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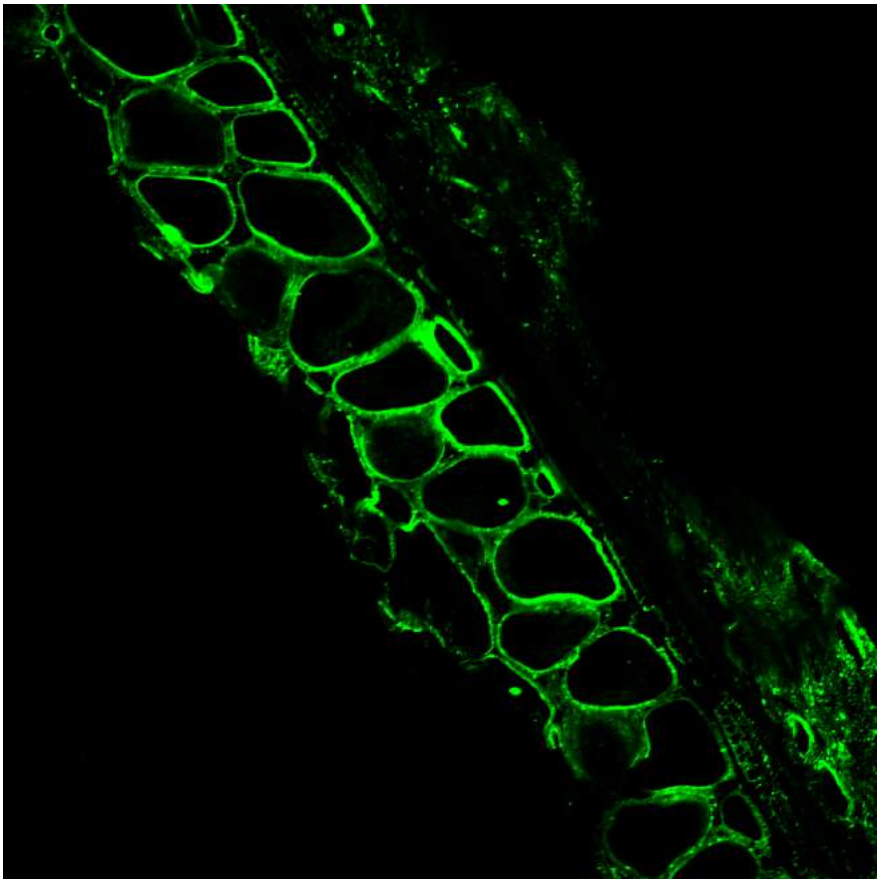

Examination of changes in cell wall structure in bran after enzymatic treatment

A study based on analysis by light- and confocal laser scanning microscopy.

Master's thesis in Biotechnology

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Department of Biology and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2017

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Department of Biology and Biological Engineering
Division of Food and Nutrition Science
Thesis performed at: Research Institutes of Sweden (RISE)
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Master's Thesis 2017:NN
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Cover: Examination of changes in cell wall structure in bran after enzymatic treatment

Typeset in LaTeX
Gothenburg, Sweden 2017

Sammanfattning

Aleuronelagret i vetekli består av protein som skulle kunna användas som växtbaserad proteinkälla med låg miljöpåverkan. På grund av svårigheten för människor att bryta ner vetekli, används kliet ofta som bulkprodukt i djurfoder. Det här projektet har i syfte att undersöka mikrostrukturen i cellväggarna hos vetekli efter enzymatisk behandling för att kunna utveckla metoder för behandling av vetekli.

Två urval av vetekli av olika partikelstorlek inkuberades i vatten med ration 30:70 (w/v) i 4, 8 och 24 timmar, vid olika temperaturer (30°C, 35°C, 45°C och 55°C), med och utan ett kommersiellt producerat enzym. Enzymet benämns i denna rapport som enzyme 1 (E1) på grund av överenskommelse gällande konfidentialitet. Synliga effekter av bioprocessering av vetekli vid sura förhållanden undersöktes med hjälp av ljusmikroskopi (LM). LM analyserna visade att högre temperatur i kombination med längre inkubationstid var gynnsamt för enzymets förmåga att bryta ned cellväggarna i aleuronelagret. Proteinet från aleuronelagret var dock ej, vid dessa förhållanden, helt degraderat och verkade förekomma i förslutna paket. Inga visuella skillnader mellan neutralt pH och lågt pH kunde observeras med LM. Immunoinmärkning av arabinoxylan (AX) och β-glukan (BG) utfördes för att undersöka mikrostrukturen i cellväggarna, och analyserades med hjälp av konfokalmikroskop (CLSM). Resultaten visade att BG är lokaliserat i områden av cellväggen som ligger närmare cellumen medan AX ligger längre ut. CLSM-analys visade att AX degraderades vid tillsättning av enzymet. Projektet bestyrker tidigare studier på mikrostrukturen hos vetekli, men ytterligare studier krävs för att fullständigt förstå mikrostrukturen i cellväggarna hos vetekli efter enzymatisk nedbrytning.

