.2% (w/w)	0.48	0.48	0.48±0.00[A]
20			0.73	0.67	0.70±0.04[A]
30			0.54	0.59	0.56±0.03[A]
40			0.62	0.7	0.65±0.06[A]
50			0.50	0.68	0.59±0.13[A]
60			0.6	0.51	0.55±0.06[A]
10	135	Citric acid 0.2% (w/w)	0.05	0.07	0.06±0.01[B]
	145		0.18	0.13	0.16±0.04[B]
	160		0.48	0.48	0.48±0.00[A]
	170		0.57	0.58	0.58±0.01[A]
	180		0.61	0.64	0.63±0.02[A]
	200		0.56	0.71	0.63±0.11[A]
10	160	Malic acid 0.2% (w/w)	0.63	0.60	0.62±0.02[*]
20			0.80	0.81	0.80±0.00[**]
10	180		0.89	0.97	0.93±0.06[**]

The average that does not share the same lowercase letter (a-c), uppercase letters (A-B) and symbols (*-**) are statistically different (Tukey's test, $p<0.05$).

Table B2. Rhamnose content of pectic hydrolysates.

Time (min)	Temperature (°C)	Solvent	Rhamnose concentration (g/g pectin)		Average content (g/g pectin)
			Rep1	Rep2	
10	160	Water	0.05	0.05	0.05±0.002[a]
20			0.05	0.05	0.05±0.001[a]
30			0.05	0.07	0.06±0.012[a]
40			0.05	0.05	0.05±0.001[a]
50			0.05	0.05	0.05±0.002[a]
60			0.05	0.05	0.05±0.001[a]
10	135	Water	0	0	0
	145		0	0	0
	160		0.05	0.06	0.05±0.002[a]
	170		0.05	0.04	0.05±0.008[a]
	180		0.05	0.06	0.05±0.003[a]
	200		0	0	0
10	160	Citric acid 0.2% (w/w)	0.05	0.05	0.05±0.00[A]
20			0.06	0.06	0.06±0.002[A]
30			0.06	0.06	0.06±0.001[A]
40			0.06	0.07	0.06±0.005[A]
50			0.07	0.07	0.07±0.002[A]
60			0.07	0.07	0.07±0.001[A]
10	135	Citric acid 0.2% (w/w)	0	0	0
	145		0	0	0
	160		0.05	0.05	0.05±0.00[C]
	170		0.06	0.06	0.06±0.00[B]
	180		0.08	0.07	0.08±0.002[A]
	200		0.05	0.05	0.05±0.00[D]
10	160	Malic acid 0.2% (w/w)	0.06	0.06	0.06±0.001[*]
20			0.07	0.06	0.07±0.007[*]
10	180		0.08	0.08	0.08±0.001[**]

The average that does not share the same lowercase letter (a), uppercase letters (A-D) and symbols (*-**) are statistically different (Tukey's test, $p<0.05$).

Table B3. $DPPH^{\cdot}$ scavenging activity of pectic hydrolysates.

Time (min)	Temperature (°C)	Solvent	Scavenging activity (%)		Average (%)
			Rep1	Rep2	
10	160	Water	84.61	85.95	85.28±0.95^{a}
20			78.72	79.73	79.23±0.71^{b}
30			75.95	77.04	76.49±0.77^{bc}
40			75.78	76.96	76.37±0.83^{bc}
50			71.15	71.83	71.49±0.48^{d}
60			73.93	71.99	72.96±1.37^{cd}
10	135	Water	74.35	80.82	77.59±4.58^{ab}
	145		73.17	71.40	72.29±1.25^{bc}
	160		84.61	85.95	85.28±0.95^{a}
	170		74.60	73.84	74.22±0.54^{bc}
	180		68.21	70.23	69.22±1.43^{c}
	200		67.62	67.87	67.75±0.18^{c}
10	160	Citric acid 0.2% (w/w)	90.58	90.66	90.62±0.06^{A}
20			87.22	87.13	87.17±0.06^{B}
30			82.84	82.93	82.88±0.06^{CD}
40			85.28	84.02	84.65±0.89^{C}
50			81.08	81.67	81.37±0.42^{D}
60			80.91	81.92	81.41±0.71^{D}
10	135	Citric acid 0.2% (w/w)	40.71	40.45	40.58±0.18^{F}
	145		71.24	70.23	70.73±0.71^{E}
	160		90.58	90.66	90.62±0.06^{A}
	170		83.26	84.19	83.73±0.65^{B}
	180		81.16	81.16	81.16±0.00^{C}
	200		77.54	78.30	77.92±0.54^{D}
10	160	Malic acid 0.2% (w/w)	93.15	91.99	92.57±0.82*
20			91.49	91.41	91.45±0.06*
10	180		87.28	87.45	87.37±0.12**

The average that does not share the same lowercase letter (a-d), uppercase letters (A-D) and symbols (*-**) are statistically different (Tukey's test, $p<0.05$).

