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Aalto University
School of Chemical
Engineering

Pinja Pöri

ENZYMATIC MODIFICATION OF OAT PROTEIN CONCENTRATE FOR INCREASED FIBRILLATION DURING HIGH-MOISTURE EXTRUSION COOKING

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Supervisor   Prof. Jan Deska

Instructors   M.Sc. Anni Nisov
                       D.Sc. Emilia Nordlund

A? Aalto University School of Chemical Engineering

Author Pinja Pöri	

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Abstract

This thesis was done at VTT Technical Research Centre of Finland Ltd as a part of the OatHow project funded by Business Finland. The aim of this thesis was to increase fibrillation between oat proteins during high-moisture extrusion cooking by enzymatic modification of oat protein concentrate (OPC). Protein concentrates do not typically fibrillate well due to their low protein content and the presence of other components e.g. starch. Enzymatic modification of oat proteins and hydrolysis of starch may lead to improved fibrillation due to the creation of a stronger protein network. Three commercial enzymes were used to modify the OPC. Transglutaminase (WM enzyme) was used to cross-link oat proteins, a mix of transglutaminase and protein-glutaminase (SYG enzyme) was used to cross-link as well as deamidate oat proteins, and α-amylase (BAN enzyme) was used to hydrolyse the starch from the OPC. Different treatment conditions were tested e.g. preheating, temperature, enzyme dosage, and incubation time. Protein solubility, viscosity, particle size distribution, and SDS-PAGE were analysed from the modified OPC. The modified OPC samples were extruded in a high-moisture extrusion cooker and tensile strength as well as free thiol groups were analysed from the extrudates.

The results showed that both protein-modifying enzymes (WM and SYG) were able to induce the cross-linking reaction in the oat proteins. The highest cross-linking reaction was observed in the BAN+WM sample, where the starch had been hydrolysed. The samples treated with SYG had increased protein solubility, and decreased particle sizes. Viscosity was increased in the preheated SYG 5 U and the preheated BAN+SYG 5 U samples. The WM modified samples had a slight decrease in protein solubility and decreased particle sizes, although less than in the SYG treated samples. In addition, WM had no effect on the viscosity when compared to the control. When the samples were studied in the extrusion, two extrudates (SYG 0.5 U and WM 0.5 U) showed indications of fibrillation when compared to the extruded reference sample. All the non-preheated extrudates showed a rigid structure probably due to the formation of more disulphide bonds during the extrusion, while fewer disulphide bonds were formed in the preheated extrudates. The preheated SYG 5 U sample had the strongest structure according to the tensile strength results, probably since the proteins gelatinized in the elevated temperatures and formed a strong protein network. The viscosity result also supported this hypothesis. The BAN treated extrudates had a brittle structure, probably due to the strong hydrolysis of starch.

Overall, this thesis provided new information about the functional properties of the modified oat proteins and the high-moisture extrusion cooking of the oat proteins. It was observed that a stronger protein network can be formed by enzymatic modification, however, a clear fibrillated structure was not observed. In the future, higher protein content OPC, partial hydrolysis of starch, and larger variety of the extrusion parameters could be tested to increase the fibrillation of the OPC.

Keywords Cross-linking, oat protein concentrate, high-moisture extrusion cooking

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Tiivistelmä

Tämä lopputyö tehtiin Teknologian tutkimuskeskus VTT Oy:lle, OatHow projektiin, jonka on rahoittanut Business Finland. Työn tavoitteena oli parantaa kauraproteiinin säikeistymistä märkäekstruusion aikana, muokkaamalla kauraproteiinikonsentraattia (KPK) entsyymeillä. Proteiini konsentraatit eivät tyypillisesti muodosta säikeitä hyvin, koska proteiini-pitoisuus on alhainen ja ne sisältävät muita komponentteja, kuten tärkkelystä. Proteiinien entsymaattinen muokkaus ja tärkkelyksen hydrolysointi saattavat parantaa proteiinien säikeisyyttä muodostamalla vahvan proteiiniverkoston. Kolmea kaupallista entsyymiä käytettiin KPK:n muokkaukseen. Transglutaminaasia (WM-entsyymi) käytettiin kauraproteiinien ristisilloitukseen ja transglutaminaasin ja proteiini-glutaminaasin sekoitusta (SYG-entsyymi) käytettiin ristisilloitukseen, sekä deamidaatioon. Amylaasia (BAN-entsyymi) käytettiin KPK:n sisältämän tärkkelyksen hydrolysoimiseen. Erilaisia käsittelyolosuhteita testattiin, kuten esikuumennusta, lämpötilaa, entsyymin annostusta ja inkubointi-aikaa. Proteiinin liukoisuus, viskositeetti, partikkelikoko-jakauma sekä proteiiniprofiili analysoitiin muokatusta KPK:sta. Muokatut KPK-näytteet ekstruudattiin korkeassa vesipitoisuudessa ja niistä tutkittiin vetolujuus sekä vapaiden tioli-ryhmien määrä.

Tulokset osoittivat, että molemmat proteiineja muokkaavat entsyymit (WM ja SYG) pystyivät ristisilloittamaan kauraproteiineja. Eniten ristisilloittumista havaittiin BAN+WM -näytteestä, missä tärkkelys oltiin hydrolysoitu. Proteiinin liukoisuus kasvoi ja partikkelikoko pieneni SYG-entsyymillä. Viskositeetti kasvoi esikuumennetuissa SYG 5 U ja BAN+SYG -näytteissä. WM-muokatuissa näytteissä proteiinien liukoisuus laski hiukan. Myös partikkelikoko laski, mutta vähemmän kuin SYG:llä käsitellyissä näytteissä. Lisäksi, WM-entsyymillä ei ollut vaikutusta viskositeettiin, kun sitä verrattiin kontrolliin. Ekstruusiossa huomattiin kahden näytteen (SYG 0.5 U ja WM 0.5 U) näyttävän mahdollisia merkkejä säikeisyydestä, kun niitä verrattiin referenssi näytteeseen. Kaikilla ekstrudaateilla, joita ei oltu esikuumennettu, havaittiin jämäkkä rakenne, mahdollisesti ekstruusion aikana muodostuneiden rikkisidosten takia. Esikuumennetuissa ekstrudaateissa rikkisidoksia muodostui ekstruusion aikana vähemmän. Vetolujuus-tulosten mukaan vahvin rakenne oli esikuumennetulla SYG 5 U -ekstrudaatilla, mikä johtui luultavasti proteiinien geeliytymisreaktiosta korkeassa lämpötilassa, joka muodosti vahvan proteiiniverkoston. Viskositeetti-tulos tuki myös kyseistä hypoteesia. BAN-käsitellyillä näytteillä oli hauras rakenne, joka johtui mahdollisesti tärkkelyksen voimakkaasta hydrolysoitumisesta.

Kaiken kaikkiaan tämä työ antoi uutta tietoa muokattujen kauraproteiinien funktionaalisista ominaisuuksista sekä kauraproteiinien märkäekstruusiosta. Työssä todettiin, että vahvempi proteiiniverkosto voidaan saavuttaa entsymaattisen muokkauksen avulla, mutta selkeää säikeistä rakennetta ei havaittu ekstrudaateista. Tulevaisuudessa voitaisiin testata korkeamman proteiinipitoisuuden KPK:a, osittaista tärkkelyksen hydrolysointia ja useampia ekstruusio-parametrejä lisäämään KPK:n säikeisyyttä.

Avainsanat Ristisilloitus, kauraproteiini konsentraatti, märkäekstruusio

Preface

Helsinki, 23rd of November 2020

Pinja Pöri

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Abbreviations

BAN	BAN 480 L (α-amylase)
BSA	Bovin serum albumin
EDTA	Ethylenediaminetetraacetic acid
FPC	Faba protein concentrate
FPI	Faba protein isolate
HMEC	High-moisture extrusion cooking
L	Lengthwise direction
NaP	Sodium phosphate
OPC	Oat protein concentrate
OPI	Oat protein isolate
PG	Protein-glutaminase
RT	Room temperature
SPI	Soy protein isolate
SYG	Activa SYG (protein-glutaminase / transglutaminase mix)
TG	Transglutaminase
W	Widthwise direction
WM	Activa WM (transglutaminase)

1. Introduction

Avena sativa L., common oat, belongs to the *Gramineae* family and is one of the oldest cultivated crop (Lásztity, 1998). Almost 60% of oats are produced in Northern Europe since oat is a robust crop that tolerates wet weather and acidic soils. Furthermore, it grows optimally in moderate temperature and enjoys long day length (Mäkinen et al., 2016; Arendt, Elke K. & Zannini, 2013).

Most of the produced oats are going to animal feed (50-90%), while the rest is used for human consumption e.g. as flours and flakes. Many health beneficial components have been recognized from oats, including cholesterol lowering beta-glucan and other dietary fibers as reviewed by Sterna et al. (2016), Lásztity (1998) and El Khoury et al. (2012). The protein level of oats (15-20%) is the highest among the cereals and its amino acid composition is superior due to the high amounts of lysine and threonine. Oats are also a good source of fats, minerals, and antioxidants (Klose & Arendt, 2012; Peterson, 2001).

The demand for plant proteins has increased due to the sustainability issues related to animal proteins. Soybean has been one of the most important plant protein source for a long time, but issues related to GMO, sustainability, and allergenicity forces the manufacturers to investigate other plant protein sources (Mäkinen et al., 2016). The interest to use oats to replace animal derived food products has become popular. Oat protein composition lacks gluten, thus it is a suitable ingredient for most people with celiac disease. However, oat proteins have poor solubility and other functional properties that restrict their use in food applications. Improving the functionality of oat proteins is necessary for their utilization in food products (Nivala et al., 2017).

This thesis concentrates to the modification of oat protein concentrate (OPC) by enzymes to improve its functionality and especially the performance during the high-moisture extrusion cooking (HMEC). The HMEC is commonly used in the formation of meat mimicking structures through proteins. During the HMEC

proteins denaturate, unfold, realign, and cross-link as a result of the heat, pressure, shear, and cooling (Zahari et al., 2020).

In this thesis a protein concentrate is defined to have a maximum of 70% protein, where as a protein isolate is defined to have over 70% protein content. The OPC used in this thesis was modified by a protein modifying enzymes, transglutaminase (TG) and a mixture of TG and protein-glutaminase (PG), that are commercial food grade enzymes known to alter the structure and functional properties of oat proteins (Nivala et al., 2017; Jiang et al., 2015; Siu et al., 2002). Additionally, α-amylase was used to hydrolyze the starch from the OPC. These modifications were expected to lead to the improvement of oat proteins' functional properties and to the creation of a strong protein network which may enhance the proteins aligment during the extrusion. This thesis is divided into two parts; the first part reviews the related literature and the second part discusses the experimental part of the thesis.

LITERATURE PART

2. Composition of oat

Oat grains are nutritionally valuable since they have naturally great amounts of beneficial nutrients and micronutrients e.g. soluble fibers, insoluble fibers, proteins, unsaturated fatty acids, vitamins, minerals, and antioxidants (Klose & Arendt, 2012). The four main components of oat grain are hull, bran, starchy endosperm, and germ, i.e. embryo. These components are represented in Figure 1.
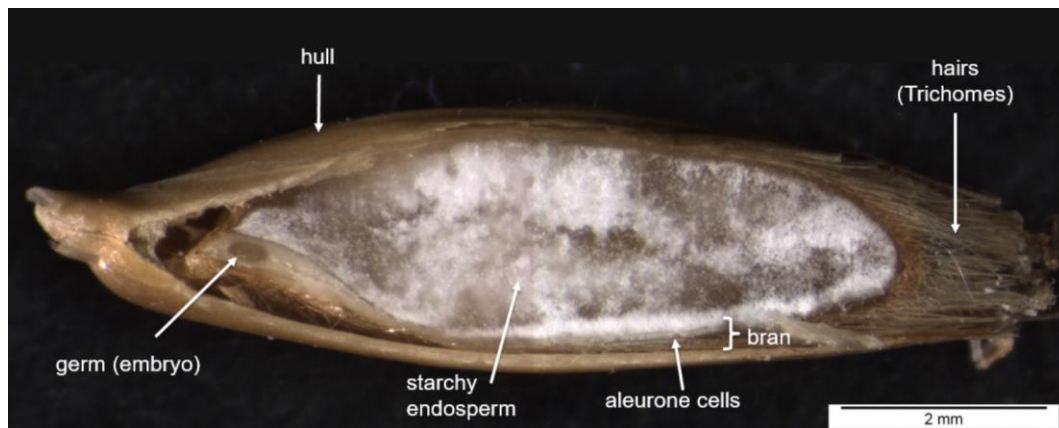
Figure 1. Structure of oat grain. Courtesy of VTT Technical Research Centre of Finland Ltd / Ulla Holopainen-Mantila.

The hull of the oat grain consist of a lemma and palea. The function of the hull is to protect the grain. Chemically the hull is composed mostly from cellulose and hemicellulose. Some minor amounts of lignin or other phenolic compounds are also present (Webster & Wood, 2011). The bran is the outer layer of the grain. It consist of a pericarp, seed coat, nucellus, aleurone layer, and a part of starchy endosperm. Inside the bran there are protein bodies, neutral lipids, β-glucans, phenolics, niacins, phytins, and aromatic amines (Webster & Wood, 2011). The starchy endosperm forms the largest part of a kernel. It has reserves for starch, protein, lipids, and β-glucan. These are all hydrolyzed by enzymes from the

aleurone layer, during the germination, to provide nutrients to the developing embryo (Klose & Arendt, 2012). The germ is made of an embryonic axis and a scutellum. It is a structure that has metabolic activity. The mature plant rises from the germ. The germ has notable amounts of proteins and lipids, but only a little bit of starch. Every protein body is enriched by lipid bodies in the germ (Webster & Wood, 2011). The amino acid composition differs notably between the germ and the starchy endosperm, e.g. germ has almost 90% higher lysine content, but 37% lower glutamic acid content (Klose & Arendt, 2012).

2.1 Oat proteins

Depending on the growing conditions of the oat and its genotype, the grain can include protein of approximately 15 to 20% of its weight. Of all physiological parts of the kernel, the germ has the highest protein content of over 30%. The protein content in the bran is approximately 20%, in the starchy endosperm 10%, and in the hulls about 2%. However, out of the total protein content in the oat grain, the germ provides only 3% whereas the bran and the starchy endosperm provides 49% and 45%, respectively (Klose & Arendt, 2012).

Storage proteins are the largest group of proteins in oat grains. The most common storage protein types in oats are albumin, globulin, avenin (prolamin), and glutelin. Plant proteins are often classified by the Osborne classification, meaning they are classified according to their solubility in different solvents (Arendt & Zannini, 2013; González-Pérez & Arellano, 2009). In food applications, solubility is a critical factor as proteins that are soluble provide homogenous dispersability in colloidal systems and improve the interfacial properties (Mäkinen et al., 2016). The Osborne classification of oat proteins is presented in table 1.

Table 1. Protein content, molecular weight, isoelectric point, solubility, and amino acid content of lysine and glutamic acid of oat fractions represented by the Osborne fractionation. Lysine and glutamine (represented as glutamic acid) contents are shown since transglutaminase induced cross-linking reaction between these residues. Modified from Klose & Arendt, 2012 and Klose et al., 2009.

Protein fraction	% of total protein	Molecular weight (10^3)	Isoelectric point	Protein solubility	Lysine (mg/g)	Glutamic acid (mg/g)
Albumin	1-12	14-17 / 20-27 / 36-47	pH 4-7	Water	13.91	39.00
Globulin	50-80	20-35 / 50-60	pH 5.5 and pH 8-10	Salt	18.89	127.37
Avenin	4-15	17-34	pH 5-9	Alcohol	4.84	125.23
Glutelin	< 10	9	-	Base/Acid	7.50	37.76

Oat albumins are water soluble storage proteins which represents 1 to 12 % of the total protein content in oat. Albumins amino acid balance shows high contents of lysine, asparagine-aspartic acid, and alanine, compared to globulins, avenins, and glutelins. Oat albumins have a low amount of glutamine-glutamic acid. Most often lysine is located in the embryo, which suggest that albumins occur in and around the embryo (Klose & Arendt, 2012).

Globulins are the largest group of oat storage proteins (50-80%) and consist of different polypeptides. They are soluble in saline solutions. Oat globulins can be separated to three main fractions through sedimentation coefficients: 3S, 7S, and 12S (monomer, trimer, and hexamer structure, respectively). The 12S fraction is the major component. It is an oligomeric protein with a quaternary structure that consist of basic (β) and acidic (α) polypeptides, with molecular weights of 22 and 32 kDa, respectively. Together these polypeptides form a dimer of 54 kDa. The hexamer structure is formed with six of these dimers. Acidic polypeptide is rich in glutamic acid and basic polypeptide is rich in asparagine. The structure of 12S fraction is similar to the legume storage protein 11S. The 3S and 7S oat globulins are vicilin-like proteins. The 3S fraction consists of two major components that have molecular weights of 15 and 21 kDa. The 7S fractions major component has a molecular weight of 55 kDa (Arendt & Zannini, 2013; Klose & Arendt, 2012). Typically vicilin-like proteins are trimeric proteins that have subunits of different molecular weights and they lack cysteine residues and therefore cannot form

disulphide bonds (González-Pérez & Arellano, 2009). The tertiary structure of oat globulins is sensitive to the modification of pH and ionic strength since they influence the association or dissociation action of the subunits. Oat globulin has a denaturation temperature of 110 °C, which is high compared to other plant seed globulins. The strong hydrophobic interactions with subunits, get stronger in elevated temperatures. This may explain why the denaturation temperature is high (Mäkinen et al., 2016).

Oat avenins (prolamins) represent about 4 to 15% of the total protein content. They are soluble to a 70% ethanol and can withstand the ethanol without losing disulphide bonds. Oat avenins are rich in sulphur, they have small amounts of basic amino acids but lots of glutamic acid and glutamine. Avenins are presumable present in protein bodies in the endosperm. Oat avenins molecular weight varies between 17 to 34 kDa (Klose & Arendt, 2012). Generally, avenins contain small amounts of charged amino acid residues, which can result in proteins that have a low net charge at acidic or alkaline conditions (Mäkinen et al., 2016). The effect of low avenin and high globulin composition in an oat grain results in a better essential amino acid balance compared to other cereal proteins (Klose & Arendt, 2012).

Oat glutelin represents less than 10% of the total protein content and its molecular weight is approximately 9 kDa. Glutelin is partly soluble to acidic or alkaline solutions, and only fully soluble when disulphide bonds are reduced. The amount of glutelins in the oat grain is related to the extraction of the other storage proteins: albumin, globulin, and avenin. As a result, the reported glutelin values can differ significantly (Klose & Arendt, 2012).

2.2 Other components in oats

Starch is the largest component in the oat grain. It occurs mostly as aggregated individual granules, unlike in other cereals. Its amount in oat grain differs between cultivars from 43% to 64% (Klose & Arendt, 2012). Most of the oat starch is stored

in the starchy endosperm and its mainly composed of amylose and amylopectin (Arendt & Zannini, 2013).

The amount of lipids in oat grain is much higher compared to other cereal grains. On average, oats total lipid amount is about 7% whereas the total lipid amount of e.g. wheat, rye, corn, barley, and rice varies between 1.7-3.6%. In oat, the fat is spread throughout the starchy endosperm, while in other cereals it is mostly centered in the germ (Arendt & Zannini, 2013). Nearly all of the oat fats are neutral lipids including some amounts of glycolipids and phospholipids. The main fatty acid is linoleic acid, though some amounts of oleic and palmitic acids have also been detected (Webster & Wood, 2011).

Oats are a good source of dietary fibers with β-glucan and arabinoxylans being the most abundant. Other dietary fibers found in oats include e.g. celluloses, glucomannans, and mannoses (Klose & Arendt, 2012). Oats β-glucan is a soluble fiber that have numerous health benefits documented (El Khoury et al., 2012). β-glucans are non-starch polysaccharides, that are formed by D-glucose monomers linked by β-glycosidic bonds. As a result of the β-linkages, the β-glucans are nondigestible (El Khoury et al., 2012).

Oats contain approximately 2 to 3% of minerals. Minerals in oat kernel include potassium, phosphorus, magnesium, calcium, iron, manganese, and copper. Additionally, oats contain a variety of different vitamins, such as vitamin E, pantothenate, niacin, thiamin, vitamin B6, riboflavin, folate, and biotin. Especially, thiamin and pantothenate are found in high levels compared to other cereals (Arendt & Zannini, 2013). Oats have also compounds that show antioxidant activity. These include phenolic compounds, vitamin E, phytic acid, and avenanthramides which are the most common antioxidants present in oats. Most of these compounds are located in the outer layers of the kernel. Antioxidants aid the processed oat products to maintain their stability e.g. they can help to prevent the rancidity of oils and fats (Peterson, 2001).

Oats can contain proteinase inhibitors that are antinutritional enzymes (Klose & Arendt, 2012). These enzymes are a general problem with plant protein raw materials. Plant proteins have naturally antinutritional components that hinder the bioavailability or metabolism of nutrients. This can affect the nutritional potential of plant proteins. Formation of the antinutritional components might occur also in food processing due to heating, extreme pH treatments, organic solvents or oxidizing agents (González-Pérez & Arellano, 2009). In addition to proteinase inhibitors, phytic acid is an example of an antinutritional component that is found in oats. Phosphorus is stored as phytic acid in the plant seeds and the bran. Phytic acid inhibits the absorption of essential minerals by binding to the minerals to form phytates. Phytates can also prohibit absorption of proteins by forming complexes and thus prohibit the bioavailability of proteins (Gupta et al., 2013).

3. Functionalization of plant proteins for improved structure formation

As already stated in Chapter 1, the functional properties of proteins have great impact on the alteration of food structures. The functional properties of plant proteins are affected by the composition and conformation of the protein and also by the presence and reactivity of disulphide bonds. Emulsifying, foaming, gelling, and water solubility are the most crucial functional properties in oat proteins (Jing et al., 2016). Protein solubility is known to affect foaming, gelling, and emulsifying properties, so by improving solubility, other properties of food proteins can be improved as well.