Nyckelord: vetekli, enzymatisk behandling, cellväggsnedbrytning, arabinoxylan, β-glukan.

Abstract

The aleurone layer within wheat bran consists of protein that could be used as a plant-based sources of protein with low environmental impact. Due to restricted digestibility of wheat bran, it is commonly used as a bulk product in animal feed. This project aimed to investigate micro structure of cell walls in wheat bran after enzymatic treatment, in order to develop techniques for bioprocessing of wheat bran that can expand its application. Two samples of wheat bran of different particle size were incubated with water at a ratio of 30:70 (w/v) for 4h, 8h and 24h at varying temperatures (30°C, 35°C, 45°C and 55°C) with and without a commercially produced enzyme, referred to as enzyme 1 (E1) due to confidentiality agreement. The visible effects of bioprocessing of wheat bran at acidic conditions was investigated using light microscopy (LM). LM analysis showed that a higher temperature with a long incubation time was favorable for the enzyme to degrade the cell walls of the aleurone layer. However, proteins were not fully degraded and appeared to remain in enclosed packages. No visible differences between a neutral pH and acidic pH could be observed using LM. Immunolabelling of arabinoxylan (AX) and β-glucan (BG) was performed to investigate the microstructure of the cell walls, analyzed with confocal laser scanning microscopy (CLSM). Results showed that BG is located in regions of the cell wall close to the cell lumen and AX further out. CLSM analysis showed that AX was degraded by addition of the enzyme. The project confirms previous studies about micro structure in wheat bran but further studies are required to fully understand the micro structure of cell walls in wheat bran after enzymatic degradation.

Keywords: wheat bran, enzymatic treatment, cell wall degradation, arabinoxylan, β-glucan.

Acknowledgements

First, I would like to thank my supervisor Annika Krona at RISE, for all her help and support throughout the project despite her limited time sometimes.
I would further like to thank all staff in the group of Product design and perception at RISE for all the support and inspiration. Thank you for always taking your time to answer my endless questions and for bringing joy into every working day at RISE. This includes all the diploma workers which all contributed to that my time at RISE, has become the best it could possibly be. Without all of you, it would not have been possible to complete this project. Special thanks to Annika Altskär and Camilla Öhgren for always taking your time to help me with the microscopes.
Furthermore, I would like to thank my wonderful friends Ida Alexandersson and Linnea Johansson, for always supporting me and for that you always manage to put a smile on my face.
Last but not least, of course, I would like to thank my dear Alexander for always believing in me and for your endless patience.

Kajsa Malmberg, Gothenburg, June 2017

Contents

List of Figures

1
Introduction

This master thesis was performed at RISE (Research Institutes of Sweden) at the department for Food and Agriculture at the division of *Product design and perception*. The thesis is a part of a larger project at RISE, in cooperation with Lantmännen Ceralia AB, that aims to improve the deconstruction of bran in order to increase profitability, minimize environmental impact and create new healthy foods. This master thesis has contributed to this project by investigating the effect of enzymatic treatment on wheat bran cell wall. The project included method optimization of methods used for examination changes in cell wall of bran. Methods that were optimized are in different ways improving knowledge about micro structure in bran and also investigating changes in cell wall of bran after enzymatic treatment. Lantmännen Ceralia AB has provided RISE with two samples of wheat bran of different particle sizes that are by-products from the milling process of white wheat flour.

1.1 Background

Cereal grains constitute a major source of dietary nutrients in large parts of the world. Even though grains can be used in foods as whole grains, they are often processed. The grains are modified into a specific product, often by removing unwanted parts which will end up as by-products. Wheat belongs to one of the most commonly produced cereals and it is mainly processed by milling the grains into white flour. One of the obtained by-products during the milling process is bran. Depending on the variety of wheat and the cultivation conditions, the milling process give rise to a variation of flour extraction rates, hence the rate of by-products will also vary. [1, 2]

According to a various number of studies, consumption of whole grain-foods and cereal dietary fiber have an effect in lowering the risk of chronic diseases, in particular cardiovascular disease, metabolic syndrome, type 2 diabetes and certain cancers. It is therefore desirable to includ by-products such as bran into more foods in order to increase its nutritional value. [3, 4]