Table B4. Ferric reducing antioxidant power of pectic hydrolysates.

Time (min)	Temperature (°C)	Solvent	Concentration (μM)		Average concentration (μM)
			Rep1	Rep2	
10	160	Water	716.55	1003.16	859.85±202.66^{c}
20			961.97	868.68	915.32±65.97^{bc}
30			1006.52	1029.21	1017.87±16.05^{abc}
40			1284.73	1259.51	1272.12±17.83^{a}
50			1321.71	1384.75	1353.23±44.57^{a}
60			1227.57	1218.33	1222.95±6.54^{ab}
10	135	Water	166.86	170.22	168.54±2.38^{b}
	145		302.18	206.36	254.27±67.75^{b}
	160		716.55	1003.16	859.85±202.66^{a}
	170		1077.96	1043.50	1060.73±24.37^{a}
	180		1178.82	1156.13	1167.48±16.05^{a}
	200		828.33	987.19	907.76±112.33^{a}
10	160	Citric acid 0.2% (w/w)	1015.76	971.22	993.49±31.50^{C}
20			1425.09	1422.57	1423.83±1.78^{A}
30			1436.02	1382.22	1409.12±38.04^{A}
40			1406.60	1359.53	1383.06±33.28^{A}
50			1327.59	1359.53	1343.56±22.58^{A}
60			1223.37	1169.58	1196.47±38.04^{B}
10	135	Citric acid 0.2% (w/w)	229.89	207.20	218.55±16.05^{C}
	145		216.45	171.90	194.17±31.50^{C}
	160		1015.76	971.22	993.49±31.50^{B}
	170		1487.29	1542.76	1515.02±39.23^{A}
	180		1467.11	1555.37	1511.24±62.40^{A}
	200		1425.93	1462.07	1444.00±25.56^{A}
10	160	Malic acid 0.2% (w/w)	767.82	828.33	798.07±42.79*
20			1000.64	998.95	999.79±1.19*
10	180		1466.27	1440.22	1453.25±18.42**

The average that does not share the same lowercase letter (a-c), uppercase letters (A-C) and symbols (*-**) are statistically different (Tukey's test, $p<0.05$).

Appendix C. Hydrolysis of pea protein concentrate in subcritical water media with addition of citrus pectin and citric acid.

Table C1. Degree of hydrolysis of pea protein hydrolysates.

Time (min)	Temperature (°C)	Protein:catalyst ratio	Degree of hydrolysis (%)		Average (%)
			Rep1	Rep2	
10	180	Protein:Pectin 1:1 (w/w)	24.01	26.81	25.41 ± 1.98^{B}
20			34.53	33.21	33.87 ± 0.93^{B}
30			44.08	43.64	43.86 ± 0.31^{A}
40			53.81	47.15	50.48 ± 4.71^{A}
50			48.81	47.15	47.98 ± 1.18^{A}
60			47.32	46.45	46.88 ± 0.62^{A}
10	160	Protein:Pectin 1:1 (w/w)	15.77	9.98	12.88 ± 4.09^{B}
	180		24.01	26.81	25.41 ± 1.98^{B}
	200		23.66	27.16	25.41 ± 2.48^{B}
	220		63.36	66.17	64.76 ± 1.98^{A}
	240		68.53	55.04	61.78 ± 9.54^{A}
10	180	Protein:citric acid 1:0.2 (w/w)	26.05	25.12	$25.58\pm0.66^{\alpha}$
20			24.42	22.57	$23.50\pm1.31^{\alpha}$
30			27.82	26.82	$27.32\pm0.71^{\alpha}$
40			28.36	29.21	$28.79\pm0.60^{\alpha}$
50			29.68	29.45	$29.56\pm0.16^{\alpha}$
60			23.73	31.38	$27.55\pm5.41^{\alpha}$
10	160	Protein:citric acid 1:0.2 (w/w)	23.50	22.34	$22.92\pm0.82^{\beta}$
	180		26.05	25.12	$25.58\pm0.66^{\beta}$
	200		22.26	24.58	$23.42\pm1.64^{\beta}$
	220		29.21	25.12	$27.17\pm2.90^{\beta}$
	240		34.86	34.47	$34.66\pm0.27^{\alpha}$
10	180	Protein (control)	26.59	25.04	25.81 ± 1.09^{d}
20			33.46	28.13	30.80 ± 3.77^{bcd}
30			25.51	25.81	25.66 ± 0.22^{cd}
40			40.88	40.03	40.46 ± 0.60^{a}
50			30.37	34.31	32.34 ± 2.79^{bc}
60			37.87	39.57	38.72 ± 1.20^{ab}
10	160	Protein (control)	22.18	23.34	22.76 ± 0.82^{c}
	180		26.59	25.04	25.81 ± 1.09^{c}
	200		28.36	31.53	29.95 ± 2.24^{b}
	220		37.95	39.65	38.80 ± 1.20^{a}
	240		39.96	42.27	41.12 ± 1.64^{a}

The average that does not share the same lowercase letter (a-d), uppercase letter (A-B), Greek letter (α-β), or symbol (*-****) are statistically different (Tukey's test, $p<0.05$).