Food structure is an important factor that affects many aspects such as the mouth feel and the appearance of the food. Food structure can be modified by physical and (bio)chemical techniques, such as covalent cross-linking between food biopolymers, or by addition of hydrocolloids and thickeners (Buchert et al., 2010). Cross-linking is one of the techniques used to modify the protein structure and functionality. Gerrard and Brown (2002) have described protein cross-linking as follows:

"Protein cross-linking refers to the formation of covalent bonds between polypeptide chains within a protein (intramolecular cross-links) or between proteins (intermolecular cross-links)" (Gerrard & Brown, 2002).

Cross-links can be formed either by physical or chemical methods or by enzymes. Physical cross-linking is achieved by altering reaction conditions such as temperature, shear, and pressure. Irradiation can also be used. Chemical methods include cross-linking agents that are often double-headed reagents that affect the side chains of the proteins. However, chemical cross-linking is rarely suitable for food applications. Enzymatic cross-linking is favored because enzymes have milder reaction conditions, high specificity, and they are unlikely to produce toxins (Gerrard and Brown, 2002).

Amino acids have a significant role in the formation of cross-links. Different amino acids in food proteins can influence to the type of the protein cross-link. Table 2 represent a summary of different reactive groups and protein cross-links that may form during food processing.

Table 2. Summary of cross-linking reactions that may form in food proteins (either native of denatured) during food processing. Table adapted from Gerrard 2002, Figure 1.

Amino acid	Protein cross-links
lysine	maillard cross-links
	glutamyl-lysine cross-links
glutamine	glutamyl-lysine cross-links
aspartate	isopeptide bond
cysteine	disulphides
	dehydroprotein
serine	dehydroprotein
tyrosine	dityrosyl cross-links
	quinone structures
tryptophan	uncharacterised maillard cross-links
proline	uncharacterised maillard cross-links
histidine	uncharacterised maillard cross-links
arginine	maillard cross-links

3.1 Protein and food modification by cross-linking enzymes

There are a variety of enzymes for protein cross-linking such as tyrosinases, laccases, peroxidases, sulfhydryl oxidases, and TGs. TG is a well-known enzyme often used to improve protein-rich food structures. It is a widely employed enzyme in food applications such as meat, dairy, and baked products. It is also thought that TG protects nutritionally important lysine residues from many harmful reactions in food systems (Gerrard & Brown, 2002; Dickinson, 1997). TG catalyzes an acyl transfer reaction between the γ-carboxyamide group of a glutamine residue and a primary amine, leading to (γ-glutamyl)-lysine isopeptide linkages. In the absence of amine, water functions as an acyl acceptor and deamidation of glutaminyl residues is catalyzed (Dickinson, 1997).

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In theory, cross-linking enzymes react well with proteins since proteins have various reactive groups e.g. glutamine, lysine, tyrosine, and cysteine residues. However, the cross-linking reaction is reliant on the type and activity of the enzyme, the macromolecular structure of each protein substrates, the convenience of the access of target reactive group into the biopolymer, and the conditions of the process. The access to the enzyme active site is easier to non-globular proteins than to globular proteins. For instance, caseins work well as a substrate for TG, since they have flexible and open tertiary structure (Buchert et al., 2010).

Cross-linking of plant proteins has been widely studied, and Table 3 summarizes a few of the studies on plant protein cross-linking. TG and tyrosinase seem to be the mostly used cross-linking enzymes as presented in the Table 3 and reviewed below.

Cross-linking of oat protein isolate (OPI) and faba protein isolate (FPI) by TG and tyrosinase was studied by Nivala et al. (2017) to improve the colloidal properties of oat and faba protein particles. It was found that TG could perform cross-linking to some extent and improve the colloidal stability of OPI drastically. The use of tyrosinase resulted in limited cross-linking, reduced oat protein solubility, and had no effect on the colloidal stability. TG-treated FPI had extensive cross-linking, but differences were observed between the α- and β-chain cross-linking. The electrostatic stability improved, but foaming properties and concentration of soluble protein decreased. Tyrosinase treatment of FPI resulted in limited cross-linking which led to impaired foaming. There was no effect observed on the colloidal stability. Different enzyme activities of tyrosinase did not change the results significantly. Nivala et al. (2017) concluded that cross-linking by tyrosinase could be enhanced by limited hydrolysis or partial denaturation of globular proteins.

Tang et al. (2006) reported the use of TG to improve the gelling properties of soy protein isolate (SPI). The SPI was enriched with glycinin and β-conglycinin.

Glycinin-rich SPI was more effectively polymerized or cross-linked by TG. It also formed stronger and turbid gels whereas β-conglycinin-rich SPI formed weak and transparent gels. Overall, formation of disulphide bonds in the gelling had a positive impact on the maintenance of the gel networks.

A study done by Glusac et al. (2019) investigated tyrosinase cross-linked pea protein emulsions and the impact of zein incorporation. The pea protein concentrate was treated by tyrosinase in 1:25 enzyme to protein ratio. Cross-linking occurred within 30 minutes from adding the enzyme. Cross-linking of the pea protein with tyrosinase had a positive impact on the emulsion stability, particle size, viscosity, and paste-like structure when compared with non-cross-linked pea protein. The cross-linking and incorporation of zein with pea protein resulted in significant improvement of the emulsion stability and changed the paste-like structure to gel-like structure.

Schäfer et al. (2005) reported enzymatic cross-linking of leguminous proteins and their identification and quantification with high-performance liquid chromatography-electrospray ionization mass spectrometry. Lupin protein isolate from *Lupinus angustifolius* seed was treated with TG to see the impact on gelling properties. Cross-linking was observed but the formed gel was weak. Lupin protein isolate had lowest levels of lysine but highest isopeptide content compared to other leguminous samples (pea and soy protein isolates) used in the study.

Table 3. Modification of plant proteins by cross-linking enzymes. (SPI: soy protein isolate).

Protein	Origin of grain	Protein modifications	Enzyme(s)	Enzyme activity	Use	Result	Effect	Reference
Oat protein isolate	Commercial	Alkaline extraction pH 10, isoelectric precipitation pH 5.5	Transglutaminase (*Streptomyces mobaraensis*)	100 and 1000 nkat/g	Colloidal properties	Cross-linking to some extent	Reduced oat protein particle size, increased absolute zeta-potential, colloidal stability improved	Nivala et al. (2017)
			Tyrosinase (*Trichoderma reesei*)	100 and 1000 nkat/g	Colloidal properties	Limited cross-linking	Great reducing of oat protein solubility, impaired foaming, formation of pink color, no effect on colloidal stability	
Faba protein isolate	Commercial	Alkaline extraction pH 9, isoelectric precipitation pH 4.5	Transglutaminase (*Streptomyces mobaraensis*)	10 nkat/g	Colloidal properties	Extensive cross-linking	Electrostatic stability improved, soluble protein concentration and foaming properties decreased	Nivala et al. (2017)
			Tyrosinase (*Trichoderma reesei*)	100 and 1000 nkat/g	Colloidal properties	Limited cross-linking	Impaired foaming, formation of pink color, no effect on colloidal stability	
Soy protein isolate	Commercial	Enriched with glycinin & β-conglycinin	Transglutaminase	20 U/g of protein substrate	Gelling	Cross-linking occurred	More strong and turbid gels with glycinin-rich SPI, β-conglycinin-rich SPI formed weak and transparent gels	Tang et al. (2006)
Pea protein concentrate	Commercial	Addition of zein	Tyrosinase (*Bacillus megaterium*)	n/a	Emulsions	Rapid cross-linking (within 30 min)	Stable emulsions with a paste-like structure. Incorporation of zein shifted the texture from paste to gel-like structure and improved stability	Glusac et al. (2019)
Lupin protein isolate	Produced	defatting, extraction, drying	Transglutaminase (*Streptoverticillium mobaraense*)	81-135 U/g	Gelling	Cross-linking occurred	Gelling occurred, but rather weak gels	Schäfer et al. (2005)

3.2 Other enzymes used for food protein modification

Enzymes are important tools in the modification of proteins, and there are also several other than cross-linking enzymes available for protein modification. Dickinson (1997) reviewed different enzymes that work in the food protein modification. Proteases, peptidoglutaminase, and protein kinase were reviewed. Proteases breakdown proteins into polypeptide fragments, peptidoglutaminase deamidates proteins, and protein kinase phosphorylates proteins. Especially proteases are a large group of enzymes that are utilized extensively in food processes as reviewed by (Tavano, 2013). Proteases can improve the solubility of plant proteins, at the cost of altering other functionalities and flavor, if the hydrolysis goes too far. Improvement of functionalities by proteases have been reported by Kim (Lee) et al., (1990) and Conde et al., (2005) who studied hydrolysis of SPI and sunflower proteins, respectively.

PG is an enzyme that can be used to aid in the modification of food proteins, e.g. it can be used as control agent for TG activity since it has higher reactivity towards target glutamine residues (Gu et al., 2001). PG catalyzes the deamidation of glutamine residues in the substrate protein. This results as change of glutamine residues to glutamic acid residues and as release of ammonia (Yamaguchi et al., 2001). A good example of the use of PG deamidation in a plant protein, wheat gluten, was reported by Yie et al., (2006). Alteration of conformation occurred in the gluten secondary structure. Wheat gluten has poor solubility into aqueous solutions, however protein solubility and emulsification properties were improved by the deamidation at pH 7 by PG. Reduction in allergenicity was also noticed in long-hour deamidated gluten samples.

3.3 Functionalization of oat proteins

Oat proteins have been modified for many food applications such as milks and yogurts, but have been used less for solid food models, like meat analogues. Generally, enzymes are more often applied in the functionalization of food proteins, since chemical modifications have often harsh reaction conditions and

non-specific chemical reagents. Most of the chemical modifications aim to change the net charge of the protein, which affects to the protein solubility. Enzymatic modification requires milder reaction conditions and the enzymes have high specificity, which makes them more desirable for food applications. Functionalization by enzymes often targets to modify the molecular structure of the protein (Panyam & Kilara, 1996). Table 4 shows different modifications performed to oat proteins to improve their functional properties.

Jiang et al. (2015) reported the modification of oat protein solubility and emulsion properties by enzymatic deamidation. The electronic charge in the surface of oat proteins increased by the PG-deamidation when glutamine residues were converted into glutamic acid. This modification led to improved solubility, emulsifying ability, and flexibility of the secondary structure in oat proteins.

Siu et al. (2002) studied the modification of functional properties of oat protein by calcium-independent microbial TG. Oat globulins were cross-linked which led to better solubility, foamability, gelation, and water binding as well as fat binding properties. However, emulsifying and foam stability were decreased.

Effects of succinylation and deamidation on OPI's functional properties were studied by Mirmoghtadaie et al. (2009). Succinylation alters the protein conformation and shifts the isoelectric point to lower values. It was observed that succinylation led to improved solubility and emulsifying activity. Besides foaming capacity, emulsion and foam stability were decreased. Deamidation decreases the molecular size and increases OPI's net charge. It was noticed that solubility, emulsifying activity, and foaming capacity of the OPI were improved by deamidation. However, emulsion and foam stability were decreased. The reason for the decrease by both succinylation and deamidation was probably due to reduction in protein-protein interactions as the net charge was increased.

Ma & Wood (1987) reported acylation of OPC. The acylation altered the conformation of oat proteins and increased the net charge. Significant improvement in the solubility and emulsifying properties were observed.

Furthermore, fat binding capacity, foamability and gelling properties were also improved. However, foam stability decreased with water hydration capacity. Acylation also lowered the total essential amino acid content.

Partial hydrolysis of OPI was tested to improve formation of gels by four different enzymes: Flavourzyme (mix of endoprotease and exopeptidase), alcalase, pepsin, and trypsin. Nieto-Nieto et al. (2014) reported that partial hydrolysis of oat protein could form gels that have similar water-holding capacity and mechanical strength as compared to gels formed by animal proteins. Flavourzyme and trypsin improved gel strength significantly in pH 8-9 and were able to form gels in broad range of conditions. It was observed that pepsin digested both 12S acidic and basic subunits and was not able to form gel structure under any conditions. Alcalase treatment of oat proteins at pH 9 resulted in gel formation. Oat 12S globulins basic subunit was quite resistant to Flavourzyme, alcalase, and trypsin in the hydrolysis. This was probably due to the fact that the basic subunit is not as accessible as acidic subunit since it is buried inside of the protein molecules.

Hydrolysis of an oat bran protein concentrate with trypsin was studied by Guan et al. (2007). The degree of hydrolysis affected the solubility, water-holding capacity, emulsifying activity, and foaming ability by increasing them as the degree of hydrolysis increased until to a certain extent. However, oil-holding capacities as well as emulsifying and foaming stability showed some decline. Heat treatment might have caused denaturation of larger protein components which may have affected the decline of emulsifying and foaming stability.

Addition of inulin to OPI was studied by Nieto-Nieto et al. (2015). Inulin is a non-digestible, naturally occurring polysaccharide in many edible vegetables. The aim of the study was to see if the addition of inulin in small concentrations could enhance the gelling properties of OPI. The addition of inulin improved the protein gels by increasing the hydrogen bonding and hydrophobic interactions. The results showed that the compressive stress, springiness, and gumminess of the gel increased since inulin increased the amount of junction zones which led to a highly

cross-linked network. OPI-inulin gels mechanical strength was noticed to be comparable with egg white gels.

As discussed already in the Section 3.1. Nivala et al. (2017) modified OPI with TG and tyrosinase. Cross-linking was observed to some extent by both enzymes. TG improved colloidal stability and reduced the particle size. Tyrosinase treatment had no effect on colloidal stability and protein solubility was decreased.

Table 4. Modifications performed to oat proteins. Protein fraction, protein concentration, modifications to proteins, modification mechanism, parameters and effects summarized in the table. (PG: protein-glutaminase, FBC: fat-binding capacity, EAA: essential amino acid, F: flavourzyme, A: alcalase, P: pepsin, T: trypsin, WHC: water-holding capacity, DH: degree of hydrolysis, OHC: oil holding capacity.)

Protein fraction	Protein concentration (%)	Modification	Mechanism	Parameters	Effect	Reference
Oat globulin	62.0	Enzymatic deamidation, protein-glutaminase (*Chryseobacterium proteolyticum* EC.3.5.1.44)	Catalyzes the deamidation of the side chain amino group of protein-bound glutaminyl residues.	Enzyme: 13 U/g of protein 40 °C pH 7	Improved oat protein emulsifying ability, solubility and flexibility of secondary structure. PG-deamidation increases the negative charge of the protein polypeptide by converting the glutamine residues into glutamic acid.	Jiang et al. (2015)
Oat globulin	98.9	Calcium-independent microbial transglutaminase (*Streptovericillium* sp. no. 8112)	Permanent cross-linking.	Enzyme: 0.01 U/mg of protein 37 °C pH 7.5	Better solubility (pH 4-5) and foamability, improved water- and fat-binding properties and gelation properties (pH 7). Emulsifying and foam stability were decreased.	Siu et al. (2002)
Oat protein isolate	-	Succinylation (succinic anhydride)	Increase in protein-water interactions, altering protein conformation by promoting unfolding and increasing dissociation of subunits from quaternary structure, shifting isoelectric point to lower values.	pH 8 anhydride: 20 g/100 g → pH 4-4.2 → neutralization	Improved solubility and emulsifying activity, but foaming capacity was decreased. Emulsion and foam stability were decreased also.	Mirmoghtadaie et al. (2009)
Oat protein isolate	-	Deamidation (HCl 0.5 N)	Decrease in molecular size and an increase in OPI's net charge, increase in electrostatic repulsion and decreasing hydrogen bonding.	70 °C pH 4.7 → neutralization	Improved solubility, emulsifying activity and foaming capacity. Emulsion and foam stability decreased.	Mirmoghtadaie et al. (2009)
Oat protein concentrate	60.0	Acylation	Altered conformation, increase in net charge.	-	Solubility and emulsifying properties were significantly improved. FBC, foamability and gelling property were improved. Total EAA content was lowered. Water hydration capacity and foam stability were decreased.	Ma & Wood (1987)

Oat protein isolate	91.2	Partial hydrolysis, flavourzyme, alcalase, pepsin and trypsin	Modulating the balance between the electrostatically repulsive force and the hydrophobic attractive force among polypeptide chains during the gelling process.	30 min F: ≥500 U/g, pH 7 & 50 °C A: 2.4 U/g, pH 8 & 50 °C P: ≥ 250 U/mg, pH 2 & 37 °C T: 1462 U/mg, pH 8 & 37 °C Inactivation of enzyme at 90 °C	Partially hydrolyzed oat protein could form gels with similar mechanical strength and WHC as those from animal proteins such as egg white. Flavourzyme and trypsin could significantly improve oat protein gel strength, especially at pH 8-9.	Nieto-Nieto et al. (2014)
Oat bran protein concentrate	73.4	Hydrolysis and heat treatment, trypsin (E.C.3.4.21.4)	Hydrolyzes proteins into smaller peptides or amino acids. Decrease in the molecule size of polypeptide chains. Exposure of more charged and polar groups to the surrounding water.	240 min enzyme: 1250-1450 usp-u/mg pH 8 45 °C → 100 °C DH: 4.1, 6.4, 8.3%	Solubility, WHC, emulsifying activity and foaming ability gradually increased with the increase of DH, but their OHC, emulsifying and foaming stability showed more or less decreases.	Guan et al. (2007)
Oat protein isolate	90.4	Addition of Inulin	Inulin formed nanoparticles in the void spaces of the protein network performing a filling effect and creating junction zones.	Inulin: 0.1-0.5% 100 °C pH 2.5, 5, 7	At pH 7, increased compressive stress of gels, springiness and gumminess. Highly cross-linked network with increased junction zones.	Nieto-Nieto et al. (2015)
Oat Protein Isolate	92.1	Transglutaminase (*Streptomyces mobaraensis*)	Forms enzyme/substrate complexation with protein-bound glutamine residues to initiate acyl transfer and cross-link formation between glutamine and lysine residues.	20 h, 40 °C 400 rpm enzyme: 100 and 1000 nkat/g	Reduced oat protein particle size, increased absolute zeta-potential, colloidal stability improved.	Nivala et al. (2017)
		Tyrosinase (Trichoderma reesei)	Oxidases protein-bound tyrosine residues which further reacts non-enzymatically with other tyrosine, lysine or cysteine.	20 h, 40 °C 400 rpm enzyme: 100 and 1000 nkat/g	Reduced oat protein particle size, increased absolute zeta-potential, colloidal stability improved.	

4. Use of plant proteins for meat analogue production

Meat analogue, referred also as meat substitute, is a food product often made from plant proteins. It attempts to mimic characteristics of meat, e.g. texture, appearance, and flavor. Meat analogs can be produced in different sizes and shapes. Often the structure is a striated and layered, similar to muscle meat (Asgar et al., 2010).

Dekkers et al. (2018) reviewed different strategies and techniques to create fibrous meat analogues from plant materials. Techniques such as extrusion (low-moisture and high-moisture), mixing proteins and hydrocolloids, freeze structuring, and shear cell technology are all used for modifying the structure of plant based raw materials towards meat mimicking textures (Dekkers et al., 2018). This thesis focuses on the HMEC technique.

4.1 High-moisture extrusion cooking

HMEC is an important process in food industries and one of the most used commercial technique to restructure protein based raw materials. Extrusion cooking can be defined as a thermochemical process. With pressure, heat, moisture and mass transfer, effects such as drying, melting, freezing, texturizing, cooking, and mixing are produced. Extrusion can be performed with high- or low-moisture content. High-moisture extrusion uses protein based raw materials and can result as fibrous products, whereas low-moisture extrusion uses more starch based raw materials and can result as dry and expanded products (Dekkers et al., 2018).

Twin-screw cooking extruders are more popular nowadays compared to single-screw cooking extruders. A twin-screw extruder has two screws that rotate inside the barrel, as seen in Figure 2. At the exit of the extruder barrel there is a restricted passage element, the die. It functions as a pressure release valve and transfers the

extrudates to the point where their shape is deformed (Berk, 2009). The size of the die opening influences in the protein texturization, especially by impacting the melt velocity. As the size of die opening decreases, melt velocity and shear stress increase. Screw speed and flow rate are also known to influence the texturization by affecting the barrel fill and the time that the raw material spends in the barrel (Akdogan, 1999).
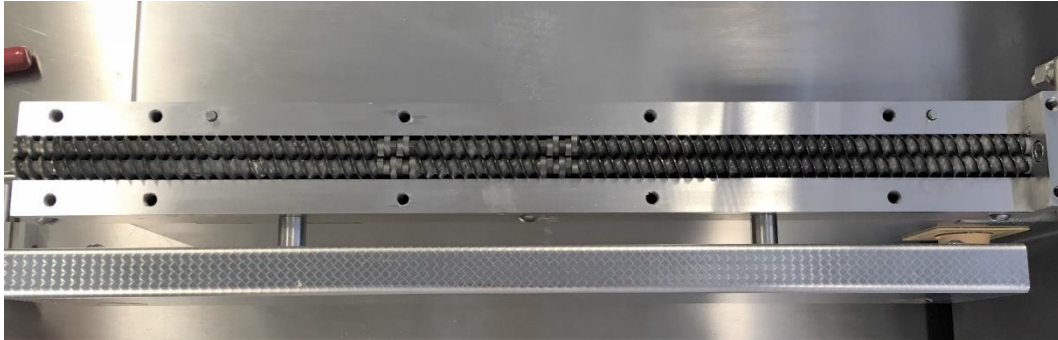
Figure 2. Twin-screws of the extruder used in this thesis. Courtesy of VTT Technical Research Centre of Finland Ltd / Anni Nisov.