Bran consists of a number of compounds considered to have a valuable nutritional status, such as a high amounts of dietary fibers and phytochemicals. Dietary fibers include cellulose, hemicellulose, lignins, pectins, gums, and mucilages which all are indigestible components [5]. Phytochemicals are naturally occurring chemical compounds in plants, and various research have been done in order to investigate their possible positive effect on human health [6]. In addition, wheat bran also stores proteins, up to 18 %, which could be used as a plant-based and inexpensive source of protein in human nutrition and thus contribute to food security and sus-

tainable food production [7, 8]. Bran is today commonly used as a bulk component in animal feed but it is desired to increase to use of bran for human consumption. However, the desire of increasing bran in other foods might be problematic since it affects the structure and taste. Wholegrain and bran contain non-volatile, flavour-active substances that are responsible and involved in the perception of flavor along with a resistant cell wall structure. These substances, together with the structure of the cell wall, are causing changes in flavor and texture during processing which might cause a decrease in consumption of the product. In wholegrain foods, different chemical constituents contribute to bitterness which also might be unwanted for the consumers [3]. In addition to sensory problems, the aspect of bioavailability of bran and its nutrients, must be considered. Whole grains and bran are rich in dietary fibers and phytic acid, which are known to have strong mineral chelating properties. Because of these properties, the nutrients within whole grain and bran are often considered to have a reduced bioavailability [9]. In order to improve the digestibility of bran proteins, among with other nutrients, and to increase the use of bran in foods it is necessary to develop techniques for bran degradation that are sustainable and cost efficient [7]. Further investigation of the structure of different bran layers is therefor of strong significance.

1.2 Relevant studies

This section includes a description of similar studies that have been used as inspiration and guidelines for this master thesis. There are a numerous studies published on investigation of micro structure of different cereal but this section will focus on the publications that have served as most relevant for this project. Together with the project aim and the objectives of this thesis, this section will give the reader a perception of how the project give additional information to current scientific literature.

Important inspiration has come from Arte *et al.* (2016), where the aim was to liberate and solubilize wheat bran proteins via cell wall degradation by using enzymes of both carbohydrate-hydrolyzing and proteolytic character. This study has provided valuable information about the method of how to bioprocess wheat bran with the addition of an exogenous enzyme. [7]

The immunolabeling of arabinoxylan (AX) and β-glucan (BG) has been influenced by protocol published in an article by Johansson *et al* (2017). The publication described methods for immunolabeling of AX and BG, including a description for double immunolabeling and these protocols have been a great inspiration for the work done in this project. [10]

Dornez *et al.* (2011) studied the grain cell wall structure by microscopic analysis with four different staining techniques. The study included figures of outer kernel layers of wheat among other cereals that have been helpful as reference pictures for my project. Also, the staining techniques used in the study have served as a great inspiration for staining the sections before investigation with a confocal laser scanning microscope. The study revealed new insight into the structure of cereal cell wall but suggested that further development of microscopic techniques would be necessary to investigate the cereal cell wall even more in detail. [11]

The impact of endogenous and exogenous enzymes in different combinations with spontaneous or controlled fermentation of wheat bran was investigated in a publication by Arte *et al.* (2015). The article focused on the release and modification of the protein fraction of wheat bran after different enzymatic and microbial bioprocessing. The authors found that bioprocessing in acidic conditions enhanced the effect of endogenous proteases and that a lower pH significantly increased solubilization of protein from wheat bran. Some of the results in this article have served as inspiration for experiments that included lowering of pH, performed in this project. [8]

2
Theory

This chapter describes the theoretical background necessary for understanding the aim and objectives of this project. The structure of cereal and bran is described, as well as the structure and composition of bran cell wall in wheat. Staining techniques, a long with information about some of the different stains that have been used throughout the project is presented. A description about immunolabeling and theory about fluorescence is described along with description of both light microscopy and confocal laser scanning microscopy, which both have been important and crucial analyzing tools throughout this project.

2.1 Cereal and bran structure

Cereals is a commonly used collection name for cultivated grasses and are, from a botanically perspective, considered as caryopsis which is a name for a fruit which only holds one seed and where the ovary wall within the fruit is joined together with the seed coat [12]. The caryopses of cereals, also known as grains, are comprised of three structural, dominant parts: the germ (2-3%), the endosperm (68-85%) and the outer layers (12-19%). However, the composition, size, shape and mass of the cereal grains varies within different species and different varieties [13], see Figure 2.1. The germ, also known as the embryo of the grain, holds the genetic information of the grain, hence the germ is a fundamental part for further development and survival of a cereal grain [12]. The germ is considered to have a high nutritional value, due to the high amounts of dietary fibers, vitamins, lipids, proteins and phytochemicals [14, 13, 15]. Structurally, the germ consists of two parts; the embryonic axis and the scutellum [16]. The latter is located closest to the endosperm and it transfers nutrients between the germ and the endosperm [15].