Table C1. *Continued*

Time (min)	Temperature (ºC)	Protein:catalyst ratio	Degree of hydrolysis (%)		Average (%)
			Rep1	Rep2	
10	180	Protein:pectin 1:0.1 (w/w)	19.62	18.66	19.14±0.68****
		Protein:pectin 1:0.5 (w/w)	16.73	16.38	16.56±0.25****
		Protein:pectin 1:1 (w/w)	24.01	26.81	25.41±1.98***
		Protein:pectin 1:2 (w/w)	39.08	38.21	38.64±0.62**
		Protein:pectin 1:3 (w/w)	64.33	64.50	64.41±0.12*

Table C2. protein/peptide/amino acid content of pea protein hydrolysates.

Time (min)	Temperature (°C)	Protein:catalyst ratio	Protein/peptide/amino acid content (mg/g protein)		Average content (mg/g protein)
			Rep1	Rep2	
10	180	Protein:Pectin 1:1 (w/w)	717.12	755.52	736.32 ± 27.15^{B}
20			812.32	859.52	835.92 ± 33.38^{AB}
30			887.52	891.52	889.52 ± 2.83^{A}
40			899.52	906.72	903.12 ± 5.09^{A}
50			881.92	867.52	874.72 ± 10.18^{A}
60			844.32	937.12	890.72 ± 65.62^{A}
10	160	Protein:Pectin 1:1 (w/w)	625.12	620.32	622.72 ± 3.39^{A}
	180		717.12	755.52	736.32 ± 27.15^{A}
	200		609.12	697.92	653.52 ± 62.79^{A}
	220		824.32	775.52	799.92 ± 34.51^{A}
	240		825.92	711.52	768.72 ± 80.89^{A}
10	180	Protein:citric acid 1:0.2 (w/w)	429.92	452.32	$441.12\pm1.58^{\alpha}$
20			461.12	445.92	$453.52\pm10.75^{\alpha}$
30			485.92	496.32	$491.12\pm7.35^{\alpha}$
40			533.12	554.72	$543.92\pm15.27^{\alpha}$
50			517.92	553.92	$535.92\pm25.46^{\alpha}$
60			463.52	579.52	$521.52\pm82.02^{\alpha}$
10	160	Protein:citric acid 1:0.2 (w/w)	425.92	392.32	$409.12\pm23.76^{\gamma}$
	180		429.92	452.32	$441.12\pm15.84^{\beta\gamma}$
	200		522.72	505.12	$513.92\pm12.45^{\alpha}$
	220		534.72	537.92	$536.32\pm2.26^{\alpha}$
	240		505.12	489.12	$497.12\pm11.31^{\alpha\beta}$
10	180	Protein (control)	401.92	417.92	409.92 ± 11.31^{cd}
20			429.12	428.32	428.72 ± 0.57^{bc}
30			385.12	381.12	383.12 ± 2.83^{e}
40			396.32	400.32	398.32 ± 2.83^{de}
50			436.32	431.52	433.92 ± 3.39^{b}
60			455.52	454.72	455.12 ± 0.57^{a}

The average that does not share the same lowercase letter (a-e), uppercase letter (A-B), Greek letter (α-γ), or symbol (*-***) are statistically different (Tukey's test, $p<0.05$).

Table C2. *Continued*

Time (min)	Temperature (°C)	Protein:catalyst ratio	Protein/peptide/amino acid content (mg/g protein)		Average content (mg/g protein)
			Rep1	Rep2	
10	160	Protein (control)	341.12	338.72	339.92±1.69[c]
	180		401.92	417.92	409.92±11.31[b]
	200		425.92	431.52	428.72±3.96[b]
	220		486.72	493.92	490.32±5.09[a]
	240		489.92	486.72	488.32±2.26[a]
10	180	Protein:pectin 1:0.1 (w/w)	465.12	478.72	471.92±9.62***
		Protein:pectin 1:0.5 (w/w)	595.52	598.72	597.12±2.26***
		Protein:pectin 1:1 (w/w)	821.12	821.92	736.32±27.15**
		Protein:pectin 1:2 (w/w)	989.92	1057.92	1023.92± 48.08*
		Protein:pectin 1:3 (w/w)	1283.52	1137.12	1210.32± 103.52*

Table C3. $DPPH^{\cdot}$ scavenging activity of pea protein hydrolysates.