In the production of meat analogues from plant proteins, HMEC is most often used. The process is dependent on the conditions of the extrusion and the properties of the protein ingredients. The ingredients are plasticized and texturized by using a cooling die. The conditions such as temperature, moisture, pressure and shear are altered during the process. These alterations result in a molecular transformation as well as in chemical reaction of the protein molecules (Osen et al., 2014). Figure 3 shows the twin-screw extruder used in the experimental part of this thesis.
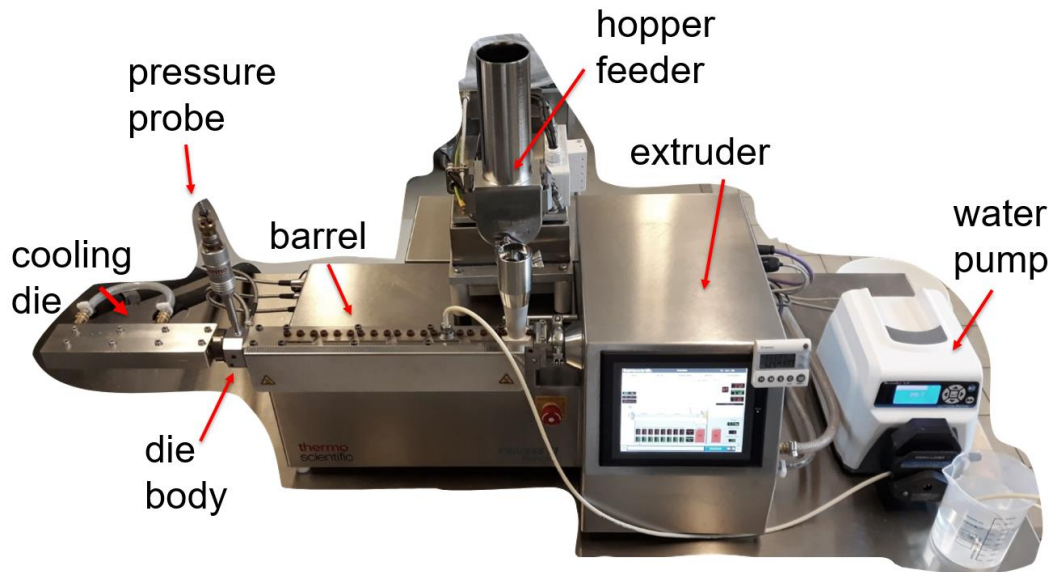
Figure 3. Process 11 Parallel twin-screw extruder (Thermo Scientific, Germany) used in this thesis. Courtesy of VTT Technical Research Centre of Finland Ltd / Pinja Pöri.

HMEC has many benefits over traditional low-moisture extrusion. The moisture content of the feed in HMEC is between 40 to 80% when in the traditional low-moisture extrusion it is often between 10 to 30%. Water functions as a lubricant, plasticizer, reactant etc., and reduces the temperature range where the polymer substrate changes from a rigid glassy material to a soft material. This ensures the melting of the protein ingredients and lowers viscosity (Chen et al., 2011). However, whether the raw material can be extruded depends on the ratio of soluble and insoluble components. If there are too many insoluble components the protein cross-linking is disturbed which leads to incoherent products (Dekkers et al., 2018).

HMEC process can be divided to three important stages as visualized in Figure 4. In the first stage, a protein powder and water are mixed together into a homogenous state. In the next stage, the mixture moves to the cooking zone where it is pressurized and heated. In the last stage, the cooked product gets pushed through from a long and narrow pipe that has external cooling system. Cooling lowers the mixture's temperature and prevents its expansion in the

extruder outlet. Texturization happens in the cooling zone where the rearrangement and cross-linking of protein molecules occur (Murillo et al., 2019).
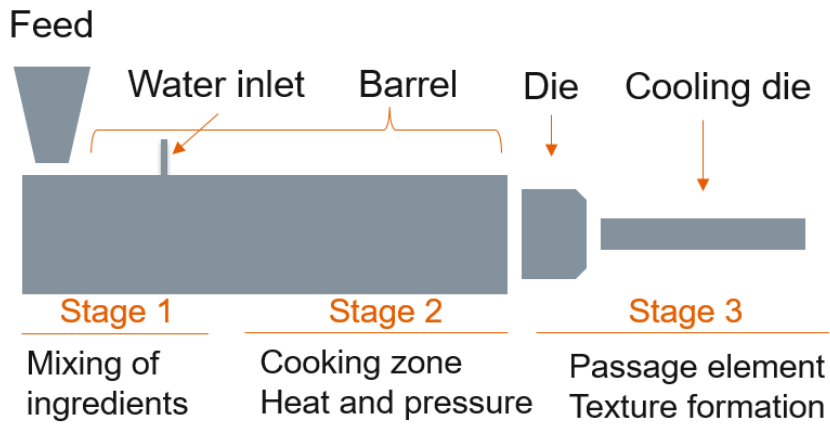
Figure 4. Illustration of the high-moisture extrusion cooking process. Adapted from Murillo et al. (2019).

4.1.1 High-moisture extrusion cooking of plant proteins

HMEC has been applied to plant proteins for over 30 years (Kitabatake et al., 1985). There is no published data available about HMEC of oat proteins, but several plant proteins such as wheat gluten, pea, and soy proteins are currently utilized for meat-analogue production. Table 5 summarizes research on HMEC of wheat, pea, and soy proteins. The protein type, its origin, process parameters, protein concentration, fibrillation temperature, and factors affecting the results are summarized in the table.

Pietsch et al. (2017) reported HMEC of wheat gluten with a twin-screw cooking extruder. In the study, the fibrillation temperature of the protein was above 90 °C and the protein concentration was 83%. The only factor that affected significantly the polymerization of wheat gluten during the HMEC, was thermal treatment of the screw section. There was no significant influence detected from die temperature, pressure, or specific mechanical energy.

Osen et al. (2014) reported HMEC of pea protein isolates. The main goal of the study was to find out which protein properties affect the product texture and

extruder responses. Fibrillation occurred in temperatures above 120 °C at a moisture content of 55%. Cooking temperature was the most significant factor that affected the extrudates texture. Below 120 °C the pea protein isolates showed a dough-like soft texture and there was no fibrillation observed. When the temperature was raised to 160 °C, it was noticed that the macrostructures had smooth surface and they were more homogenous. Additionally, Osen et al. reported that functional properties of the samples had only a minor role in the formation of fibers in the cooking temperatures above 120 °C and that the particle size from larger particles to fine powder had no effect to the texture properties.

Chen et al. (2011) investigated chemical cross-linking and molecular aggregation of soybean protein during low- and high-moisture extrusion cooking. It was detected that hydrophobic interactions, hydrogen bonds, and disulphide bonds hold the extrudates structure together. Also, regardless of the moisture content, contribution of non-covalent bonds exceeded that of covalent bonds. The increase in the moisture content resulted in the increase of the interactions of disulphide bonds with hydrogen bonds and hydrophobic interactions. This could reduce the aggregation degree as well as the difference in protein-protein interactions.

Table 5. High-moisture extrusion cooking of plant proteins. (SME: specific mechanical energy)

Protein	Origin	Process parameters	Protein concentration (%)	Fibrillation temperature (°C)	Factors affecting result	Reference
Wheat gluten	Commercial	Screw diameter 25.5 mm, length/diameter ratio 29/1, 7 barrel sections, screw speed 300 rpm, feed rate 10 kg/h, temp. 40-170 °C, die lengths 5x15x25 mm, diameter 3 mm, long cooling die 15x30x380 mm	83.0	> 90	Only thermal treatment in the screw section influenced wheat gluten polymerization. Die temperature, pressure and SME input had no significant influence.	Pietsch et al. (2017)
Pea protein isolate	Commercial	Screw diameter 16.0 mm, barrel/length diameter ratio 25/1, 5 barrel sections, die lengths 19x2x210 mm, water feed 3.4 l/min, feed rate of dry protein 0.45 kg/h, temp. 40-160 °C	84.9 87.3 83.2	> 120	Cooking temperature.	Osen et al. (2013)
Soy protein isolate	Commercial	Screw length/diameter ratio 20:1, die lengths 2x20x100 mm, 5 barrel sections, temp. 80-150 °C, feed rate 20 g/min, screw speed 160 rpm	92.7	n/a	Moisture content (60%) and specific chemical bonds.	Chen et al. (2011)

4.2 Formation of fibrous structure in plant proteins

Fibril formation is a critical factor in the creation of meat analogues from plant proteins. Fibril formation in proteins depends on many things like functional sites, protein concentration, protein solubility, other ingredients present in the protein sample, and processing conditions such as temperature and pH. Especially ionic strength has a significant role in the protein conformation and function. Typically protein contents greater than 50% are required to create a fibrous structure. Lower protein content raw materials can be texturized but that often results as a soft and weak extrudate (Keeler, 2004).

Disulphide bonds (S-S) and thiols (sulfhydryl groups) are needed in the formation of fibrous protein-based structures. New protein conformations may be formed through oxidation or reduction of disulphide bonds (Kinsella, 1976). Disulphide bonds form between thiol groups of cysteine residues under oxidizing conditions. These bonds are crucial in the stabilization of many protein structures by forming cross-links between different regions of polypeptide chains. The process of forming disulphide bond is slow because of high activation energies. A low pH may hinder the formation of disulphide bonds because free thiols are in the protonated form (De Jongh, 2007).

Denaturation of proteins, fully or partially, helps in the fibrillation process. By denaturing proteins, the structure opens and the bonding sites become exposed for cross-linking. Shear flow, high temperature, and high pressure during extrusion can lead to protein denaturation and therefore assist in the protein fibrillation (J. Zhang et al., 2019).

In the fibrous structures of protein extrudates, the formation of covalent disulphide bonds and non-covalent bonds are both important. However, results from a study done by Liu and Hsieh (2008) on soy protein mixed with gluten and starch, indicate that disulphide bonding is more significant in the holding of the rigid structure and forming of the fibrous texture. Non-covalent interactions have an important role in the formation of secondary and tertiary structures of proteins (Liu & Hsieh, 2008).

Formation of fibrous structure happens in the extrusion through protein alignment. This is visualized in Figure 5. When the protein based raw material is exposed to moisture, mechanical and thermal energy, macro-molecules lose their native, organized structure, and form a continuous, viscoelastic mass. Bonding sites are exposed by realignment. This in turn leads to disulphide formation and reformed layered and striated structure that resembles meat (Riaz, 2011).
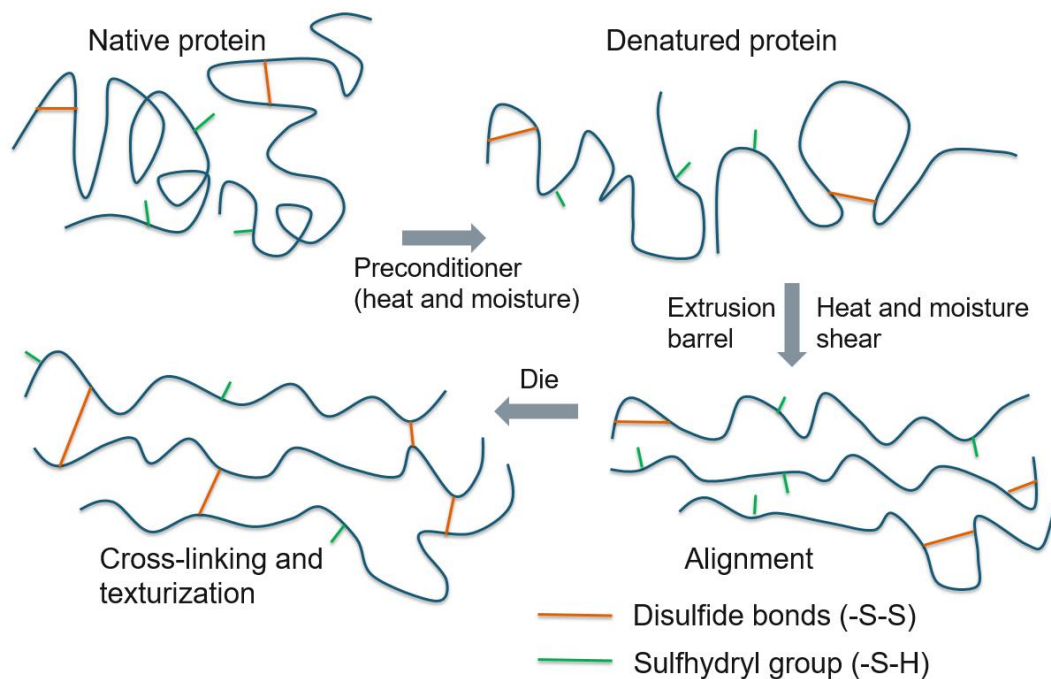
Figure 5. Denaturation of native protein during extrusion. Based on Figure 15.1 from Riaz, 2011.

4.3 Non-protein components that affect protein fibrillation in meat-analogues

The composition of the raw material affects the protein fibrillation process. Protein concentrates have often protein contents lower than 70%. Therefore there are other components present that might affect the fibrillation process by interacting with proteins and that way affecting e.g. proteins functional properties.

In HMEC, the presence of lipids can hinder the formation of fibrous structures. Lipids have lubricating effect which affects to the shear and particle alignment by decreasing them during extrusion (Akdogan, 1999). Starch is also one component that can hinder or enhance the formation of fibrous structure depending on the ratio. Already over 10 % starch content can prevent the unfolding and aggregation of protein molecules (Zhang et al. 2019). The starch amount can be reduced by using amylases. Amylases hydrolyzes starch into sugars and dextrins (Tester et al., 2006). However, sugars from the hydrolyzed starch can react with free amino acids and this in turn can lead to Maillard reactions (Zhang et al. 2019). The Maillard reaction is a complex series of chemical transformations where amino acids and reducing sugars react. The Maillard reaction gives brown color to the food product when it is cooked and affects its flavor (Akdogan, 1999).

As mentioned in Section 4.1, the solubility of the raw material can also affect to the extrusion and structure formation. Too soluble material can hinder the extrusion process and a material with a low solubility can disturb the cross-linking reaction (Dekkers et al., 2018).

Besides starch, lipids, and sugars, there are often dietary fibers present in the protein concentrates. They can be divided to a soluble and non-soluble dietary fibers. Especially the soluble dietary fibers can lead to expansion of the extrudate's structure (Robin et al., 2012). Raw materials with high fiber content also require more energy to achieve fibrous structure in the extrusion. High levels of fiber, fat, and starch in the raw material can hinder the interactions or cross-linking of proteins, which are needed in the formation of fibrous structure (Keeler, 2004).

4.3.1 Protein-carbohydrate complexes

It has been stated that addition of polysaccharides such as starch or maltodextrins might enhance the formation of fibrous structure in the extrusion (Akdogan, 1999). Zhang et al. (2020) reported HMEC of peanut protein which was enhanced with exogenous polysaccharides. Carrageenan, sodium alginate, and wheat starch

were used to improve the formation process of a fibrous structure of peanut protein. Interaction between hydrogen bonds and disulphide bonds were noticed to be the main forces holding the extrudates protein structure together. Especially, wheat starch increased the amount of hydrogen bonds and disulphide bonds. It was also noticed that fibrous degree, chewiness, and hardness would reduce if there was too much wheat starch present. Sodium alginate improved significantly the fiber orientation, tensile strength, hardness, and chewiness. Tensile strength was also improved significantly by carrageenan but it did not improve fiber orientation.

Oat protein has not been investigated in the HMEC, but Nieto-Nieto et al. (2016) reviewed thermal gelation of oat proteins at different pH's by testing the effect of dextrin, carrageenan, and chitosan polysaccharides. Dextrin and carrageenan formed phase-separated networks which improved the compressive stress significantly and therefore the gelling properties by increase in protein concentration. Oat protein gels with dextrin formed gels faster than the rate of phase separation, thus the dextrin particles were evenly distributed in the network. The gel with carrageenan showed slow aggregation process and therefore had a greater level of phase separation. The presence of chitosan had unfavorable effect on the gel properties e.g. decrease in the gel strength and the compressive stress. It was concluded that the degree of phase separation was the most important factor affecting the production of strong gels and it may be strongly related to the production of different textures (Nieto-Nieto et al., 2016).

5. Aim of the thesis

The aim of this thesis was to increase fibrillation between oat proteins by performing enzymatic treatment prior the HMEC. It is known that protein concentrates do not typically fibrillate well, due to their low protein content and high amount of other components, such as starch, that might hinder the fibrillation. Enzymatic modification may improve the functionality of the protein and create a stronger protein network which would lead to better fibrillation. In this thesis TG, a mixture of TG+PG, and α-amylase enzymes were used. TG cross-links between glutamine and lysine protein residues and PG deamidates glutamine residues to glutamic acid. Enzyme α-amylase hydrolyzes randomly 1.4-α-glucosidic linkages in amylose and amylopectin. Heating was also studied prior to the enzyme treatment since denaturation opens up the structure of the substrate protein making the active sites more easily accessible to the enzyme.

Figure 6 represents the work flow scheme of this study. The work flow was divided into two parts. First part included the finding of suitable conditions for the enzymatic treatment by testing different preheatings, parameters such as temperature, pH, and enzyme dosage as well as performing analyses to the modified raw material (Appendix A, Table A1). The second part included scale-up of the modified raw material, HMEC trials, and analyses to the extrudates (Appendix A, Table A2).
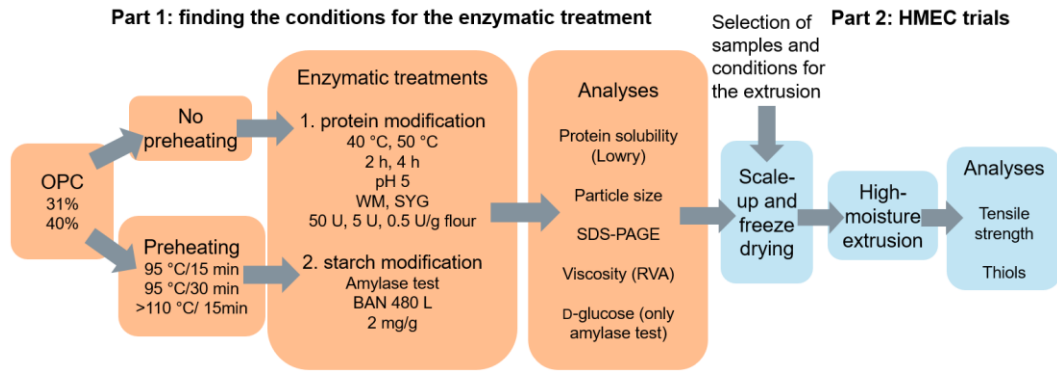
Figure 6. Process scheme of the work flow during the experimental part of the thesis. (Orange boxes represent the first part of the experimental process and blue boxes the second part. OPC: oat protein concentrate, WM: transglutaminase, SYG: a mixture of transglutaminase and protein-glutaminase, BAN 480 L: α-amylase, HMEC: high-moisture extrusion cooking)

EXPERIMENTAL PART

6. Materials and methods

6.1 Raw materials and enzymes

The OPC was produced at Hosokawa Alpine AG (Germany) and used as a raw material in this thesis. It was made in two steps based on the publication Sibakov et al. (2011). First, defatted oat flours were milled and air-classified once. From there the low β-glucan flour was air-classified for a second time and the fine fraction obtained was used as the OPC in this thesis. There were two fractions from the OPC applied in the experimental part, one had a protein content of 40% (as is) and the other 31% (as is) according to Kjeldahl (Nx6.25) method performed at VTT Ltd. Results shown in Chapter 7 are based on the 40% OPC fraction. The 31% OPC fraction was used in the first few enzymatic treatment tests where suitable conditions for the reaction were investigated (Appendix A, Table A1).

Three commercial enzymes were used in the modification of the oat protein. Two of them were cross-linking enzymes from Ajinomoto Inc. (Tokyo, Japan). Activa WM was TG and Activa SYG was a mixture of TG and PG. Both of the WM and SYG enzymes also included maltodextrins in their formulations. The third enzyme, α-amylase BAN 480 L (Novozymes A/S, Denmark), was used to hydrolyze the starch in the OPC. Table 6 represents form of the enzyme preparations, main activities, optimum temperatures, optimum pH, and food grade status.

Table 6. Information about the enzymes used in the thesis. Main activity values were obtained from manufacturers product specification sheets.

Enzyme	Form of the enzyme preparation	Main activity	Optimum temperature (°C)	Optimum pH	Food grade status
Activa WM	powder	81-135 U	55	5-8	yes
Activa SYG	powder	64-126 U, 127-218 U *	55-60	5-6	yes
BAN 480 L	liquid	480 KNU	70	5-7	yes

*First mentioned TG activity, then PG activity

6.2 Cross-linking of the oat protein concentrate

OPC's (31% & 40%) were first used to test the efficacy of the enzymatic cross-linking by TG (WM) and TG+PG (SYG). 4% (w/w) suspensions were made from the OPC with distilled water. The OPC suspensions were heated to 40 °C under constant stirring. The pH of the suspensions were measured when the solution temperature achieved 40 °C (pH ~6.3). Zero samples were taken from the suspensions and the enzymes were added to the suspensions, except to the control. Two enzymes, WM and SYG, were tested with dosages of 0.5 U, 5 U, and 50 U/g flour. The reaction was continued for 4 h with constant stirring (500 rpm). Samples were taken for SDS-PAGE, protein solubility, and particle size -analyses, at specific time points (0 h, 0.5 h, 1 h, 2 h, 4 h), to confirm the level of cross-linking.

Besides the above mentioned conditions, the effect of different incubation temperature (50 °C for 2 h), pH adjustment to 5, and preheating of OPC were also tested. Effect of preheating of the OPC was tested by keeping the OPC suspension first at 95 °C for 15 min before the 40 °C/4 h treatment and addition of enzymes. Also 95 °C/30 min and 112 °C/15 min were tested as preheating. In the tests of these conditions both enzymes, WM and SYG, were used in 5 U/g flour dosage and the used OPC had protein concentration of 31%. From these treatments protein solubility, particle size distribution, and molecular size distribution were analyzed.

6.2.1 Reducing SDS-PAGE

OPC samples (treated or controls) used in the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were mixed with 5% SDS prior placing them into a heat block (3 min at 98 °C) to end the enzymatic reaction before freezing the samples. Addition of 5 % SDS diluted the samples to 1:5.