The second dominant part of the grain, the endosperm, makes up to 85% of the whole grain depending on specie and variety of cereal [13]. It is divided into two parts, the starchy endosperm layer and the aleurone layer which are located closest to the germ and the outer layers, respectively [17, 18]. In each of the two layers, there are different cells present which differ by morphology, biochemical composition and regulation of gene expression. The starchy endosperm cells are often interpreted as rather large cells that tend to be irregularly shaped. The cells store protein bodies and grains of starch and the genes expressed within these cells are often linked to the release of starch or proteins [19]. The aleurone cells are identified to have a regular cuboidal shape, and the cells walls are often twice as thick as the cell wall of the starchy endosperm cells. Aleurone is a name of a specific protein of which

the aleurone layer and cells are rich in, hence the name. The aleurone cells lack of starch [20, 21]. The starchy endosperm, which is mostly comprised of starch and proteins, functions as a storage for nutrients and can be considered as a reserve source of essential components for the germ and is therefor of big importance for the grain and it's ability to grow. The aleurone layer transports nutrients from the outer layers, the bran, into the endosperm. The starchy endosperm layer is more prominent than the aleurone layer and it consists of starch granules together with a matrix of proteins [22, 23].

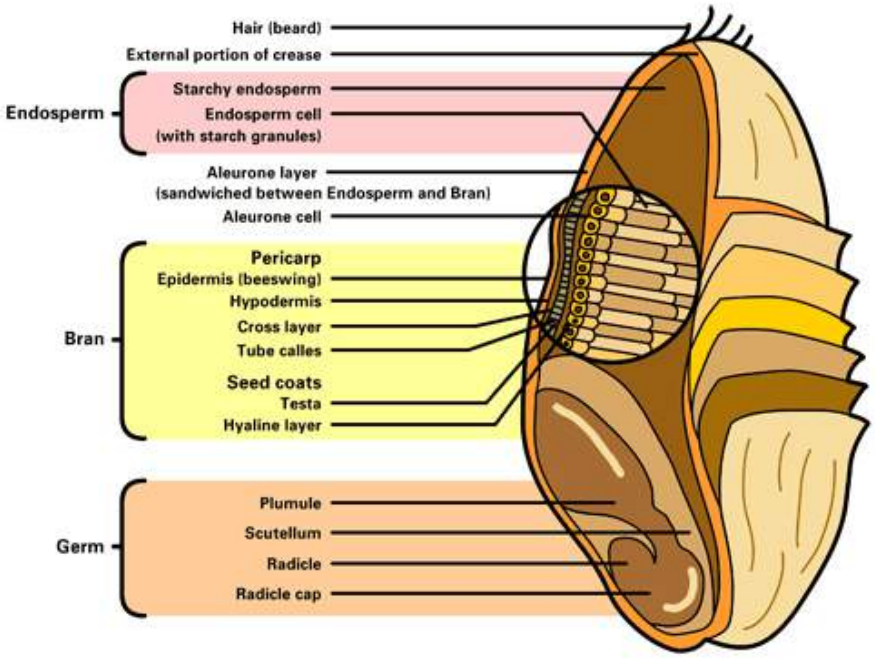


Figure 2.1: Three major parts of a wheat kernel; germ, endosperm and bran. Constituents of each part are highlighted.

The third and most outer part of the grain are the outer layers, also commonly know as bran. The bran consists of a pericarp layer, which functions as a protective layer for the kernel, and an inside layer that includes the seed coat. The bran also consist of two additional layers besides the pericarp layer named testa and aleurone, see Figure 2.2. The aleurone layer stores nutrients and tranfers nutrients from the outside of the wheat kernel to the inside and it stores lipids, minerals and proteins [24]. The aleurone layer consists of aleurone cells, which accumulate proteins within aleurone granules and lipid bodies [25]. The stored proteins within the aleurone layer is of interest, since the extraction of these proteins could possibly be used to create new plant-based protein foods. Proteins are deposited in different patterns within different endosperm storage tissues much because of the special storage properties of these tissues.

The three layers in bran are different from each other and all have their own structure, properties, thickness and composition. The bran approximately contains 19% protein, 3-5% lipids along with trace minerals, vitamins and dietary fibers [26]. Bran possess a high nutritional value and is considered as an attractive food ingredient [7]. During the production of white flour from wheat, bran is, together with the

germ and the aleurone layer, separated from the starchy endosperm and they end up as by products during the milling process [27]. In this project, the focus will we on examination of micro structure in samples from processing of wheat bran.