Time (min)	Temperature (°C)	Protein:catalyst ratio	Scavenging activity (%)		Average (%)
			Rep1	Rep2	
10	180	Protein:Pectin 1:1 (w/w)	73.09	71.44	72.27 ± 1.17^{a}
20			59.98	62.50	61.24 ± 1.78^{ab}
30			47.94	47.68	47.81 ± 0.19^{b}
40			47.51	52.41	49.96 ± 3.46^{b}
50			47.96	50.42	49.19 ± 1.74^{b}
60			52.27	64.73	58.50 ± 8.81^{ab}
10	160	Protein:Pectin 1:1 (w/w)	66.18	68.13	67.15 ± 1.38^{a}
	180		73.09	71.44	72.27 ± 1.17^{a}
	200		73.00	80.90	76.95 ± 5.58^{a}
	220		72.57	75.44	74.00 ± 2.03^{a}
	240		73.80	66.53	70.16 ± 5.14^{a}
10	180	Protein:citric acid 1:0.2 (w/w)	89.09	89.42	89.25 ± 0.23^{A}
20			91.23	90.00	90.61 ± 0.87^{A}
30			91.32	91.60	91.46 ± 0.20^{A}
40			90.85	91.04	90.94 ± 0.13^{A}
50			89.25	89.62	89.43 ± 0.27^{A}
60			88.96	87.17	88.07 ± 1.27^{A}
10	160	Protein:citric acid 1:0.2 (w/w)	76.24	75.59	75.92 ± 0.46^{D}
	180		89.09	89.42	89.25 ± 0.23^{B}
	200		90.72	91.04	90.88 ± 0.23^{A}
	220		85.49	85.70	85.59 ± 0.15^{C}
	240		84.53	85.06	84.79 ± 0.38^{C}
10	180	Protein:pectin 1:0.1 (w/w)	42.80	29.72	$36.26\pm9.25^{***}$
		Protein:pectin 1:0.5 (w/w)	39.66	39.05	$39.35\pm0.43^{***}$
		Protein:pectin 1:1 (w/w)	73.09	71.44	$72.27\pm1.17^{**}$
		Protein:pectin 1:2 (w/w)	90.21	89.48	$89.85\pm0.51^{*}$
		Protein:pectin 1:3 (w/w)	83.88	88.39	$86.14\pm3.19^{*}$

The average that does not share the same lowercase letter (a-b), uppercase letters (A-D) and symbols (*-***) are statistically difference (Tukey's test, $p<0.05$).

Table C4. Ferric reducing antioxidant power of pea protein hydrolysates.

Time (min)	Temperature (°C)	Protein:catalyst ratio	Concentration (μM)		Average concentration (μM)
			Rep1	Rep2	
10	180	Protein:Pectin 1:1 (w/w)	430.77	435.82	433.30±3.57^{bc}
20			374.46	342.52	358.49±22.58^{c}
30			473.64	444.22	458.93±20.80^{ab}
40			503.90	519.87	511.88±11.29^{ab}
50			550.97	496.33	523.65±38.63^{a}
60			419.01	441.70	430.35±16.05^{bc}
10	160	Protein:Pectin 1:1 (w/w)	331.59	345.04	338.32±9.51^{c}
	180		430.77	435.82	433.30±3.57^{a}
	200		389.59	398.84	394.21±6.54^{b}
	220		440.86	419.01	429.93±15.45^{a}
	240		383.71	381.18	382.45±1.78^{b}
10	180	Protein:citric acid 1:0.2 (w/w)	195.43	182.83	189.13±8.91^{D}
20			223.17	221.49	222.33±1.19^{CD}
30			252.59	293.77	273.18±29.12^{CD}
40			322.35	303.86	313.10±13.08^{BC}
50			393.79	482.05	437.92±62.40^{A}
60			422.37	418.17	420.27±2.97^{AB}
10	160	Protein:citric acid 1:0.2 (w/w)	159.29	173.58	166.44±10.10^{C}
	180		195.43	182.83	189.13±8.91^{C}
	200		420.69	404.72	412.70±11.29^{B}
	220		620.73	752.69	686.71±93.31^{A}
	240		500.54	534.16	517.35±23.77^{AB}
10	180	Protein:pectin 1:0.1 (w/w)	84.49	62.63	73.56±15.45*****
		Protein:pectin 1:0.5 (w/w)	129.87	136.60	133.24±4.75****
		Protein:pectin 1:1 (w/w)	430.77	435.82	433.30±3.57***
		Protein:pectin 1:2 (w/w)	747.64	751.01	749.32±2.38**
		Protein:pectin 1:3 (w/w)	1139.32	1162.85	1151.09±16.64*

The average that does not share the same lowercase letter (a-c), uppercase letters (A-D) and symbols (*-*****) are statistically different (Tukey's test, $p<0.05$).