For SDS-PAGE analysis, the frozen samples were thawed in the room temperature (RT). 60 μl of the sample was pipetted to 1.5 ml Eppendorf tube with 20 μl of 4x reducing sample buffer. Tubes were incubated in 98 °C heating block for 5 min and then centrifuged for 1 min at 14000 rpm (Eppendorf centrifuge 5424, rotor: FA-

45-24-11). 10x running buffer (pH 8.3) was diluted to 1:10 with distilled water. 4% Criterion™ TGX Stain-Free™ Precast gel (#5678094, Bio-Rad, US) was placed into the equipment. 12.5 µl of standard Precision Plus Protein™ (#161-0363, Bio-Rad, US) was pipetted into a well. 15 µl of each sample was loaded into the wells. The gel was run with Bio-Rad PowerPac™ HC for 25 min at 250 V. Once the running was stopped, the gel was positioned to an imagining tray and the tray was placed into a Bio-Rad Criterion Stain Free™ Imager machine. The gel was imaged in the equipment with Image Lab software using its default protocol.

6.2.2 Particle size distribution

Particle size distribution of the OPC samples was analyzed with Mastersizer 3000 by Malvern Instruments (United Kindom). Samples were thawed one by one to refrigerator temperature and analyzed at RT to prevent the non-inactivated enzyme to continue the cross-linking reaction. Sample was under constant stirring at RT. Drops of the sample were pipetted with a Pasteur pipette to the wet sample dispersion unit (Malvern Hydro, MV, UK) containing distilled water, until the obscuration value was between 7-8.5%. The sample was run with Fraunhofer approximation model and data collected from the software to be analyzed later. Each sample was measured at least twice.

6.2.3 Protein solubility

Protein solubility of the OPC samples was measured by using BIO-RAD DC kit (Bio-Rad Laboratories Inc., USA). The kit is based on the Lowry method (Lowry et al., 1951). Bovine serum albumin (BSA) 1.5 mg/ml, was used to make the standard curve (0, 0.25, 0.5, 0.75, 1 mg/ml). Samples were centrifuged for 15 min at 10000 G at 20 °C, with a centrifuge Heraeus Fresco 21 (Thermo Scientific, rotor: 75003424). Supernatant was diluted with MilliQ water. 25 µl of sample was pipetted into a micro cuvette (semi-micro polystyrene, VWR, Germany) with 125 µl of reagent A (an alkaline copper tartrate solution) and 1 ml of reagent B (a dilute

Folin reagent). Incubation of 17 min at RT was started. The cuvette was inverted few times with a piece of Parafilm® on top. After 17 min of incubation the absorbance of the samples were measured with an UV spectrophotometer (UV-1800, UV-VIS Spectrophotometer, Shimadzu, Japan) at the wavelength of 750 nm. The spectrophotometer was blanked with the zero sample from the standard curve. Protein content of the supernatant was calculated based on the BSA standard curve. Protein solubility was calculated as a percentage value of the protein concentration of the supernatant in relation to the total protein concentration in the initial suspension.

6.2.4 Viscosity measured with Rapid Visco Analyser

Viscosity of the OPC samples during the enzyme treatments were measured with Rapid Visco Analyzer (RVA) (Newport Scientific, Australia). Software Thermocline for Windows (Newport Scientific, Australia) was used to create the programs and control the RVA. 10% (w/w) OPC suspension was made for the analysis. The stirrer was zeroed before setting any programs. Constant stirring of 160 rpm was applied throughout the experiments.

Two different programs were used. A two hour program at 40 °C was used for the enzyme treatment whereas a 15 min program at 95 °C was used for preheating and/or end heating. The heating and cooling speed to the target temperature was approximately 10 °C/min. The enzyme treatment program was used by itself or together with preheating and/or end heating. Both SYG and WM enzymes were tested with these program combinations.

6.2.5 Amylase test

In the amylase test, starch was hydrolyzed from the OPC samples by amylase enzyme. The RVA super 4 (Newport Scientific, Australia) was used to measure the viscosity changes of the OPC during the enzyme treatments. 10% (w/w) OPC suspensions were made and enzymes, amylase BAN 480L, SYG, and WM were used

in the testing with enzyme dosages of 2 mg/g, 5 U/g and 5 U/g flour, respectively. Configurations of programs and enzymes used in the RVA experiments are represented in Table 7. Viscosity was measured throughout the RVA experiments. Enzymatic treatment in the RVA lasted from 2 h to 2.5 h, depending on the configuration. From the RVA test, samples were taken for protein solubility and D-glucose analyses.

Table 7. RVA configurations of programs and enzymes tested in the amylase test. Configurations 1 and 2 were both tested with SYG and WM enzymes, whereas configurations 3 and 4 only with SYG. Preheating and end heating were done by heating the OPC suspension to 95 °C for 15 min. (Different programs are separated with "→" symbol. WM: transglutaminase, SYG: protein-glutaminase + transglutaminase, BAN: α-amylase)

Configuration	Programs with enzymes
1	BAN (2mg/g) + SYG or WM (5 U/g) 40 °C/2 h → end heating
2	preheating → BAN (2mg/g) + SYG or WM 5 U/g 40 °C/2 h → end heating
3	BAN (2mg/g) 55 °C/30 min → SYG (5 U/g) 40 °C/2 h → end heating
4	preheating → BAN (2mg/g) 55 °C/30 min → SYG (5 U/g) 40 °C/2 h → end heating
5	preheating → BAN 40 °C/2 h → end heating

6.2.5.1 D-glucose from the amylase test

D-glucose was only measured from the amylase test performed in the RVA. D-glucose was analyzed with D-glucose (GOPOD format) assay kit (K-GLUC, Megazyme Ltd., Ireland). The samples were thawed and centrifuged (Centrifuge 5417 R, Eppendorf AG, Germany) at 10000 G for 15 min. The obtained supernatant was used for the glucose analysis.

Since GOPOD reagent had been prepared earlier, its validity was checked with spectrophotometer (UV-1800, UV-VIS Spectrophotometer, Shimadzu, Japan) against distilled water at 510 nm wavelength. The obtained sample supernatant was diluted to 1:3 with distilled water. The samples were prepared by adding 3 ml

of GOPOD reagent to a test tube with 100 μl of the diluted sample supernatant. Blank sample and standard sample were prepared the same way, but the addition of 100 μl of supernatant was replaced by 100 μl of distilled water and 100 μl of standard reagent, respectively. All of the test tubes were incubated in 50 °C water bath for 20 min and measured with the spectrophotometer at 510 nm wavelength against the blank sample. D-glucose amount in the samples was calculated with

$$D - glucose\left(\frac{\mu g}{0.1ml}\right) = \frac{dilution\ x\ sample\ abs}{standard\ abs} x\ 100,$$ (1)

where *abs* is absorbance.

6.3 Extrusion

6.3.1 Scale-up of the enzyme treatments for the extrusion trials

Scale-up process of the enzymatic treatments were performed in a Hotmix Pro Gastro mixing equipment (Metos Oy, Finland). 30% (w/w) oat suspension was made from 40% OPC with distilled water. The enzyme (SYG/WM) was diluted to distilled water and added once the targeted temperature was achieved (40 ˚C). Enzymatic treatment lasted for 2 h, except for one sample. SYG 5 U enzyme dosage was also done with 0.5 h treatment at 40 °C, besides the 2 h treatment. Preheatings were also performed in the Hotmix Pro Gastro mixing equipment by heating the OPC suspension first to 95 ˚C for 15 min and then cooling it down in an ice bath to 40 ˚C. For the preheated samples, 15% (w/w) OPC suspension was used to ensure proper mixing.

Scale-up of the amylase test was performed according to the RVA programs in Table 7. Configurations 2 and 5 were chosen, but the end heating was excluded because the extrusion step would replace it. 15% (w/w) OPC suspension was made and heated in the Hotmix Pro Gastro (Metos Oy, Finland) at 95 °C for 15 min. Then the OPC suspension was cooled down to 40 °C in an ice water bath. When 40 °C was achieved, enzymes were added and 2 h incubation at 40 °C was started.

After the treatments in the HotMix Pro Gastro mixing equipment, the OPC suspensions were rapidly frozen in a blast chiller (MonoCell AT, KOMA B.V., the

Netherlands) prior freeze drying to decrease the enzyme action rapidly to minimum. Freeze drying was performed with a freeze dryer Epsilon 2-25DS (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). The freeze dried products were collected and pulverized with a multi-purpose machine that had a cutting blade. After pulverizing, samples were stored in the flour storage (18 °C) until used in the extrusion.

6.3.2 High-moisture extrusion cooking (HMEC)

The extruder used for HMEC was Process 11 Parallel twin-screw extruder by Thermo Scientific, Germany. Cooling die was attached to the die body at the end of the barrel. The cooling die had dimensions of 5 x 20 x 250 mm (H x W x L). Screws in the barrel had length of 48 cm (Figure 2). Water feed was provided to the barrel from a water pump system (MasterFlex L/S, Cole-Parmer, US). Technical data of the extruder is represented in Table 8 and the image of the extruder is in Figure 3.

Table 8. Technical details of the Process 11 Parallel twin-screw extruder.

Technical data of the extruder	
barrel diameter	11 mm
barrel length	40 L/D (440 mm)
barrel segments	8
screw speed	10-1000 rpm
typical throughput	20-2500 g/h
torque per shaft	6 Nm
max. pressure	100 bar
temperature	RT-350 °C
dimensions (L x W x H)	860 x 490 x 400 mm

During the HMEC various parameters were adjusted. Screw speed was between 300 to 400 rpm, temperatures in the barrel sections were between 60-175 °C, and water feed was around 105-300 ml/h. Flour feed was set between 0.2-0.3 kg/h and cooling die to 30 °C. At the start of every extrusion, flour and water ratio was around 50:50 and barrel zone temperatures were between 60-100 °C. Die

temperature was 90 °C. From these starting parameters, variations were made. Representative samples were collected and frozen (-20 °C). Tensile strength and free thiol groups were analyzed from the extrudates (Appendix A, Table A2). Table 9 represents extrusion parameters from the collected extrudates. Also, FPC with a protein content of 61% (as is) (Vestkorn Milling AS, Norway) was extruded as a reference sample of a fibrillated protein concentrate.

Table 9. Parameters of the collected extrudates. Barrel temperatures are represented from the die temperature to the first heating zone (die, Z8, Z7, Z6, Z5, Z4, Z3, Z2, Z1). Melt temperature was not measured from all samples since it was broken. ("H" in the sample name refers to the preheating of the raw material). (OPC: oat protein concentrate, WM: transglutaminase, SYG: protein-glutaminase + transglutaminase, BAN: α-amylase, FPC: faba protein concentrate, T: temperature)

Sample	Barrel T (°C) zones	Flour feed (kg/h)	Water feed (ml/h)	Screw speed (rpm)	Melt T (°C)
Native OPC	155/165/165/140/120/90/70/60	0.25	204	300	-
Control	160/160/160/140/120/90/80/70	0.3	257	300	152
SYG 0.5 U	160/165/165/140/110/90/80/70	0.3	257	400	154
SYG 5 U	165/165/165/140/120/90/80/70	0.3	257	400	158
SYG 5 U 0.5 h	160/165/165/145/110/90/80/70	0.3	247	350	154
WM 0.5 U	160/170/170/140/120/90/80/70	0.3	247	400	154
WM 5 U	165/170/170/145/120/90/80/70	0.3	236	300	158
H-control	165/170/170/145/120/90/80/70	0.3	256	300	157
H-SYG	165/175/175/145/120/90/80/70	0.3	256	400	159
H-WM	170/175/175/145/120/90/80/70	0.3	226	300	163
H-BAN	125/135/135/135/90/80/70/60	0.2	155	300	-
H-BAN SYG	130/140/140/130/110/90/80/70	0.25	155	300	-
H-BAN WM	120/130/130/120/100/90/80/70	0.2	105	300	-
Reference FPC	140/150/150/135/100/90/70/60	0.3	324	350	136

6.3.3 Tensile strength

Tensile strength (kPa) and tearing force (N) values were measured with a Lloyd LS5 material testing device (Ametek Inc., USA). All extruded samples, except samples with amylase enzyme, were tested. The samples with amylase enzyme had too crumbly texture to be measured. Extruded samples were thawed and cut to 20

mm x 20 mm (L x W) bits at RT. Thickness of the samples was 0.5 mm. From each extrudates tensile strength was measured in lengthwise and in widthwise with 6 parallels in both directions. Tearing force (N) was measured as a function of sample extension (mm). Extrudates were stretched at a test speed of 2 mm/min with a 1000 N statistic load cell. Starting force was negative since the jaws compressed the samples therefore the starting force was manually readjusted to zero. Basic gauge length was set to 0.5 mm due to small dimensions of measured samples.

6.3.4 Free thiol groups

For quantifying free sulfhydryl groups, extrudate samples were freeze dried with Alpha 1-4 LSbasic freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). After the drying, the freeze dried samples, and the extrusion flours were pulverized with ball milling equipment (Mixer Mill MM 301, Retsch GmbH, Germany) to powders. The mill was set to 30 Hz frequency for 2 min.

Ellman's reagent (lot: VF299854, Thermo Fisher Scientific, USA) was used for quantifying the free sulfhydryl groups. 60 mg of milled samples were measured to 2 ml Eppendorf tubes. Ellman's reagent and cysteine standard were also measured. L-Cysteinhydrochlorid monohydrate (lot: K28120339 036, Merck, Germany) was used for preparing the cysteine standard curve. Two different buffers were tested. Buffer A had 0.1 M sodium phosphate (NaP) + 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8) and buffer B had 0.1 M NaP + 8 M Urea + 1 mM EDTA (pH 8).

Buffer A/B was added to the samples (1 ml/sample) and to Ellman's reagent. Cysteine stock was made by adding 50 ml of buffer A/B to the measured L-Cysteinhydrochlorid monohydrate. Samples were incubated at RT for 10 min. Standard solutions were prepared (0 mM, 0.05 mM, 0.1 mM, 0.25m mM, 0.5 mM and 1 mM) from cysteine stock. All samples and standard curve were made in triplicates. 2.5 ml of buffer A/B were pipetted to the test tubes with 50 µl of

Ellman's reagent. 250 µl of sample solutions were added to the test tubes and incubation of 15 min started at RT. Tubes were gently vortexed. After incubation, 2 ml of sample was pipetted from the test tubes to 2 ml Eppendorf tube and centrifuged (Centrifuge 5417 R, Eppendorf AG, Germany) at 10000 G for 10 min. Then 1.7 ml of the supernatant was pipetted to micro cuvettes and the absorbance was measured at 412 nm wavelength. To quantify the free thiol groups, the absorbance values were plotted against a cysteine standard curve in a concentration range of 0.0-1.0 mM.

7. Results

7.1 Enzymatic treatment of the oat protein concentrate

Different conditions for the enzymatic treatment of the OPC were tested (e.g. temperature and enzyme dosage) and were compiled to Appendix A. This result section represents only selected main results. The results of the protein solubility analysis are from the enzymatic treatment at 40 °C for 0 h, 1 h, and 4 h with or without preheating (95 °C/15 min). The results of the SDS-PAGE and the particle size distribution analyses are from 2 h or 4 h treatment at 40 °C with or without preheating (95 °C/15 min). The viscosity results are from 2 h or 2.5 h enzymatic treatment and some of the samples had preheating / end heating applied (95 °C/15 min). Extrusion was done to the OPC flours that had been enzymatically treated 0.5 h or 2 h with or without preheating (95 °C/15 min).

The enzymatic treatment in 50 °C did not show significantly differing results compared to the treatment in 40 °C and the enzymatic treatment in pH 5 reduced the protein solubility, therefore they are not further discussed in this thesis (Appendix B: Figure B1 and Figure B2). Furthermore, the enzyme dosage of 50 U showed similar results with 5 U dosage (Appendix B, Figure B3), therefore only the samples treated with 5 U and 0.5 U dosages were used in the represented results of the analyses.

7.1.1 Protein solubility analyzed with the Lowry method

The Lowry method was performed to evaluate how the enzymatic treatment of the OPC affected the protein solubility. It could be observed that the native OPC, control, and preheated control samples had similar and constant solubility of around 13%, 15%, and 12%, respectively (Figure 7). The OPC samples that had been treated with SYG enzyme, showed highest protein solubility at 4 h time point. The SYG 0.5 U, the SYG 5 U, and the preheated SYG 5 U had the highest protein solubility: 26%, 86%, and 77%, respectively. Especially, the SYG 5 U and the preheated SYG 5 U samples showed increase in solubility from 12% to over 70%

already after one hour treatment. Solubility of the WM treated OPC samples remained quite low throughout the 4 h treatment. At 4 h time point, the WM 0.5 U, the WM 5 U, and the preheated WM 5 U had protein solubility around 13%, 14%, and 10%, respectively. WM treated samples were similar with the control sample with constant protein solubility. Protein solubility of the preheated WM 5 U sample decreased 2% from the starting point (0 h), during the 4 h treatment.
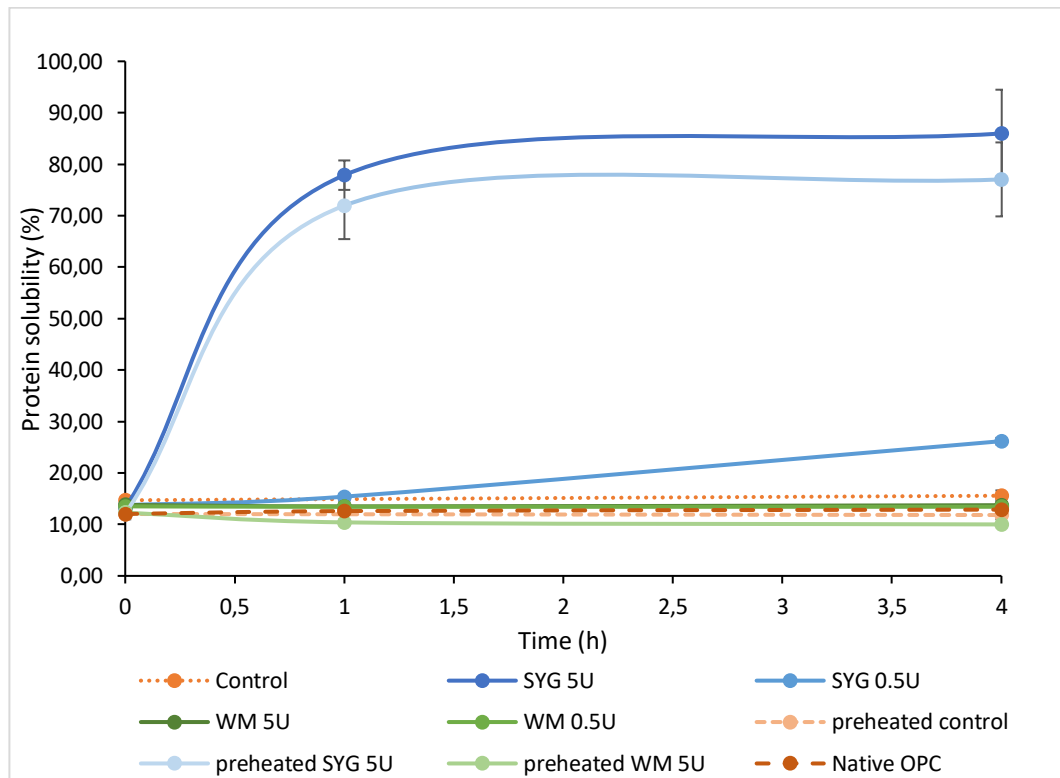
Figure 7. Protein solubility analyzed with the Lowry method from the controls and the enzymatically modified (40 °C/4 h) oat protein concentrate with and without preheating. Average of four measurements, from three different time points (0 h, 1 h, and 4 h). The error bars denote standard deviation. (OPC: oat protein concentrate, WM: transglutaminase, SYG: protein-glutaminase + transglutaminase, BAN: α-amylase)

7.1.2 Molecular weight of oat proteins by SDS-PAGE

SDS-PAGE was performed to assess the changes in the molecular size distribution of the modified OPC and to indicate the level of protein cross-linking. Larger than 250 kDa molecular aggregates cannot migrate through the gel so they stay in the well. That is observed as darker band in the well which may indicate that due to protein cross-linking, large molecular aggregates have formed.

Figure 8 (A) shows SDS-PAGE gel with samples from the enzymatic treatments; controls, WM/SYG treated samples, and preheated samples. Figure 8 (B) shows SDS-PAGE gel figure of samples that have been treated with BAN enzyme. In all of the samples in Figure 8, dominant bands were observed around 55-60 kDa, 32-35 kDa, 20-23 kDa, and 15-17 kDa.

The native OPC and the control (wells 2 and 3 of Figure 8 (A)) were not treated with enzymes so no cross-linking was observed and the loading wells were empty. Six protein bands were observed in both samples, molecular sizes ranging from 15 to 55 kDa. In the well 4, the SYG 0.5 U sample protein bands were also quite similar to the native OPC and the control. Band around 40-45 kDa was fainter compared to the control. Between the SYG 0.5 U and the SYG 5 U samples, (wells 4 and 5 of Figure 8 (A)) there were small differences observed. The SYG 5 U had slightly larger band around 35 kDa, while the SYG 0.5 U had a similar band around 32 kDa. The SYG 5 U had also a lower band around 37 kDa, while the SYG 0.5 U had a corresponding band around 40 kDa. Both samples had smears and larger molecular aggregates (> 250 kDa) observed in the wells. The WM 0.5 U and the WM 5 U samples had similar protein bands observed as the native OPC and the control had, however, also marks of occurred cross-linking were observed from the larger molecular aggregates in the wells and the smears (wells 6 and 7 of Figure 8 (A)). The WM 5 U sample had a slightly fainter band around 40-45 kDa when compared to the control.

The preheated control (well 8 of Figure 8 (A)) had protein bands identical to the bands of the native OPC (well 2 of Figure 8 (A)). The preheated SYG 5 U had similar protein bands and larger molecular aggregates observed in the well (well 9 of Figure 8 (A)), as the SYG 5 U (well 5 of Figure 8 (A)), but also a little bit more intense smear. It also seemed that 45 kDa band from the control was absent from both SYG 5 U and preheated SYG 5 U samples. The preheated WM (well 10 of Figure 8 (A)) had similar bands observed as the preheated control (well 8 of Figure 8 (A)) but also larger molecular aggregates (> 250 kDa) were observed from the well.