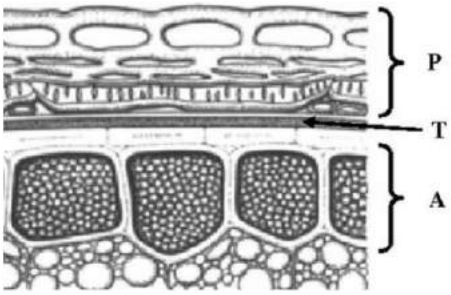


Figure 2.2: Cross section of wheat bran. P: pericarp, T:Testa and A: aleurone.

2.2 Cell wall in wheat bran and important polysaccharides

After maturation, the wheat grain mainly comprises three components, starch, proteins and cell wall polysaccharides. Together, these components accounts for approximately 90% of the total weight of the wheat grain [28]. The most predominant hemicellulose group of plant cell walls is called xylans, which refers to polysaccharides that are composed of a -(1-4)-D-xylopyronanose backbone in combination with a variety of side chains. In cereal grains, arabinoxylan is a hemicellulose that is present in both primary and secondary cell walls and. AX consists of copolymers of two pentose sugars - arabinose and xylose which can vary in level between different cereals and species. AX contents in wheat kernels vary between 4.0 to 9.0 %, [29, 30, 31]. Another important group of polysaccharide present in wheat bran is glucans. Together with AX, the glucan named β-glucan are the most nutritionally important cell wall polysaccharides within cereal and there have been numerous of studies published on both the polysaccharides. β-glucan consist of a mixture of β-(1-3)- and β-(1-4)-linked glucose residues. Oat and barley are known for being rich in β-glucans, while other cereal such as rye and wheat holds a lower concentration. The total amount of β-glucans also varies between species and their genetics but also on environment. [32, 31]

AX and BG are both nonstarch polysaccharides that are present in cereal cell wall and together AX and BG make up 2-8% of a whole cereal grain. Of total dietary fibers, it is known that human intestinal enzymes only can degrade starch hence AX and BG are resistant to human digestive enzymes. Since both AX and BG are hemicelluloses, they make up the noncellulosic part of the cereal cell wall and they contribute to the total intake of dietary fibre. [31, 29]

2.3 Polysaccharide and protein staining

Most cells, in their natural state, are not detected with a light microscope. In order to make them visible with a microscope, different staining methods of the cells of often performed. The cells are by rule often fixed or sectioned on a microscopy slide before staining is performed. By using one stain by itself or in combination with others, it is possible to perform many different staining methods, including double and triple staining procedures. Different combinations of stains will make it possible to detect and localize different components within the cells, for example lipid, proteins and carbohydrates. [33, 34]

2.3.1 Lugol

Lugol's iodine solution is comprised of iodine and potassium-idodide, hence its other name I_2KI. The solution contains free idoine and it can be used to visualize starch and proteins within plant cells, revealed as purple and yellow, respectively. This visualization is possible due to the formation of I^{3-} from I_2 and I^- which can react with polysaccharides starch. The solution should, at all times, be kept in dark since light induces I^- to form I^0, which will reduce the intensity of the staining agent [35].

2.3.2 Light Green

Light green staining is commonly used for plant tissues when it is desired to visualize proteins. In the case of staining cereals, it is for instance useful in order to localize the aleurone layer, since this layer is rich in protein. The Light Green staining dye, as the name reveals, stain the proteins green. The light green staining dye molecule contains two side groups of sulphate, which are charged negatively due to the acidic pH of the light green solution. When negatively charged, these side groups can interact with positively charged groups, such as amino acids in protein[35].

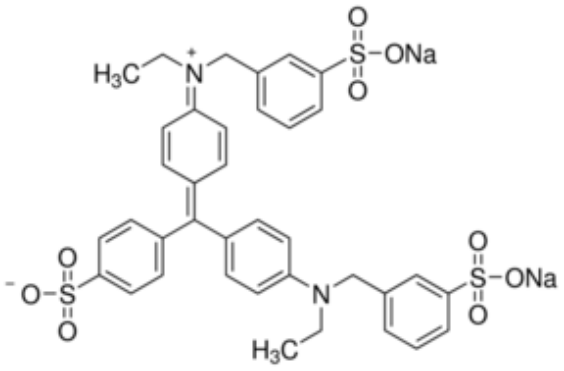


Figure 2.3: Light Green staining dye molecule. The negatively charged side groups of sulphate interact with positively charged amino acids in proteins, hence proteins can be visualized as green with a light microscope.

2.4 Immunolabeling

Immunolabeling, also known as immunohistochemistry, is a method for localizing is a technqie used for identifying and localizing of specific antigens in tissues based on the interaction between antibody-antigen. It is referred to as a specific method for localization compared to other staining techniques that are used for staining of particular macromolecules such as fat, proteins or carbohydrates, hence not specific methods. In the immunolabeling method it is possible to use either one or two antibodies for detection and localization of a specific antigen, referred to primary- and secondary antibody. However, regardless the use of sole primary antibody or in combination with a secondary antibody, the most important step of the immunolabeling technique to achieve good quality of the staining is the binding of the primary antibody to its antigen. After interaction between antigen and primary antibody, it is possible to detect the antigen within a tissue if the primary antibody is labeled with fluorescent dye, biotin or enzymes for example. This method is referred to as a direct method of immunolabeling, since it only involves one step of adding antibodies. In order to gain a higher sensitivity and more specific result of the immunolabeling, a secondary antibody if often used. This method is referred to as indirect immunolabeling and requires two steps of addition of antibodies - a primary and a secondary. In the indirect method, the secondary antibody is labelled and not the primary antibody. In the indirect method, the first step includes addition of a primary antibody which bound to a specific antigen within the tissue. Subsequently, a labelled secondary antibody that binds to the primary antibody is added. The indirect method is potentially more sensitive than the direct method, thus it is often more used than the direct method, [36, 37]. A description of direct and indirect immunolabeling is described in Figure 2.4. In this project, indirect immunolabeling is used do to a higher sensitivity. In order to avoid unspecific binding of the primary antibody, an incubation step with a blocking solution is always used, preventing the primary antibody to bind to any other antigen except for the targeted one.

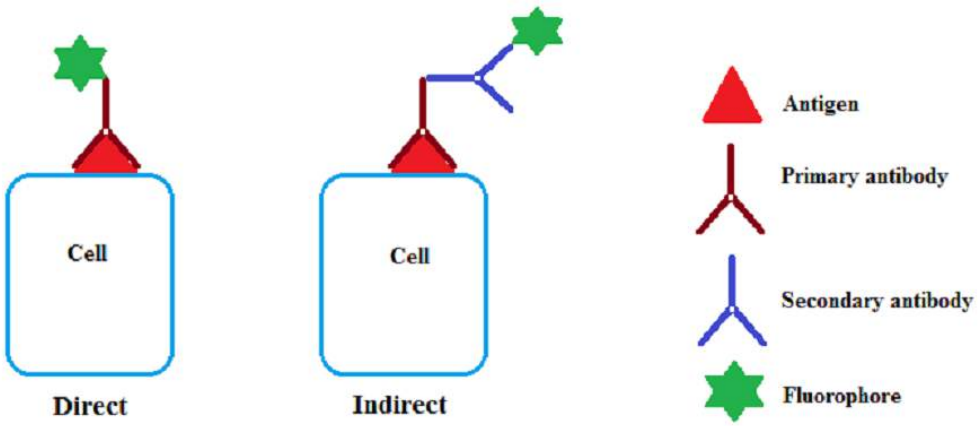


Figure 2.4: Direct and indirect immunolabeling. The direct method only includes a labeled primary antibody while the indirect method includes both a primary- and secondary antibody, where the secondary antibody carries a label. The label is often a fluorophore and can be detected with fluorescence.

2.4.1 Fluorescence

When a molecule absorbs energy, for example in the form of light and its energy is raised from the ground state to an excited state, an absorption spectrum results, see Figure 2.5. The absorption of energy causes a rearrangement of an electron from one orbital to another which gives rise to an electronic transition to a higher energy state for the absorbing atom or molecule. When the excess energy of the molecule is re-emitted, it will regress to the ground state, E_0. The return to the ground state, the lowest state of energy of a molecule and also the preferred state, results in an emission spectrum. When atoms or molecules absorb energy in form of light and re-emits this energy of electromagnetic radiation, the phenomena that appears is referred to as fluorescence. A chemical compound that has the ability to re-emit light upon light excitation, is commonly referred to as a fluorophore and can for example be used in order to study different biological processes with a UV-light. Fluorophores are typically aromatic compounds. [38, 39, 40]

Different components within a wheat kernel can be visualized due to autofluorescence. Examples of constituents that express autofluorescence are the aleurone tissue, due to the presence of ferulic acid, and the pericarp layer. Depending on the excitation and emission spectra of the components responsible for the fluorescent, the layers can be visualized at different wavelengths [41].

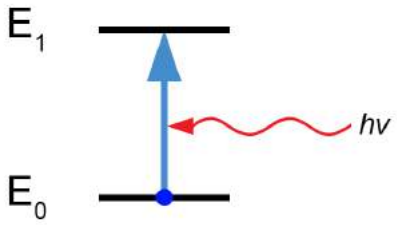


Figure 2.5: Excitation of an atom or a molecule when absorbing energy (hv). The atom or molecule excites from ground state, E_0, to the next level of energy, E_1, also referred to as the excited state.