In Figure 8 (B) the OPC samples with BAN enzyme had been preheated in 95 °C/15 min and incubated for 2 h at 40 °C. The BAN treated OPC sample (well 2 from Figure 8 (B)), worked as a control since there was no cross-linking enzyme present. Molecular weights of the BAN treated OPC were similar with controls from Figure 8 (A), but the six protein bands that were observed were more intense. The well 3 in Figure 8 (B) had a sample that was modified with BAN and SYG enzymes. Similarities in the molecular weights could be observed when comparing the BAN+SYG sample to the preheated SYG sample from Figure 8 (A) (well 9). However, the observed bands were more intense in the BAN+SYG sample. Here also the absence of a band around 45 kDa was noticed. In the well 3 (Figure 8 (B)) there were faintly larger molecular aggregates observed. The BAN+WM sample (well 4 of Figure 8 (B)) had clearly larger molecular aggregates and smear observed in the well. When comparing the well 4 with the well 2 in Figure 8 (B), it could be seen that most of the observed bands were similar, but the well 4 had slightly lighter bands and there was a new band observed around 70 kDa.
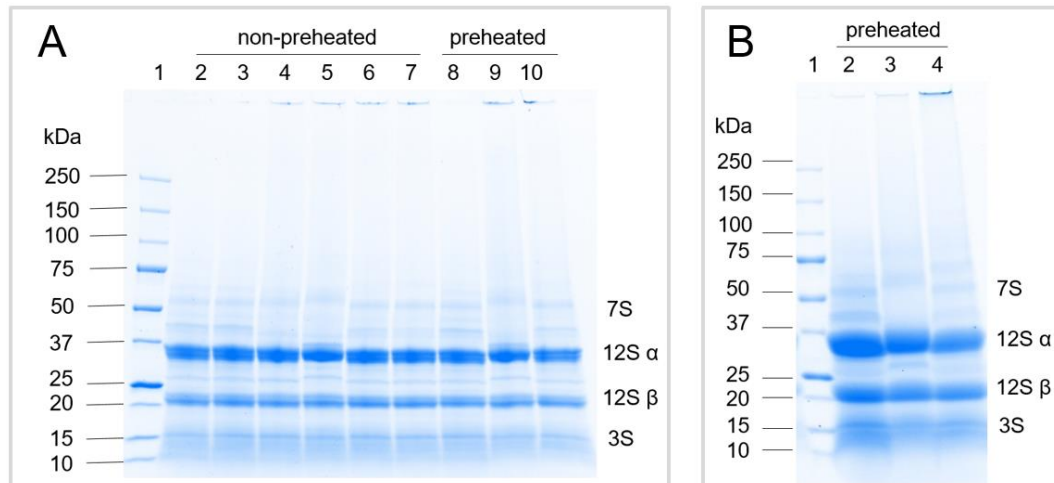
Figure 8. Enzymatically treated samples and their controls visualized in the SDS-PAGE gel. (A) 4 h samples from enzymatic treatments at 40 °C visualized in the reduced SDS-PAGE gel. (1: precision blue standard (Bio-Rad), 2: native OPC, 3: OPC control, 4: SYG 0.5 U, 5: SYG 5 U, 6: WM 0.5 U, 7: WM 5 U, 8: preheated control, 9: preheated SYG 5 U, 10: preheated WM 5 U). (B) 2 h samples from the amylase test scale-up process visualized in the reduced SDS-PAGE gel. (1: precision blue standard (Bio-Rad), 2: preheated BAN 2mg/g, 3: preheated BAN 2mg/g + SYG 5 U, 4: preheated BAN 2mg/g + WM 5 U). (OPC: oat protein concentrate, WM: transglutaminase, SYG: protein-glutaminase + transglutaminase, BAN: α-amylase)

7.1.3 Particle size distribution of the oat protein concentrate samples

Particle size distribution was measured to see how the enzymatic modification affected the particle sizes of the controls and the modified OPC samples. Figure 9 and 10 represents the volume weighted distribution of the OPC samples where the contribution of each particle relates to its volume.

The native OPC had a particle size distribution, where two peaks were observed (Figure 9). The smaller peak had particle sizes around 0.5-12 µm and the larger peak had particle sizes around 12-250 µm. From the particle size distribution of the native OPC sample, the most abundant particle size was 81 µm, with a relative volume of 6% and the second most abundant particle size was 5 µm (2.3%). For the control sample (treated without enzyme) there were also two peaks observed. The larger peak had particle sizes ranging from 10.5 to 225 µm and the smaller peak had a particle sizes around 0.5 to 10.5 µm. The most abundant particle size was 48 µm with a relative volume of 5.5% and the second most abundant particle size was 5 µm (3.5%). Enzymatic modification of the OPC showed larger amount

of smaller particles in the distribution compared to the native OPC and the control samples. The SYG 0.5 U sample had particle sizes between 0.5 and 80 µm and the most abundant particle size was 5 µm with a relative volume of 7.5%. The SYG 5 U sample had a large peak around 0.5-55 µm and its most abundant particle size was 4 µm with a relative volume over 10%. The WM 0.5 U and the WM 5 U samples both had two similar peaks. The smaller peak ranged from 0.5 to 15 µm. The larger peak in the WM 0.5 U sample started from 15 µm and ended in 150 µm, while the WM 5 U peak ended in 170 µm and had slightly wider particle size distribution. The most abundant particle size for the WM 0.5 U and the WM 5 U samples was approximately 5.5 µm with a relative volume of 5.5% and the second most abundant particle size was 42 µm with a relative volume of 3%.
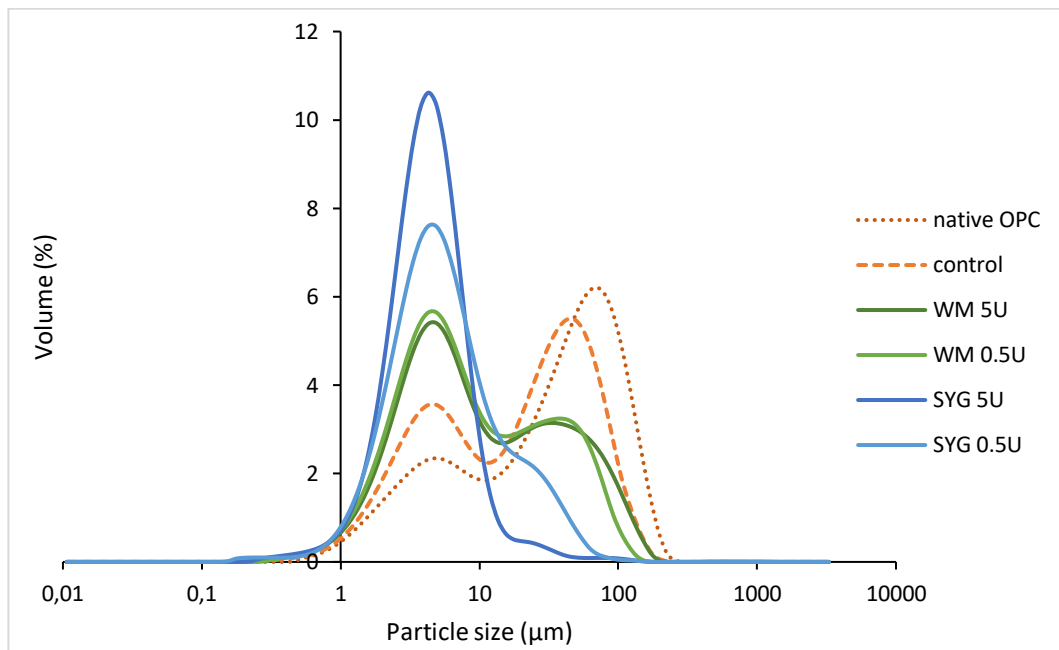
Figure 9. Particle size distribution of the controls and enzymatically modified oat protein concentrate samples from 4 h at 40 °C treatment, according to the Fraunhofer approximation. Results are average of two parallel samples (average of 20 measurements). (OPC: oat protein concentrate, WM: transglutaminase, SYG: transglutaminase + protein-glutaminase).

Figure 10 shows the particle size distribution of the native OPC and the preheated samples (control, WM 5 U, and SYG 5 U). The native OPC sample is the same one shown already in Figure 9. Preheated control had a peak where particle sizes were

between 3 µm to 1000 µm and the most abundant particle size was 118 µm with a relative volume of 6%. As was found without preheating, enzymatic modification of the OPC resulted again in larger amount of smaller particles. The preheated SYG 5 U sample had a peak between 3 µm to 250 µm and the most abundant particle size was 29 µm with 8% relative volume. The preheated WM 5 U sample had a peak between 3 µm to 400 µm and the most abundant particle size was 43 µm with relative volume of 6.5%.
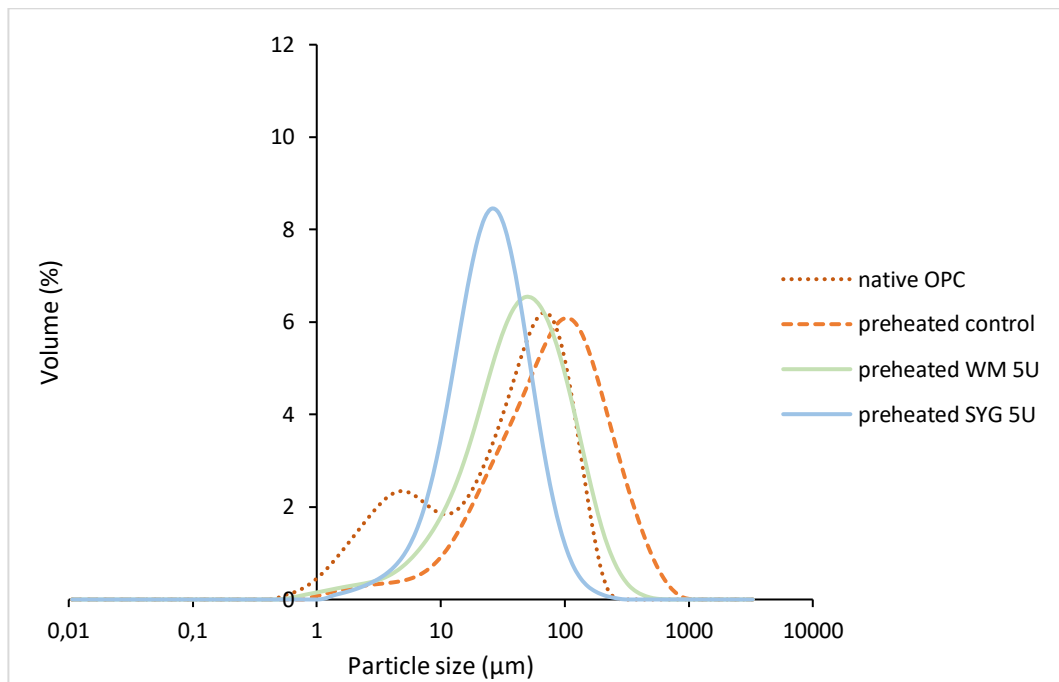
Figure 10. Particle size distribution of the native oat protein concentrate and the preheated oat protein concentrate samples from 4 h treatment at 40 °C, according to the Fraunhofer approximation. Results are average of two parallel samples (average of 20 measurements). (OPC: oat protein concentrate, WM: transglutaminase, SYG: transglutaminase + protein-glutaminase).

7.1.4 Viscosity of the modified oat protein concentrate and the amylase test

Viscosity was measured to evaluate how the enzymatic treatment of the OPC affected its viscoelastic properties. Viscosity measurement was also used as an indication of how the starch of the OPC could behave during the extrusion cooking. Viscosity of the non-preheated OPC samples (control, SYG 0.5 U, SYG 5 U, WM 5 U) were also tested with the RVA but the results are not shown. The viscosity level

remained under 20 cP throughout the treatment of 40 °C for 2 h, in all of the samples.

Figure 11 shows the RVA viscosity curves of the preheated OPC samples (control, SYG 5 U, and WM 5 U) during the treatment. It was seen that viscosity rose the most during the cooling phases. When the preheated control and the preheated WM 5 U samples were compared it was seen that the viscosity profiles were similar and that the addition of WM enzyme had insignificant effect to the OPC viscosity, with 5 U enzyme dosage. During the 2 h incubation at 40 °C the viscosity started to lower with both samples and lowered rapidly when heating to 95 °C had started. When 95 °C had been reached the viscosity stayed steady at around 100 cP until the cooling started. With both the preheated control and the preheated WM 5 U samples the viscosity rose to about 500 cP during the last cooling phase. For the preheated SYG 5 U sample, the viscosity started to slowly rise during the 2 h treatment in 40 °C and lowered slightly when the temperature started to rise to 95 °C. When 95 °C temperature had been reached, the viscosity started to rise again rapidly and during the cooling phase it reached viscosity of about 1300 cP. The preheated SYG 5 U resulted in approximately 800 cP higher viscosity compared to the preheated WM 5 U and the preheated control viscosities.
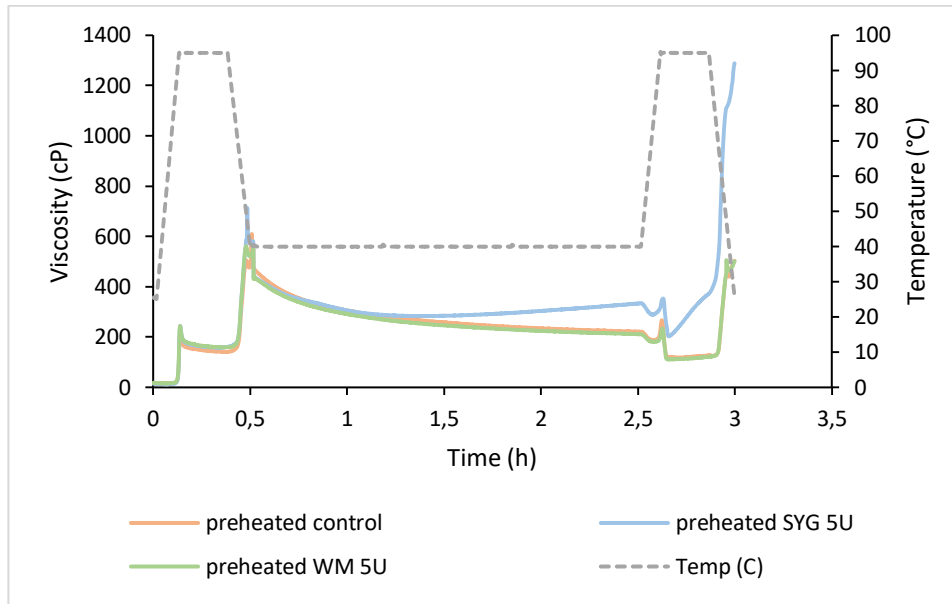
Figure 11. RVA viscosity curves of the preheated samples made from 10% oat protein concentrate suspension. Enzyme has been added around 0.5 h. (Data from separate programs (preheating, 40 °C incubation for 2 h, end heating) have been concatenated here). (WM: transglutaminase, SYG: transglutaminase + protein-glutaminase).

Figure 12 shows the RVA viscosity curves of the amylase test. Preheating of the samples to 95 °C for 15 min prior the addition of enzymes was applied here as well. Addition of amylase enzyme had clear effect to the viscosity profile when the results are compared to samples without amylase treatment (Figure 11). All samples had similar viscosity curves during the preheating phase, until the enzymes were added at around 0.5 h. Addition of BAN enzyme rapidly lowered the viscosity from around 700 cP to around 25 cP in all samples. Samples with just BAN and BAN+WM enzymes did not show any viscosity changes after dropping to 25 cP. However, viscosity started to differ in the sample with BAN and SYG enzymes. There the viscosity began to rise slowly during the 2 h treatment in 40 °C and at the start of the end heating, viscosity rose from 50 cP to around 350 cP.
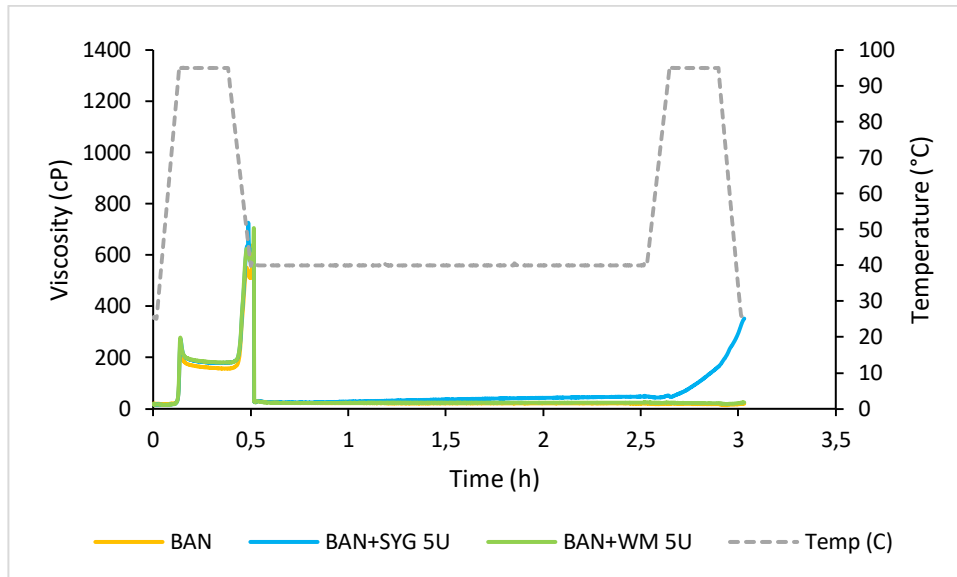
Figure 12. RVA viscosity curves of the preheated oat protein concentrate samples from 10% oat protein concentrate suspension with amylase enzyme. SYG and WM enzymes are used in 5 U/g flour dosage whereas BAN enzyme is used in 2 mg/g flour dosage. Addition of enzyme(s) happens around 0.5 h. (Data from separate programs (preheating, 40 °C incubation for 2 h, end heating) have been concatenated here). (BAN: α-amylase, WM: transglutaminase, SYG: transglutaminase + protein-glutaminase).

The D-glucose and the protein solubility was measured from the samples that were treated either with BAN+SYG or with BAN+WM enzymes and taken from specific points during the RVA measurements. Some of the taken samples from the end of the RVA measurements could not be analyzed since they were too thick. In all of the measured samples the D-glucose value was approximately 120 µg/0.1 ml after the 2 h enzymatic treatment (BAN + WM/SYG) at 40 °C (data not shown). The protein solubility of the same samples were high (> 80%) when SYG enzyme was present and low (< 16%) when WM enzyme was present (data not shown).

7.2 Evaluation of the oat protein concentrate samples after the high-moisture extrusion cooking

HMEC was performed to evaluate if the enzymatic modification of the OPC would improve the formation of a fibrillar, meat mimicking structure during the extrusion. Figure 13 shows FPC that has been extruded in the HMEC. Faba protein

fibrillated well and similar result was hoped from the extrusion of the modified OPC samples.

Figure 13. High-moisture extrusion cooked native faba protein concentrate (protein content 61% as is).

Figure 14 shows the extrudates obtained from HMEC of the OPC flours (native and treated). Overall, small indications of fibrillar structure were observed in some of the samples as presented below. The SYG 0.5 U and the WM 0.5 U extrudates had a scattered sides which resembles slightly the extruded FPC (Figure 13). Most textures where quite stiff but still soft (e.g. native OPC, controls, SYG 5 U, and WM 5 U). The preheated WM 5 U had very soft and silky texture. During the extrusion, sweet roasted odor was observed from most of the samples at temperatures over 150 °C in the die section. Preheated extrudates with BAN enzyme had a different texture compared to other extrudates. Textures were crumbly and not holding together well. Also during the extrusion of the BAN treated samples, similar temperatures with other extrudates were not achieved since the extruder got jammed every time at 140 °C temperature and the extrusion had to be stopped.

Extrudates differed in their color. Most of the samples had light brown color. The SYG 5 U, the preheated WM 5 U and the preheated BAN+SYG extrudates had a darker brown color. The SYG 5 U extrudates color differed from the color of the preheated SYG5 U even though the cooking temperatures were around the same level (see the barrel temperature zones from Table 9). The preheated SYG 5 U had

10 °C higher maximum temperature in the cooking zone 8 when comparing to the SYG 5 U sample, but the die temperature was the same (165 °C).
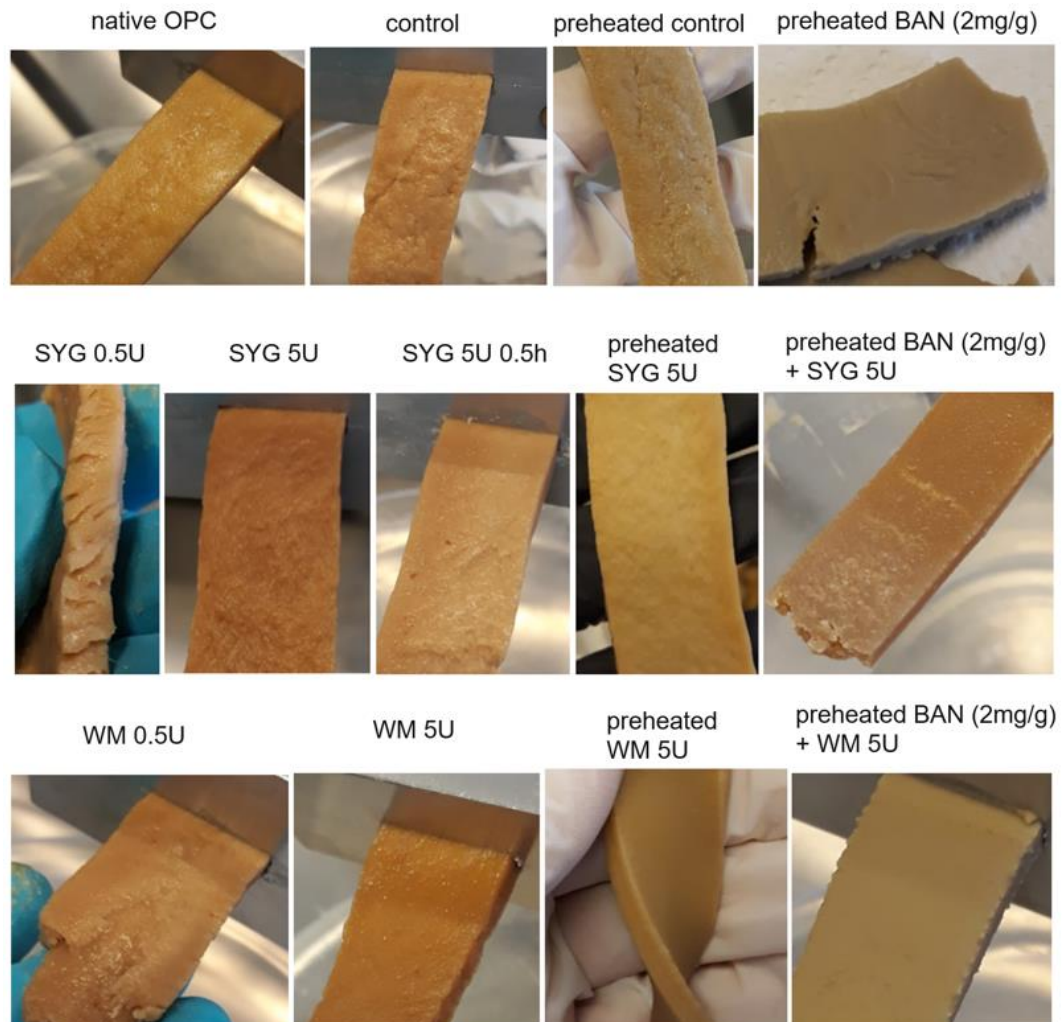
Figure 14. Pictures of the collected extrudates. 1st row has the pictures of controls, 2nd row has the pictures of all SYG treated samples, and 3rd row has the pictures of all WM treated samples. (From left to right, 1st row: native oat protein concentrate, control, preheated control, preheated BAN 2 mg/g. 2nd row: SYG 0.5 U, SYG 5 U, SYG 5 U 0.5 h, preheated SYG 5 U, preheated BAN 2 mg/g + SYG 5 U. 3rd row: WM 0.5 U, WM 5 U, preheated WM 5 U, preheated BAN 2 mg/g + WM 5 U). (OPC: oat protein concentrate, BAN: α-amylase, WM: transglutaminase, SYG: transglutaminase + protein-glutaminase).