2.5 Cryo sectioning

Cryo sectioning is also referred to as frozen section procedure and is a technique that prepares the sample before microscopy. The technique is often used when rapid microscopy analysis of a sample is desired. The sample, in this case the bran flakes, are mixed with a specific glue suitable for cryo sectioning. The mix is then frozen in liquid nitrogen by dipping several times. The frozen sample is sliced in a microtome by a sharp blade. The cryo sectioning allows for very thin slicing of the samples which is desired for further investigation with microscopy. [42, 43]

2.6 Microscopy

Microscopy is a commonly used tool for analyzing numerous aspects of food. Among these aspects is the location of nutrients within cereals and bran [44]. Various forms of microscopy and imaging techniques could be used to localize these nutrients, but they differ by the type of signal that is detected and by the resolution. In this project, for examination of the effect of enzymatic treatment on the cell wall of bran, two different types of microscopy techniques will be used, light microscopy (LM) and confocal laser scanning microscopy (CLSM).

2.6.1 Light microscopy, LM

LM is a useful and often occurring tool when examining the composition of different cereals and bran. LM is the simplest form of microscopy and it consist of a single lens. A lens in a microscope enables for parallel rays to meet at a certain point, which often is referred to as the focus of the lens. The distance between the lens and its focus is referred to as the focal length and the shorter this distance is, the higher power can the objective serve. If the focal length becomes shorter, this will mean that the objective is positioned closer to the sample and this will give rise to a more enlarge image compared to if the focal length was longer. [45]

2.6.2 Confocal laser scanning microscopy, CLSM

CLSM is a useful tool for examination of bulk samples and thin sections of a sample is not necessary, compared to investigation with LM. Confocal means that there are two focal points in the microscope set up, also referred to as pinholes, that coinciding. Compared to LM, where light is used as energy source, the CLSM uses laser as energy source. The conventional set up of CLSM consist of one entrance pinholes, in which a laser beam passes. After the first entrance pinhole, a lens (condenser lens) focuses the laser beam on small, fluorescent (mostly) spot of the sample. A second lens (objective lens) then collects the fluorescent light of a chosen wave length. The last step of a conventional CLSM set up consists of a second pinhole, located after the objective lens, which focuses the light of the spot and transmits it to a detector. [45, 46, 47] A schematic description of a CLSM set up is displayed in Figure 2.6.

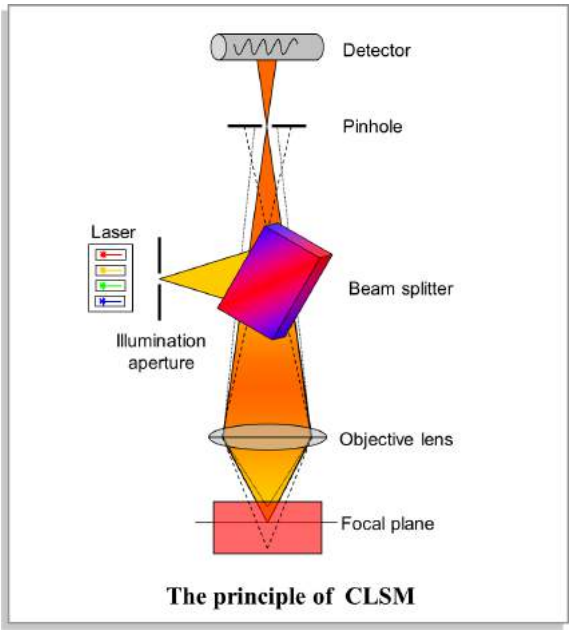


Figure 2.6: Schematic set up CLSM. Image courtesy of Annika Altskär, RISE.

3
Aim

3.1 Project Aim

The aim of this project is to examine changes in cell wall structure in bran after enzymatic treatment.

3.2 Objectives

Wheat bran is a by-product when producing white flour from wheat through milling. The bran contains many nutrients that are of interest since they could contribute to create more healthy and sustainable foods. However, with the methods used today it is difficult to bioprocess bran in order to increase bioavailability of such nutrients. In order to optimize bioprocessing of bran it is desired to increase knowledge about the micro structure of bran and it's components. In addition, it is desired to find new, less expensive and more sustainable methods of how to bioprocess bran, for example with enzymatic treatment.

This project will contribute to increase the knowledge of micro structure in bran, before and after treatment with the commercially produced enzyme, E1. The results found in this project can possibly be used by the industry for the optimization and improvement of bioprocessing of bran in order to create more healthy foods in a sustainable way.