7.2.1 Tensile strength measured from the extrudates

Tensile strength was measured to evaluate the maximum tensile stress that the extrudates can withstand. This could give indication of the extrudates textural

properties e.g. toughness. Extrudates with amylase enzyme were not measured, since their structure was too crumbly and not holding well together, and thus, they were not possible to be analyzed by the method used.

Figure 15 shows the measured tensile strengths (kPa) of the extrudates in widthwise (W) and lengthwise (L) directions. Overall, the widthwise tensile strength varied between 33 kPa to 109 kPa and the lengthwise tensile strength varied between 20 kPa to 114 kPa, in the measured extrudates. Most of the extrudates had higher tensile strength in lengthwise direction. Some deviation between the tensile strength of parallel measurements was also observed. Highest load (N) strength peaks from the extrudates were obtained around 1-3 mm extension in both directions, except for the preheated WM 5 U extrudate, less than 1 mm extension was enough for both directions (Appendix C).

Native OPC extrudate had the weakest structure lengthwise (20 kPa). Control extrudate had quite even tensile strengths in both pulling directions (W: 55 kPa, L: 57 kPa). SYG 0.5 U extrudate had higher tensile strength in widthwise than in lengthwise (W: 68 kPa, L: 53 kPa). SYG 5 U extrudate had lower widthwise tensile strength (42 kPa), but better lengthwise tensile strength (66 kPa). SYG 5 U extrudate that had been enzymatically treated only 0.5 h had almost equal tensile strengths in both pulling directions (W: 69 kPa and L: 70 kPa). When the SYG 0.5 U, the SYG 5 U, and the SYG 5 U 0.5 h samples are compared to control sample, it can be observed that the tensile strength do not differ much. However, the WM 0.5 U and the WM 5 U samples showed some differences compared to the control. The WM 0.5 U extrudate had widthwise tensile strength of 92 kPa and lengthwise of 65 kPa. The WM 5 U extrudate had widthwise tensile strength of 33 kPa and lengthwise of 38 kPa. The WM 0.5 U extrudate had strongest structure among the non-preheated samples and when the WM 5 U extrudate had the weakest.

Preheated control had very similar tensile strengths as non-preheated control, in both directions (W and L around 52 kPa). Preheated SYG 5 U extrudate showed highest tensile strength in both direction among all the studied extrudate samples

(W: 109 kPa, L: 114 kPa). However, it also showed most deviation between the parallel measurements. The SYG 5 U extrudate had almost half weaker structure compared to the preheated SYG 5 U extrudate. Preheated WM 5 U had lower widthwise tensile strength compared to the lengthwise tensile strength (W: 40 kPa, L: 64 kPa) and the results were similar with the preheated control.
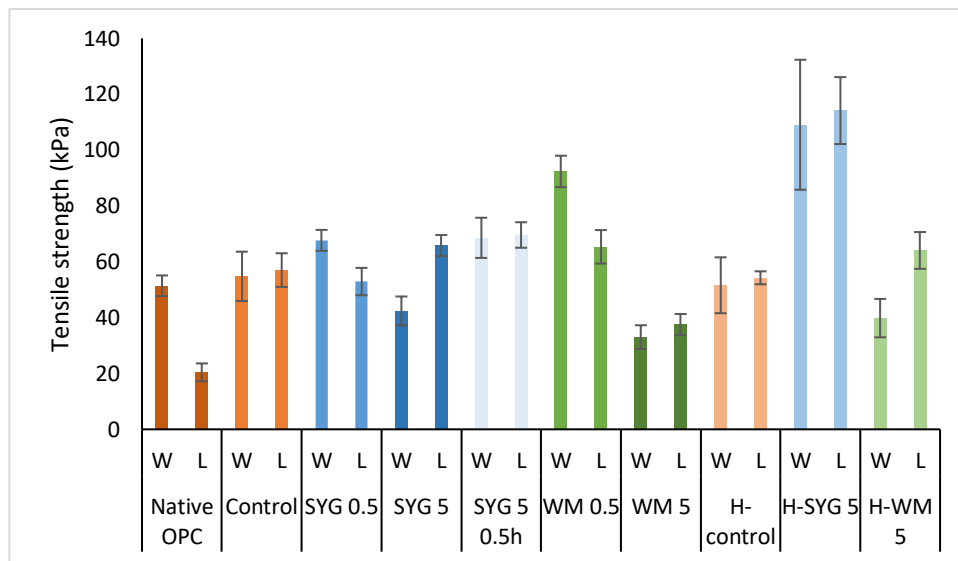
Figure 15. Tensile strength (kPa) of the extrudates. Results are average of six parallel measurements measured in widthwise and lengthwise directions. The error bars denote standard deviation. (W: width, L: length, OPC: oat protein concentrate, BAN: α-amylase, WM: transglutaminase, SYG: transglutaminase + protein-glutaminase. "H" in the sample names refers to the preheating of the oat protein concentrate (95 °C/15 min)).

7.2.2 Free thiol groups measured from the extrudates and the treated oat protein concentrate powders

Free thiol groups were quantified to see how the extrusion affected their availability. Disulphide bonds are needed in the formation of fibrous structures and from the amount of the free thiol groups, between the treated OPC samples (powder format) and the extrudates made from the OPC samples, formation of disulphide bonds targeted to be evaluated. Two buffers were tested, one with urea (buffer B) and one without (buffer A). Urea denaturates proteins by breaking the non-covalent bonds which in turn opens up the proteins' globular structure (Liu & Hsieh, 2008). As a result, Ellman's reagent has better accessibility to the free

thiol groups. By comparing the results made with urea buffer and without, it is possible to indicate the amount of thiol groups that were hidden inside the globular protein structure.

Figure 16 shows the results obtained by quantifying the amount of free thiol groups with buffer A (sodium phosphate) from the treated OPC powders and the extrudates. The buffer A gave results where the powders had smaller amount of free thiol groups than the extrudates (Figure 16). It looks like the availability of free thiol groups increased after the extrusion step. Free thiol group amounts from the powders analyzed with buffer A varied between 1.2 to 3.8 µmol/g and the extrudates free thiol group amounts varied between 1.8 to 4.4 µmol/g. In most of the samples the amount of free thiol groups increased during the extrusion almost by half (control, SYG 0.5 U, SYG 5 U 0.5 h, WM 5 U, preheated BAN). The amount of free thiol groups was increased by over 50% during the extrusion in the preheated control, the preheated SYG 5 U, and the preheated WM 5 U extrudates. Smaller differences in the amount of the free thiol groups between powders and extrudates were observed from the native OPC, the SYG 5 U, the heated BAN SYG, and the heated BAN WM samples.

Figure 16. Sodium phosphate buffer (buffer A) quantified thiol groups from powders before extrusion and from the extrudates extruded from the oat protein concentrate samples. Average of three parallel samples. The error bars denote standard deviation. (P: powders, E: extrudates, OPC: oat protein concentrate, BAN: α-amylase, WM: transglutaminase, SYG: transglutaminase + protein-glutaminase. "H" in the sample names refers to the preheating of the oat protein concentrate (95 °C/15 min)).

Figure 17 shows the results obtained by quantifying the amount of free thiol groups with buffer B from the treated OPC powders and the extrudates. From Figure 17 it was noticed that results were opposite to the results obtained with buffer A (Figure 16). The amount of free thiol groups dropped after the extrusion step, in all of the analyzed samples. Urea in the buffer B denatured the globular oat protein and revealed more thiol groups from the inside. Results with the buffer A were also in lower scale compared to the buffer B results.

The native OPC powders had the largest amount of free thiol groups available and after the extrusion the availability of free thiol groups dropped by half (Figure 17). Similar results were also noticed from the control, SYG 0.5 U, SYG 5 U, SYG 5 U 0.5 h, WM 0.5 U, and WM 5 U -samples. Free thiol group amounts from the powders of these samples varied between 4.5 to 6.4 µmol/g and extrudate results varied between 2.3 to 3.1 µmol/g. The preheated samples had fewer free thiol groups available in the powders to begin with and the drop after extrusion was not that

great as with the non-preheated samples. Especially, with the preheated BAN+WM sample the amount of free thiol groups seemed to be almost equal between the powders and the extrudate. The amount of free thiol groups of the preheated powders varied between 3.2 to 3.8 µmol/g and the amounts from preheated extrudates varied between 2.3 to 3.3 µmol/g. Generally, the amount of free thiol groups of the extrudates were at similar level in all of the samples quantified with buffer B (urea) ranging from 2.3 to 3.3 µmol/g.
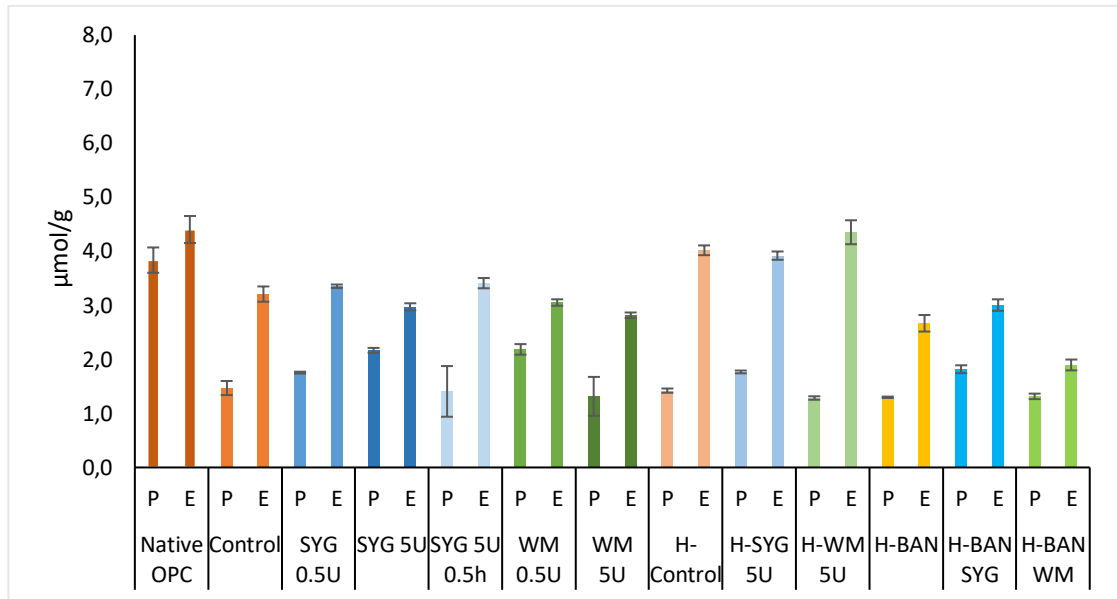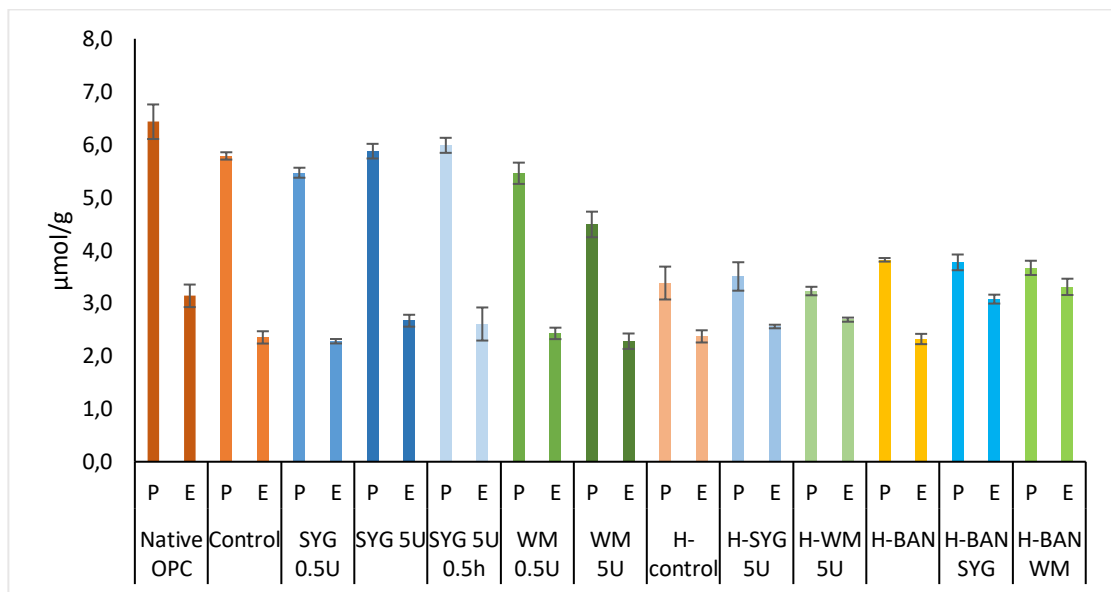

Figure 17. Urea buffer (buffer B) quantified thiol groups from powders before extrusion and from the extrudates extruded from the oat protein concentrate samples. Average of three parallel samples. The error bars denote standard deviation. (P: powders, E: extrudates, OPC: oat protein concentrate, BAN: α-amylase, WM: transglutaminase, SYG: transglutaminase + protein-glutaminase. "H" in the sample names refers to the preheating of the oat protein concentrate (95 °C/15 min)).

8. Discussion

8.1 Enzymatic treatments of the oat protein concentrate

Commercial food grade enzymes (WM, SYG, and BAN) were used to increase the oat proteins' ability to fibrillate during HMEC. By creating a stronger protein network, fibrillation of oat proteins may improve. WM and SYG enzymes were used for the protein modification. WM enzyme had TG activity, whereas SYG had TG and PG activity. TG induces cross-linking between glutamine and lysine residues. PG deamidates glutamine residues to glutamic acid, which changes the isoelectric point to lower pH. Besides the modification of proteins, hydrolysis of starch by BAN enzyme (α-amylase) was also tested. Too high levels of starch may interfere the fibrillation of proteins and therefore the OPC was also modified by α-amylase which hydrolyses the starch to sugars and dextrins. Protein solubility, SDS-PAGE, viscosity, and particle size distribution were analyzed to evaluate how the enzymatic treatments affected the OPC raw material.

8.1.1 Solubility of the oat proteins

The native oat proteins are known to have a low protein solubility into aqueous solutions since the most abundant protein fraction is a salt soluble globulin and since the oat globulin has a compact molecular structure as well as a high denaturation temperature (Jiang et al., 2015). The native OPC and the control samples were not modified by protein-modifying enzymes and they had a low protein solubility (≤ 15%). This was in line with the study of Nivala et al. (2017) where the solubility of the non-enzyme-treated OPI was 16%. The WM-modified samples showed a slight decrease on their protein solubility when compared to the control. Nevertheless, the decrease was not significant. Nivala et al. (2017) reported that TG treatment increased the OPI protein solubility, although the increase was insignificant. The observed increase was suggested to occur due to the pH difference between the non-enzyme-treated OPI and the TG treated OPI. Nivala et al. (2017) reported also that the protein solubility of the FPI decreased after the TG treatment. Siu et al. (2002) reported that the TG treatment improved

the protein solubility of the oat globulin at pH 4-5. The WM-modified OPC samples had a higher pH (pH > 6) than in the oat globulins reported by Siu et al. (2002), which may have affected the slight decrease of the protein solubility.

The SYG enzyme increased the protein solubility in the OPC samples. The SYG 5 U sample had the highest protein solubility among the samples in Figure 7. The SYG enzyme was a mixture of TG and PG enzymes, which both can cause protein deamidation. Deamidation and the improvement of oat protein solubility has been studied by Jiang et al. 2015. In the study it was concluded that deamidation caused by PG increased the structural flexibility and net charge of the protein, which was assumed to be the mechanism behind the improvement of the protein solubility in water. The PG deamidation introduced negatively charged carbonyl side chains to the oat protein which caused increase in the oat protein molecules electronic repulsion forces. Most likely in the present study, the protein net charge changed and the oat proteins isoelectric point shifted lower. This made the protein-water interactions more favorable in the pH of the enzymatic reaction than the protein-protein interactions which occur near the isoelectric point.

Preheating of the OPC had a decreasing effect on the protein solubility (Figure 7). All of the preheated samples had a lower protein solubility than their corresponding non-preheated samples. This was probably caused by partial denaturation and aggregation of the proteins. Protein denaturation results as changes in the conformation which further affects the net charge of the protein molecules. Mann et al. (2014) reported the effect of heat treatment on wheat flours. The heating of the wet flour at 90 °C for 30 min decreased the protein solubility significantly. The denaturation and aggregation of wheat gluten was suspected to be the reason for the decrease in the protein solubility.

8.1.2 Cross-linking of the modified oat protein concentrate

From the SDS-PAGE analysis, it was noticed that oat globulins were cross-linked by both WM and SYG enzymes (Figure 8). Both enzymes induced the cross-linking

reaction already at 0.5 h (Appendix D). Although cross-linking occurred and high molecular weight aggregates were formed (> 250 kDa), the overall effect was moderate as detected by remaining protein bands in the SDS-PAGE gel. As mentioned in Section 3.1, Nivala et al. (2017) successfully created strong cross-links with the FPI and some with the OPI by modifying both protein isolates by TG. The results obtained with the TG modified OPI were similar to the ones that were obtained in the present study with the WM- and SYG-modified OPC. Cross-linking occurred only to some extent. In the case of the FPI it was suspected that the extensive cross-linking reaction was result of the folding of legumin proteins and the differences in the amino acid composition. In addition, more soluble proteins were better available for the cross-linking reaction. These reasons could also have an effect in the present study of the modified OPC. The strong cross-linking reaction of FPI faded some of the protein bands in the gel and induced the smear under the well. From Figure 8 it was seen that the cross-linked OPC samples (wells 4, 5, 6, 7, 9, 10 from A and wells 3, 4 from B) had a faint smear and only some protein aggregates were observed in the well. Additionally, the observed protein bands did not fade significantly in any of the samples. Preheating of the OPC did not increase or decrease the cross-linking reaction nor affected the formation of large molecular aggregates when compared to the non-preheated samples (Figure 8 (A)).

The dominant bands from Figure 8 were identified to be the oat globulin subunits as also noted by Nieto-Nieto et al. (2014). The band around 32-35 kDa was identified to be the 12S α-subunit and the 12S β-subunit was identified to be around 20-23 kDa. The bands between 40-70 kDa were identified as 7S subunit and 3S subunit was detected around 15-17 kDa.

The 12S β-subunits molecular sizes were not affected by the enzymatic treatments (Figure 8). The oat globulins α-subunit might be more exposed on the surface of the molecule, while the β-subunits might be inside the core, which could explain the reason that the α-subunits were affected but the β-subunits were not, as also mentioned by Nieto-Nieto et al. (2014). The TG enzyme induced cross-linking

reaction in the modified OPC samples. The PG enzyme in the SYG-modified samples, with 5 U enzyme dosage, probably affected the observed bands in wells 5 and 9 (Figure 8 (A)). In both samples, the 12S α-subunit band had an increase in the molecular weight from around 32 kDa to 35 kDa and the 7S subunit had a band from around 40 kDa most probably cross-linked and become > 250 kDa molecular aggregate, when compared to the controls (wells 3 and 8 of Figure 8 (A)). The reason why SYG 5 U treated samples had the 12S α-subunits molecular weight increased a little is not clear, but it may be related to the deamidation by PG (ionic strength) and to the balance between intra- and intermolecular cross-links in the protein.

The samples in Figure 8 (B) had large bands around 32-35 kDa, which were identified to be the 12S α-subunit based on the study of Nieto-Nieto et al. (2014). The sample modified by both BAN and SYG enzymes had increase in the molecular size of the 12S α-subunit, as in the samples modified by the SYG 5 U in Figure 8 (A). Moreover, the 7S subunit band from around 40 kDa also seemed to cross-link and become a large molecular aggregate. The sample modified by both BAN and WM had intense smear and large molecule aggregates in the well (Figure 8 (B)). The observed bands seemed to be fainter when compared to the sample modified with only BAN enzyme. Also a band around 70 kDa was observed unlike in the other BAN treated samples which could be due the stronger cross-linking reaction. Among all of the samples in Figure 8 the BAN+WM sample seemed to have most intense cross-linking reaction. The hydrolysis of starch probably enhanced the cross-linking reaction by TG. The BAN+SYG sample did not have that intense cross-linking reaction probably since PG deamidation used also glutamine residues, therefore there was not as many residues available as the BAN+WM sample had.

8.1.3 Particle size distribution of the modified oat protein concentrate

Enzymatic modification of the OPC had an effect on the particle size distribution when compared to the native OPC and the control samples (Figure 9). The SYG modified OPC samples showed higher ratio of smaller particles and a more uniform particle size distribution compared to the WM modified OPC samples.

However, WM also shifted the particle size distribution towards smaller particles when compared to the control. Nivala et al. (2017) reported that TG reduced the particle size of oat protein particles probably due to intra-particle cross-links which may prevent aggregation. The smaller particle sizes of the SYG samples could be possibly explained by the PG's deamidation effect. This has been reported also by Jiang et al. (2015) where the PG deamidated oat proteins showed a higher amount of small particles (< 10 μm) and more uniform particle size distribution. It should be noted that in the particle size analysis soluble particles are not measured, therefore affecting the particle size distribution.

Particle size distribution decreased as the SYG enzyme dosage increased from 0.5 U to 5 U, this was probably due to the higher deamidation degree caused by the higher enzyme dosage. Same effect caused by the enzyme dosage was not observed between WM 5 U and WM 0.5 U samples even though Nivala et al. (2017) reported that the particle size dispersion decreased as a function of TG dosage. However, the difference in the WM dosages were smaller than the TG dosages used in the study of Nivala et al. (2017). In the present study the particle size distributions between the WM 0.5 U and the WM 5 U were close to identical (Figure 9).

From Figure 10 it was seen that the preheating of the OPC affected the results by increasing the particle sizes, when compared to the corresponding samples without preheating (Figure 9). The preheated SYG sample had higher amounts of smaller particles in the particle size distribution than the other preheated samples. The preheated WM samples particle size distribution was between the preheated SYG and the preheated control. Increase of the particle size distribution towards larger particles was probably due to the protein aggregation and the swelling of the starch during the 95 °C preheating. Lagrain et al. (2005) reported that increase of the wheat gluten suspensions viscosity at 95 °C temperature occurred due to formation of large protein aggregates. Kim et al. (2004) reported swelling of wheat starch granules due to gelatinization that occurred in elevated temperatures, which caused increase in the particle size distribution.

8.1.4 Viscosity analysis of the modified oat protein concentrate

RVA analysis is often used for the measurement of starch-related physicochemical properties. Furthermore, it can also inform about the protein gelling (Balet et al., 2019). RVA gave information about the behavior of starch in the OPC during 95 °C heating and enzymatic treatment of 2 h at 40 °C. From Figure 11 it could be seen that the preheating step increased the viscosity. This was probably due to the swelling of the starch components in the samples and the partial denaturation of the proteins. The preheated control and the preheated WM samples had identical viscosity results, which was probably due to their low protein solubility. It could be concluded that TG had no effect on the viscosity. The preheated SYG on the other hand had viscosity curve differing from the preheated control. This was probably due the heat-induced gelation of proteins. Increase in the protein solubility increases the gelling ability. Heat-induced denaturation of globular proteins results as formation of cross-links which leads to the formation of protein aggregates that increase in size during gelation, which also results as increase in the viscosity (Nicolai & Chassenieux, 2019). The preheated SYG sample had a high protein solubility (Figure 7), which could explain the increase in the viscosity though the gelation of the proteins. Also, swelling of starch increased the viscosity.

The use of amylase enzyme to hydrolyze starch, was tested in the RVA. SYG and WM enzymes were both tested with BAN enzyme. From Figure 12 it was seen that the amylase treated samples had similar effect in the viscosity development as the preheated samples from Figure 11. The OPC sample with just BAN and the OPC sample with BAN and WM had almost identical viscosity curves. Addition of the amylase enzyme, after the preheating, lowered the viscosity instantly to low values. However, the OPC sample with both BAN and SYG enzymes showed increase in the viscosity, although the increase was not as high as in the preheated SYG sample. These results also indicate that TG in the WM sample had no significant effect on the viscosity. The PG activity in the SYG enzyme was probably the main contributor on the increased viscosity of the SYG-modified samples. Protein solubility was not measured from this specific amylase test. Nevertheless,

it was measured from the samples (rehydrated flours) made in the scale-up process (Appendix E). Mainly the proteins were affecting the increase of the viscosity since the starch was hydrolyzed from the sample. The improved protein solubility probably led to improved gelling of proteins and that resulted as increased viscosity.

8.2 Extrusion of the modified oat protein concentrate

The native and modified OPC flours were used in the HMEC. The quality of the extrudates obtained from the HMEC with similar extrusion parameters, varied. In addition to visual observation, tensile strength and free thiol groups were analyzed from the extrudates to evaluate the structural properties of the extrudates.

With the tested extrusion parameters, clear fibrous structure, as in the reference sample (Figure 13), was not observed from the OPC extrudates. Nevertheless, the SYG 0.5 U and the WM 0.5 U extrudates showed some indications of possible fibrous structure with scattered sides, but they still had quite soft texture (Figure 14). The native OPC, the SYG 5 U, the SYG 0.5 U 0.5 h, the WM 0.5 U, the preheated control, and the preheated SYG 5 U extrudates had rigid but still quite soft textures. The WM 5 U extrudate had a very silky and smooth texture that did not become rigid in the high extrusion temperatures. The textural differences between the extrudates are mostly affected by the molecular interactions e.g. non-covalent and covalent interactions, cross-linking, and protein-lipid-starch interactions (Dalbhagat et al., 2019).

Generally the extrudates had similar color as the native OPC extrudate (Figure 14). However, some color variations were observed. Darker brown color was observed especially from the extrudates SYG 5 U and WM 5 U. Bakalov et al. (2016) mentioned that in the extrusion of bean-based products, the extrudates showed darker colors compared to their raw-blends due to the formation of brown pigments through the Maillard reaction. However, in the present study, there is no clear correlation observed between the Maillard reaction and the extrudates color. In addition to the Maillard reaction, the reflection of light from the

extrudate with differing textures could influence the differences observed in the colors. However, the color differences between the extrudates are difficult to explain.

The preheated SYG 5 U extrudate had a light brown color. It was surprising that in the temperatures over 170 °C, the Maillard reaction did not turn the color darker. The preheated WM 5 U extrudate had a lighter color than the non-preheated WM 5 U extrudate. It would seem that the preheating process of the OPC affects the color formation during the extrusion.

The OPC samples that were treated with amylase enzyme, were difficult to extrude in the HMEC. With the tested parameters, the extrusion cooker got jammed and higher temperatures above 140 °C could not be tested. This was probably due too high water and flour feed during the extrusion. It was interesting to notice that preheated BAN+SYG extrudate had a darker color than the preheated SYG 5 U even though its extrusion temperatures were lower than 150 °C. This was probably due the Maillard reaction, since there was more free sugars available for the reaction due the hydrolysis of starch. The hydrolysis of starch also resulted as extrudates that had a brittle texture that cracked easily (Figure 14).

8.2.1 Tensile strength of the extrudates

The differences in the tensile strength between the pulling directions were insignificant in the control, the SYG 5 U 0.5 h, the WM 5 U, the preheated control, and the preheated SYG 5 U -samples (Figure 15). The native OPC, the SYG 0.5 U, and the WM 0.5 U extrudates showed stronger structure in widthwise than in lengthwise direction. On the other hand, the SYG 5 U and the preheated WM 5 U extrudates had stronger structure in lengthwise direction. Usually, the other pulling direction has been observed to be stronger than the other when the extrudate has fibrous structure (Dekkers, 2018). This could indicate that at least some fibrils were formed in some of the extrudates (e.g. SYG 0.5 U and WM 0.5 U extrudates). However, more tests should be performed to verify this.

The differences in the SYG enzyme dosages with the 2 h treatment, resulted in the change of the lower pulling direction. The SYG 0.5 U extrudate had lower tensile strength in lengthwise and the SYG 5 U had lower tensile strength in widthwise (Figure 15). The fiber orientation might have influenced the observed difference. With the non-preheated WM-modified extrudates it was observed that the structure weakened with higher enzyme dosage. The WM 0.5 U extrudate had the strongest structure among the non-preheated extrudates according to the tensile strength measurements (Figure 15). It probably had more covalent bonds formed during the extrusion than the other non-preheated extrudates, which generated a stronger structure.

Among the preheated samples, the preheated control did not differ from the non-preheated control. The preheated SYG 5 U extrudate had the strongest structure among all of the tested extrudates according to the tensile strength results. As discussed in the section 8.1.4 the preheated SYG 5 U had also higher viscosity due to the aggregation of proteins. The gelation of proteins probably created a stronger protein network which lead to a stronger structure for the preheated SYG 5 U extrudate (Nicolai & Chassenieux, 2019). The preheated WM 5 U had slightly stronger structure in lengthwise compared to the non-preheated WM 5 U extrudate. It could be concluded that preheating had significant effect to the preheated SYG 5 U extrudates structure and a slight impact on the preheated WM 5 U extrudates structure.

It is known that extrusion cooking temperatures and moisture content influence the tensile strength of the extrudates (Zahari et al., 2020; Osen et al., 2014). Zahari et al. (2020) noticed from a high-moisture meat analogues made from a hemp protein concentrate that a high moisture content in the extrusion can result as a structure that breaks more easily and that high temperatures can increase the tensile strength in the lengthwise direction. These observations might explain some differences between the OPC extrudates, such as the weak structure of the WM 5 U extrudate and the increase of the lengthwise tensile strength in the SYG 5 U and the preheated WM 5 U extrudates.

8.2.2 Free thiol groups quantified from the extrudates and the oat protein concentrate powders

Liu & Hsieh, (2008) stated that disulphide bonds have a significant role in the holding of the rigid structure and in the formation of fibrous texture of the extrudate. The quantification of free thiol groups gave indication of the formation of new disulphide bonds. In the present study, two buffers were used to see the difference between the free thiol groups of the globular non-denatured oat protein and the denatured oat protein, before and after the extrusion. The results obtained by urea buffer showed that the amount of free thiol groups reduced after the extrusion, which means that possible new disulphide bonds were formed during the extrusion (Figure 17).

The results obtained without protein denaturation (buffer A) showed the opposite result where the free thiol groups increased after the extrusion (Figure 16). In the extrusion barrel, temperatures are sharply increased. This can result in the disruption of intramolecular disulphide bonds and the formation of new intermolecular disulphide bonds. In temperatures over 150 °C, the new disulphide bonds can afterwards be disrupted and consequently increase the amount of free thiols groups (Zhang et al. 2019). This could explain some of the variations between the samples, since all, except amylase treated samples, were extruded at temperatures over 150 °C.

Preheating of the samples influenced the amount of free thiol groups. It is known that the formation of disulphide bonds can be increased by heating (Visschers & De Jongh, 2005). Some disulphide bonds were already formed during the preheating of the OPC. This would explain why the preheated samples had lower amounts of free thiol groups compared to the non-preheated samples (Figure 16 and Figure 17).

Especially the powders without preheating, quantified with urea buffer, had almost 50% more free thiol groups compared to the amount of free thiols after the extrusion (Figure 17). From the textural point of view, the non-preheated extrudates all showed quite rigid texture, which is in agreement with the free thiol

group results since more disulphide bonds formed during the extrusion. The preheated samples did not show that significant difference between free thiol groups of the powders and the extrudate samples. It also seems that changing the enzyme dosage of SYG and WM from 0.5 U to 5 U had only slight effect to the amount of free thiol groups. Differences between the SYG- and the WM-modified extrudates were also quite small. Clear connection between the tensile strength results and the free thiol group results were not observed. The preheated SYG 5 U extrudate that had the highest tensile strength, showed free thiol group results that were similar with the preheated control and the preheated WM 5 U, which had almost 50% lower tensile strength compared to the preheated SYG 5 U. The preheated WM 5 U extrudate had a smooth texture, which was probably due to low amount of new disulphide bonds formed during the extrusion and its low protein solubility.

8.3 Limitations of the study

The protein solubility measurements were done according to the Lowry method and it should be noted that starch and sugars can interfere with the colorimetric measurement by over rating the results. Phenolic compounds could have also affected the analysis. However, the Kjeldahl method was used to confirm some of the results and the Kjeldahl results indicated that results from the Lowry method were similar (Appendix F). Furthermore, it should be noted also that the preheating of the OPC could create some starch clumps that do not solubilize during the 4 h incubation in the water bath. This could also explain the deviation between the parallel results.

Particle size distribution measurements had some variation in the samples and therefore, it should be investigated how much the freezing and thawing of the sample affected the particle size distribution compared to if the sample would have been measured right after the enzymatic treatment. The enzymes were not inactivated from the protein solubility and the particle size distribution samples, which might also affect to the results. However, both enzymes (WM and SYG) had

an optimum temperature of 55 °C according to the manufacturer, so the measurements in the RT should not have significant impact to the results.

Possible problems in the viscosity measurement could come from the enzyme addition step where the RVA machine had to be stopped and a new program started. This resulted as "cleaned" data where the data from these separate programs had been concatenated for the result figures. The used enzymes also had 1-2 min to be in the sample suspension before the mixing and the viscosity measurement started again. This could already affect to the viscosity, but it was not measured. However, it was impossible to add the enzyme any other way than by ending the program.

It should be noted that the extrudates represented in Figure 14 shows photos that were not taken in similar conditions so the brightness of the room may change the colors and the camera can also influence the colors of the photos. It should be also noted that there was a limited amount of the modified OPC sample flours made, which limited the testing of different extrusion parameters. In addition, optimization of the extrusion parameters during extrusion should be practiced more.

The small size of the extrudates limited the tensile strength measurements since the gauge length had to be short. In addition, some of the cut extrudates were slightly less than 2 cm in width, which could affect the variation of the parallel measurements. However, several parallel measurements were performed in lengthwise and widthwise pulling directions to obtain more trustworthy results.

Some problems occurred during the free thiol group analysis. Samples were difficult to solubilize to the urea buffer and since the urea denaturates proteins, the solution became viscous and harder to pipette. Longer vortexing time was needed with the urea buffer samples than with the sodium phosphate buffer samples. This resulted as a slight foaming of the samples before pipetting. However, the standard deviations of the results were acceptable and differences between the samples could be discussed.

9. Conclusions

In this thesis, the OPC was modified by enzymes to increase oat proteins fibrillation during the HMEC. Generally, protein concentrates have poor fibrillation due to the low protein content and the presence of other components e.g. starch. The hypothesis in this work was that the cross-linking of oat proteins by WM and SYG enzymes might result as a strong protein network which might increase the fibrillation during the extrusion cooking. Hydrolysis of starch by BAN enzyme could also increase the fibrillation of oat proteins and enhance their cross-linking reaction when treated together with the WM and SYG enzymes.

Different treatment conditions were explored for the enzymatic modification of the OPC. Temperature, enzyme dosage, pH, treatment time, and preheating were tested and the modified OPC was analyzed by SDS-PAGE, protein solubility, viscosity, and particle size distribution -analyses. These analyses were performed to see how the enzymatic modification affected to the OPC, and most representative samples could be selected for the HMEC. The modified OPC samples were then extruded by the HMEC and tensile strength as well as free thiol groups were analyzed.

The results showed that cross-linking reactions were induced by both protein-modifying enzymes. The preheated BAN+WM sample had the strongest cross-linking reaction, probably since the starch was hydrolyzed and that enhanced the cross-linking by WM. However, the SYG enzyme had greater impact on the functional properties of the oat proteins than the WM enzyme. This was most probably due to the PG activity in the SYG. Most likely the deamidation caused by PG increased the protein solubility significantly, which further affected the other functional properties e.g. gelling. Increase in the viscosity was observed in the preheated SYG 5 U and the preheated BAN+SYG samples probably due the gelation reaction of proteins at high heating temperature. It was confirmed that the rise of viscosity at the end heating was not only due the swelling of starch, since the viscosity increased also in the BAN+SYG sample where the starch had been hydrolyzed. Further studies are needed to confirm if the cross-linking

reaction affected to the gelation or if it was the PG activity that increased the viscosity. It was observed that the WM enzyme had no effect to the viscosity when it was compared to the control. This was probably since both had similar protein solubility. The deamidation probably influenced the particle size distribution in the SYG treated samples by decreasing it. The particle size distribution of the WM treated samples also decreased towards lower particles, but not as much as with the SYG treated samples. The preheating of the OPC increased the particle size distribution, probably due to the swelling of starch and the protein denaturation.

These detected modifications to the OPC resulted also differences in between the extrudates produced by the HMEC. Color and textural differences were observed by visual observation. The SYG 0.5 U and the WM 0.5 U extrudates showed possible signs of fibrous structure as compared to the extruded reference (FPC). Otherwise, clear fibrous structures were not observed. The non-preheated samples had a rigid structure, which was probably caused by the formation of disulphide bonds during the extrusion. The results from the free thiol group analysis supported the hypothesis. The preheated extrudates had less disulphide bonds formed during the extrusion since during the preheating some disulphide bonds were already formed. In the tensile strength analysis, the preheated SYG 5 U extrudate showed highest tensile strength in both pulling directions, which was in agreement with the viscosity results. Most probably, the gelation of oat protein at high temperature resulted as a strong protein network which gave strength to the preheated SYG 5 U extrudates structure.

All in all, this thesis provided initial results about the HMEC of the modified oat proteins and additional information about the functional properties of modified oat proteins. The obtained results can be used for the future experiments of the production of meat analogues and other oat innovations. Formation of a stronger protein network by the enzymatic modification was observed, however, extrudates did not show a clear fibrillated structure. Further optimization of the process might result with fibrillated oat proteins.

10. Future prospects

These results indicated that formation of a stronger protein network is possible by the enzymatic treatment. However, further experiments are needed to achieve fibrillated structures from oat proteins. In the future, the extrusion of the OPC could be performed with a wider variety of different extrusion parameters to find the optimum conditions for the possible fibrous structure formation. Optimization of water feed could also enhance the formation of fibrillated structure during the HMEC. Furthermore, extrusion of a higher protein content OPC could enhance the formation of fibrous structure during the HMEC. In addition, partial hydrolysis of starch could be tested since in this study the strong hydrolysis resulted as too brittle texture in the extrudates and there were too many soluble components present which also affected the extrusion.

In the future, proper color measurements could also be performed to the extrudates (e.g. by Minolta). Other textural properties besides tensile strength e.g. hardness and chewiness could be also evaluated from the extrudates to get better insight of the texture. Furthermore, sensory evaluations could give important information about the extrudates texture, appearance, and taste.

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APPENDICES

Appendix A. Summary of the performed treatments and analyses in the experimental part of the thesis

Table A1. Conditions, samples, and analyses tested in the 1st experimental part of the thesis (enzymatic treatments of the oat protein concentrate).
(OPC: oat protein concentrate, RT: room temperature, T: temperature, t: time, SYG: transglutaminase + protein-glutaminase, WM: transglutaminase, BAN: α-amylase)

Raw material	Protein content (as is)	Preheating (+ end heating) (T/t)	Enzymatic treatment time (h) (not applied in viscosity)	Sample	Enzyme activity	Other parameters	Analyses				
							Lowry (protein solubility)	Particle size	Reducing SDS-PAGE	D-glucose	Viscosity*
OPC (VTT's fraction)	31%		4	Control		40 °C	x	x	x		x
			4	WM	50 U	40 °C	x	x	x		
			4	WM	5 U	40 °C	x	x	x		x
			4	WM	0.5 U	40 °C	x	x	x		
			4	SYG	50 U	40 °C	x	x	x		
			4	SYG	5 U	40 °C	x	x	x		x
			4	SYG	0.5 U	40 °C	x	x	x		x
		95 °C/15 min	4	WM	5 U	40 °C	x	x	x		x
		95 °C/15 min	4	WM	0.5 U	40 °C	x	x	x		x
		95 °C/15 min	4	SYG	5 U	40 °C	x	x	x		x
		95 °C/15 min	4	SYG	0.5 U	40 °C	x	x	x		x

			4	WM	5 U	pH 5, 40 °C	x	x	x		
			4	WM	0.5 U	pH 5, 40 °C	x	x	x		
			4	SYG	5 U	pH 5, 40 °C	x	x	x		
			4	SYG	0.5 U	pH 5, 40 °C	x	x	x		
			2	Control		50 °C	x				
			2	WM	5 U	50 °C	x	x	x		
			2	WM	0.5 U	50 °C	x	x	x		
			2	SYG	5 U	50 °C	x	x	x		
			2	SYG	0.5 U	50 °C	x	x	x		
		95 °C/15 min	4	Control		40 °C	x	x	x		x
		95 °C/15 min	4	Control		40 °C	x				
		95 °C/30 min	4	Control		40 °C	x	x	x		
		95 °C/30 min	4	WM	5 U	40 °C	x	x	x		
		120 °C/15 min	2	Control		40 °C	x	x	x		
		120 °C/15 min	2	WM	5 U	40 °C	x	x	x		
		120 °C/15 min	2	SYG	5 U	40 °C	x	x	x		
	40%		4	Control		40 °C	x	x	x		
		95 °C/15 min	4	Control		40 °C	x	x	x		
			4	WM	5 U	40 °C	x	x	x		
			4	SYG	5 U	40 °C	x	x	x		
		95 °C/15 min	4	SYG	5 U	40 °C	x	x	x		
	31%		4	Control		40 °C	x				
		95 °C/15 min	4	Control		40 °C	x				
			4	SYG	5 U	40 °C	x				
		95 °C/15 min	4	SYG	5 U	40 °C	x				
	40%		4	Control		40 °C	x	x			
		95 °C/15 min	4	Control		40 °C	x	x			x
			4	SYG	5 U	40 °C	x	x			

	95 °C/15 min	4	SYG	5 U	40 °C	x	x			x
31%		4	Native OPC		RT	x				
40%		4	Native OPC		RT	x	x			
		4	SYG	0.5 U	40 °C	x	x			
		4	SYG	0.5 U	40 °C	x	x			
		4	WM	0.5 U	40 °C	x	x			
		4	WM	0.5 U	40 °C	x	x			
		4	WM	5 U	40 °C	x	x			
	95 °C/15 min	4	WM	5 U	40 °C	x	x			x
	95 °C/15 min	4	WM	5 U	40 °C	x	x			
40%		4	Native OPC		RT	x	x	x		
		4	Native OPC		RT	x	x	x		
40%		2	BAN; SYG	2mg/g; 5 U	40 °C	x			x	x
	95 °C/15 min + 95 °C/15 min	2	BAN; SYG	2mg/g; 5 U	40 °C	x			x	x
		0.5, 2	BAN; SYG	2mg/g; 5 U	55 °C, 40 °C	x			x	x
	95 °C/15 min + 95 °C/15 min	0.5, 2	BAN; SYG	2mg/g; 5 U	55 °C, 40 °C	x			x	x
		2	BAN; WM	2mg/g; 5 U	40 °C	x			x	x
	95 °C/15 min + 95 °C/15 min	2	BAN; WM	2mg/g; 5 U	40 °C	x			x	x
	95 °C/15 min	2	BAN	2mg/g	40 °C			x		
	95 °C/15 min	2	BAN; SYG	2mg/g; 5 U	40 °C			x		
	95 °C/15 min	2	BAN; WM	2mg/g; 5 U	40 °C			x		

*Viscosity analyses have approximately 2 h enzymatic treatment.

Table A2. Conditions, samples, and analyses tested in the 2nd experimental part of the thesis (the extrusion trials). (OPC: oat protein concentrate, HMEC: high-moisture extrusion cooking, RT: room temperature, T: temperature, t: time, SYG: transglutaminase + protein-glutaminase, WM: transglutaminase, BAN: α-amylase)

Raw material	Protein content (as is)	Preheating (T/t)	Enzymatic treatment time (h)	Enzymatic treatment T (°C)	Sample	Enzyme activity	HMEC	Analysis from the flours before HMEC	Analyses from the HMEC	
								Lowry (protein solubility)	Tensile strength	Free thiol groups
OPC	40%		2	RT	Native OPC		x	x	x	x
			2	40	Control		x	x	x	x
			2	40	SYG	0.5 U	x	x	x	x
			2	40	SYG	5 U	x	x	x	x
			0.5	40	SYG	5 U	x	x	x	x
			2	40	WM	0.5 U	x	x	x	x
			2	40	WM	5 U	x	x	x	x
		95 °C/15 min	2	40	Control		x	x	x	x
		95 °C/15 min	2	40	SYG	5 U	x	x	x	x
		95 °C/15 min	2	40	WM	5 U	x	x	x	x
		95 °C/15 min	2	40	BAN	2mg/g	x	x		x
		95 °C/15 min	2	40	BAN; SYG	2mg/g; 5 U	x	x		x
		95 °C/15 min	2	40	BAN; WM	2mg/g; 5 U	x	x		x

Appendix B. Protein solubility of oat proteins treated in different conditions
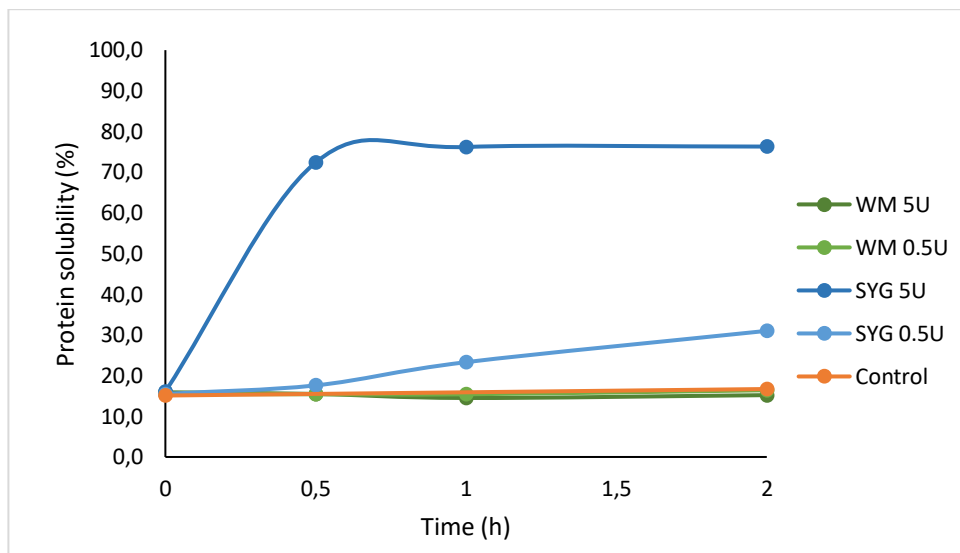

Figure B1. Protein solubility of enzymatically treated (50 °C/2 h) of 31% oat protein concentrate. (WM: transglutaminase, SYG: transglutaminase + protein-glutaminase)
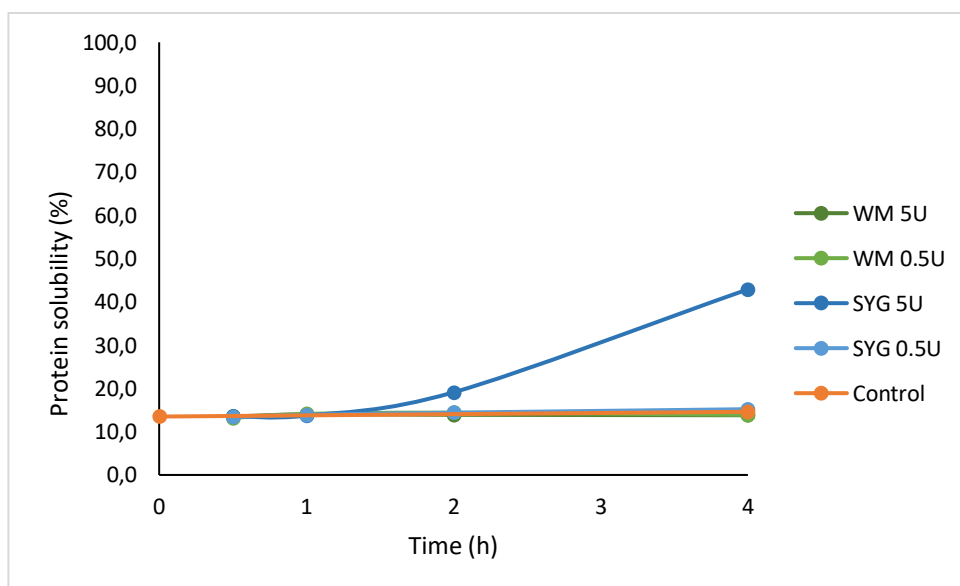

Figure B2. Protein solubility of enzymatically treated (pH 5/ 40 °C /4 h) of 31% oat protein concentrate. (WM: transglutaminase, SYG: transglutaminase + protein-glutaminase)
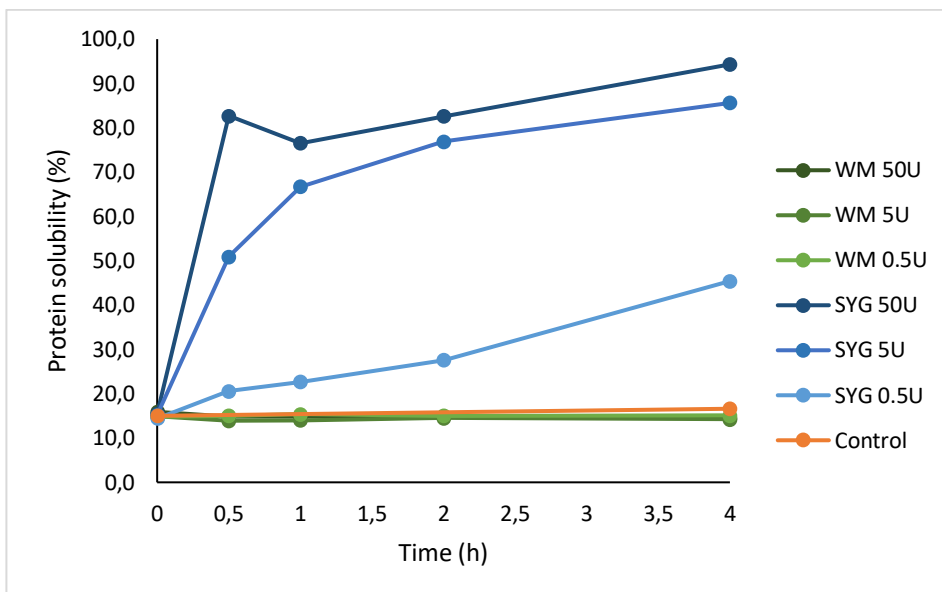
Figure B3. Protein solubility of enzymatically treated (40 °C/4 h) 31% oat protein concentrate. Three different enzyme dosages: 0.5 U, 5 U, and 50 U. (WM: transglutaminase, SYG: transglutaminase + protein-glutaminase)

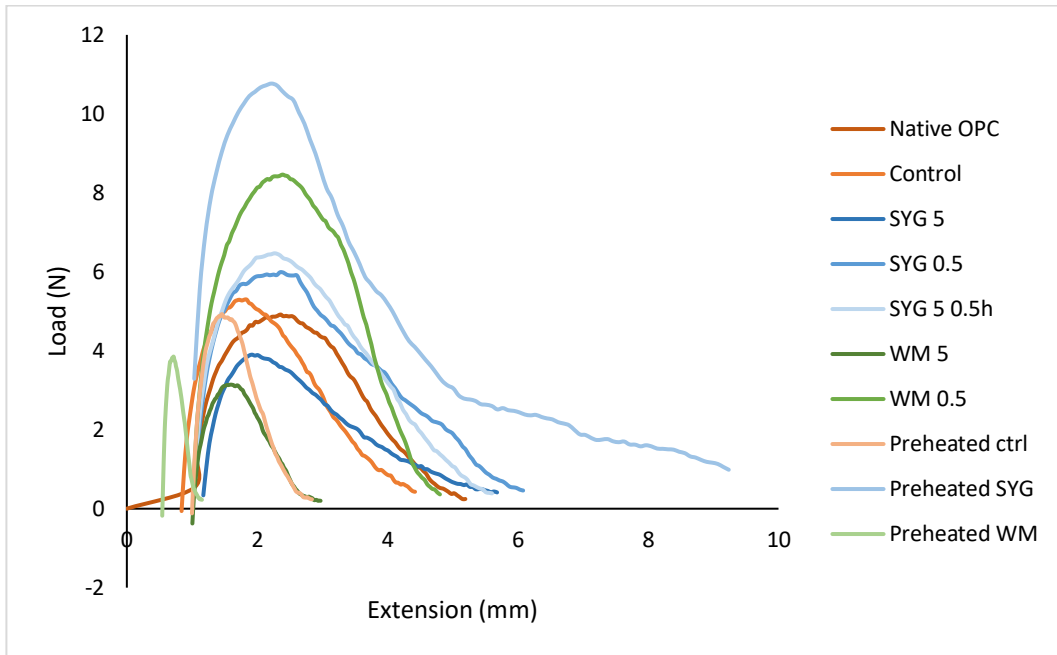# Appendix C. Load from the tensile strength measurements of the extrudates

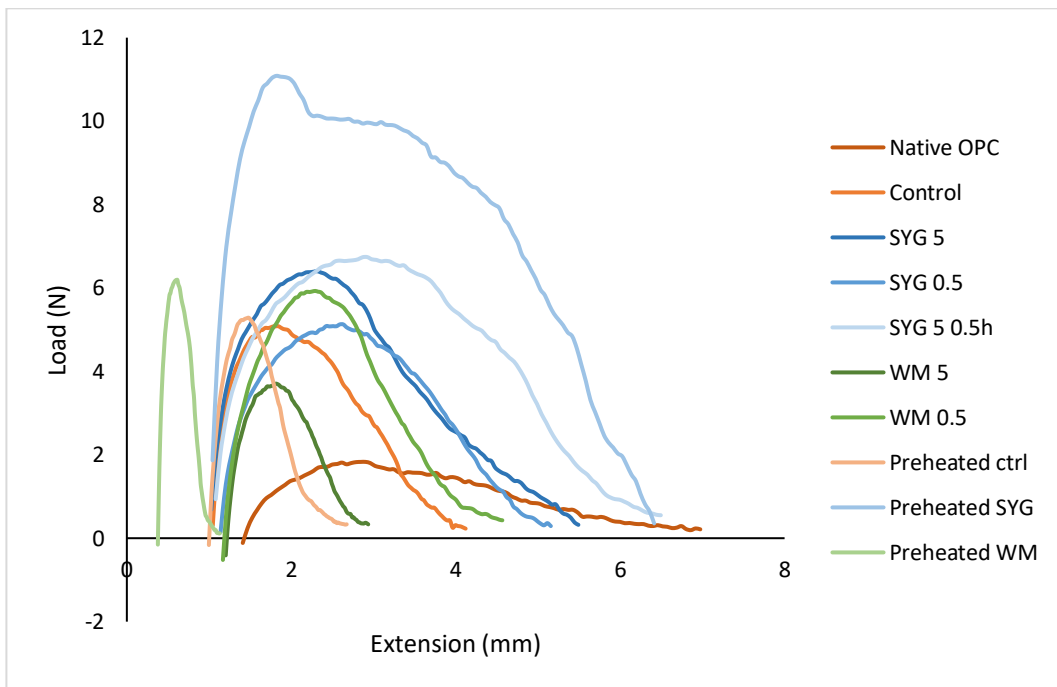Figure C1. Widthwise measurement of the load (N) from the oat protein concentrate extrudates.

Figure C2. Lengthwise measurement of the load (N) from the oat protein concentrate extrudate.

Appendix D. Reducing SDS-PAGE gels from enzymatically modified oat protein concentrates
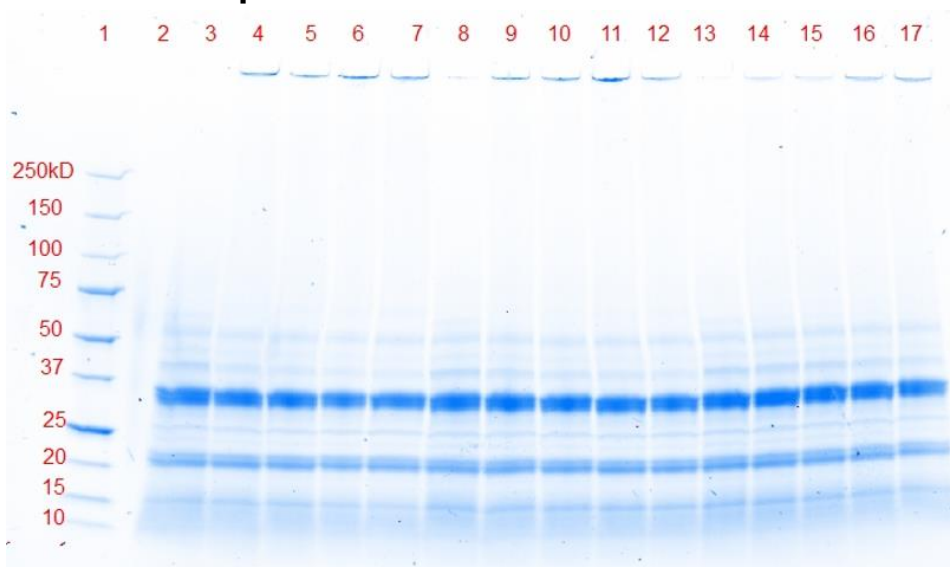

Figure D1. Reducing SDS-PAGE gel of WM-modified oat protein concentrate (40 °C/ 4 h). (1: Standard, 2: empty, 3: WM 50 U 0 h, 4: WM 50 U 0.5 h, 5: WM 50 U 1 h, 6: WM 50 U 2 h, 7: WM 50 U 4 h, 8: WM 5 U 0 h, 9: WM 5 U 0.5 h, 10: WM 5 U 1 h, 11: WM 5 U 2 h, 12: WM 5 U 4 h, 13: WM 0.5 U 0 h, 14: WM 0.5 U 0.5 h, 15: WM 0.5 U 1 h, 16: WM 0.5 U 2 h, 17: WM 0.5 U 4 h). (WM: transglutaminase).
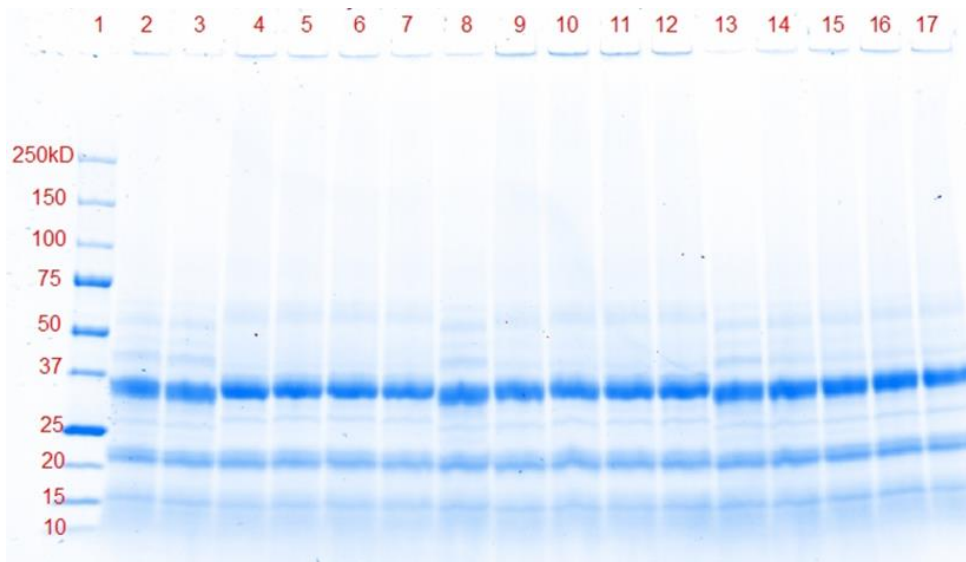

Figure D2. Reducing SDS-PAGE gel of SYG-modified oat protein concentrate. (1: Standard, 2: Control 4 h, 3: SYG 50 U 0 h, 4: SYG 50 U 0.5 h, 5: SYG 50 U 1 h, 6: SYG 50 U 2 h, 7: SYG 50 U 4 h, 8: SYG 5 U 0h, 9: SYG 5 U 0.5 h, 10: SYG 5 U 1 h, 11: SYG 5 U 2 h, 12: SYG 5 U 4 h, 13: SYG 0.5 U 0 h, 14: SYG 0.5 U 0.5 h, 15: SYG 0.5 U 1 h, 16: SYG 0.5 U 2 h, 17: SYG 0.5 U 4 h). (SYG: protein-glutaminase + transglutaminase).

Appendix E. Protein solubility from the rehydrated enzymatically modified oat protein concentrate flours

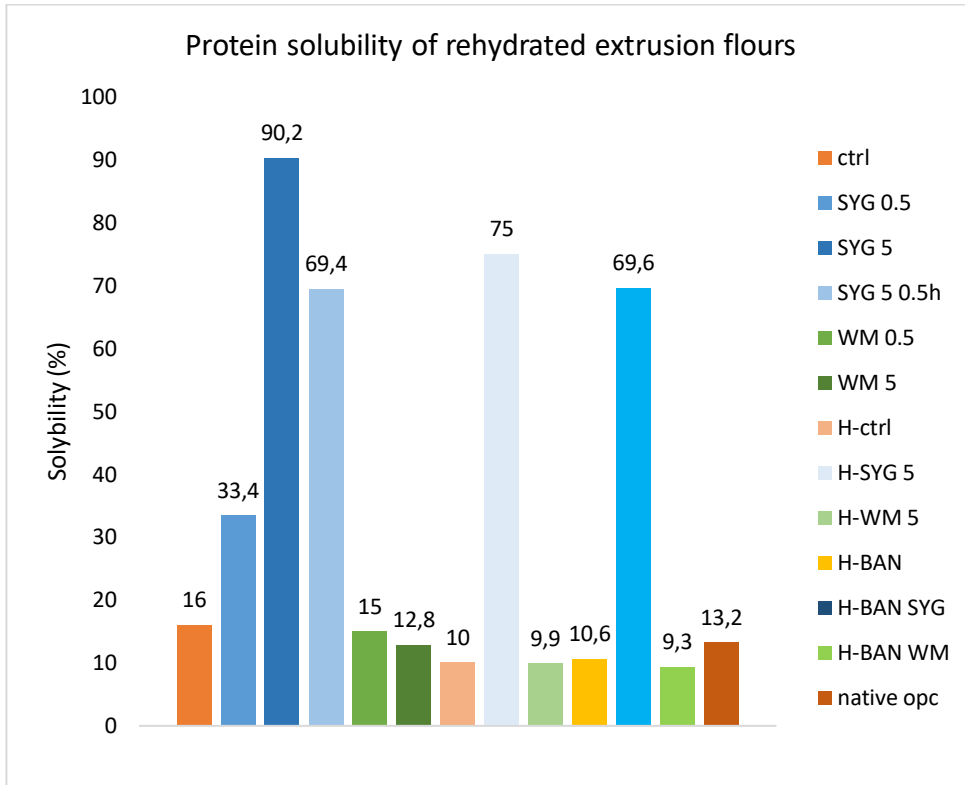Figure E1. Protein solubility of the rehydrated enzymatically modified oat protein concentrate flours used in the high-moisture extrusion cooking. "H" in the sample names refer to the preheating (95 °C/15 min). (WM: transglutaminase, SYG: transglutaminase + protein-glutaminase, BAN: α-amylase)

Appendix F. Protein solubility with Kjeldahl and Lowry methods

Table F1. Kjeldahl and Lowry protein solubility results from the selected samples. (OPC: oat protein concentrate, SYG: transglutaminase + protein-glutaminase)

Protein content of the OPC (%)	Sample	Kjeldahl (%)	Lowry (%)
31	Native OPC	8.8	13.8
	Control 4 h	11.6	15.0
	SYG 5 U 4 h	80.9	83.2
	Preheated control 4 h	7.0	11.8
	Preheated SYG 5 U 4 h	74.6	70.6
40	Native OPC	8.2	11.3
	Control 4 h	11.0	14.8
	SYG 5 U 4 h	79.4	78.9
	Preheated control 4 h	6.5	11.3
	Preheated SYG 5 U 4 h	59.3	70.8