In the first part of the project we investigated how the cell wall structure in bran changes with time and temperature before and after enzymatic treatment. The enzyme will, due to confidentiality agreement be referred to as enzyme 1 (E1). Different conditions of varying time and temperature was investigated. Different staining techniques was used on order to enable characterization of different components within bran. In order to visualize the effect of the enzymes, light microscopy (LM) was used.

The second part of the project focused on investigation of micro structure in bran with fluorescently labeled antibodies by confocal laser scanning microscopy (CLSM) analysis. Primary antibodies was used to bind to arabinoxylan or β-glucan, which are two present components within wheat bran cell walls. The incubation step of primary antibodies was followed by addition of secondary antibodies labeled with a fluorophore. The fluorescent signal allowed for analysis of the micro structure with CLSM, before and after enzymatic treatment. Throughout this part of the project, attempts to optimize the method of immunolabeling of AX and BG were performed.

The third objective of the project was to investigate the effect of acidic conditions. The hope was to be able to investigate if endogenous enzymes within wheat bran cells became activated be this conditions and if this could enhance the degradation of the cell walls. Experiments both with and without the addition of the exogenous enzyme, E1, were performed at two acidic conditions. The samples were analyzed with LM and CLSM.

4

Method and material

This section will include information about the raw material, equipment and protocols that have been used throughout the project in order to investigate and analyze each of the objectives, described in section 3.2.

4.1 Raw material and Enzymes

Two different fractions of wheat bran samples were investigated throughout the project. The samples were provided by Lantmännen Cerealia AB (Stockholm, Sweden) and were at different sizes. From Lantmännen Ceralia AB, information about the composition of *Kruskakli* and *Kruskakli milled* was given. The two side stream samples are identical in composition in terms of proteins, fat, carbohydrates and fibers. The *Kruskakli milled*, is as the name indicate, a milled form of *Kruskakli*, hence a sample with smaller fractions of wheat bran.

4.1.1 Bioprocessing of bran

A ratio of 30:70 (bran/H_2O mixture, w/v) and an enzyme dosage (ED) of 500 nkat/g bran according to their xylanase activity were used in all experiments. The bran was weighed and placed in an Eppendorf tube followed by adding destilled water (pH 7)The tubes were then incubated in a oven with set temperature for a given time before stopping the incubation by freezing the samples in liquid nitrogen. Three temperatures (30°C, 35°C and 45°C) and two incubation times (8h and 24h) were varied for two different side stream samples provided by Lantmännen AB, *Kruskakli* and *Kruskakli milled*. An incububation time of 4h was varied at 30°C, 35°C and 55°C for both the side stream samples. For each trial, a control without the addition of the exogenous enzyme E1 was performed. Controls were only including destilled water (pH 7and bran at a ratio of 30:70 (bran/H_2O mixture, w/v).

4.1.2 Acidic condition

Wheat bran samples of Kruskakli with a ratio of 30:70 (bran/H_2O mixture, w/v) and an enzyme dosage (ED) of 500 nkat/g bran according to their xylanase activity was performed in lower pH than previous described experiments. Destilled water was titrated with HCL (1 M) and NaOH (0.1 M) to pH of 4.0 and 2.9, the pH was measured with a pH meter . Before measuring the pH meter was calibrated with buffer solution of pH 7.0 and pH 4.01. The samples were incubated at 35°C for 24 h

with pH 2.9 and pH 4.0 to study the potential impact of endogenous enzymes with and without the effect of the exogenous enzyme E1 at acidic conditions. For each trial, a control without the addition of the exogenous enzyme E1 was performed. Controls were only including destilled water (pH 7) and bran at a ratio of 30:70 (bran/H_2O mixture, w/v).

4.2 Microscopic investigation

For investigation with LM and CLSM, the incubation and enzyme activity were stopped by freezing the sample in liquid nitrogen (AGA, Lindingö, Sweden). Before freezing the sample was mixed with an cryo embedding medium (Pelco Prod. No. 27300, ted Pella Inc, Redding, USA). The samples were then set to rest in a microtome, Leica CM 1900 (Cellab Nordia AB, Sollentuna, Sweden), at -13°C for at least 20 minutes. For sectioning a microtome blade (Leica 819, Leica Biosystems, Nussloch, Germany) was used. The samples were sectioned in 10 µm sections and placed on either Thermo Scientific polysine microscope slides (Mezel-Gläser, Braunschweig, Germany) or Thermo Scientific SuperFrost microscope slides (Mezel-Gläser, Braunschweig, Germany).

4.2.1 LM investigation

LM research was performed with a Nikon Microphot-Fxa microscope (Bergman Labora AB, Danderyd, Sweden) connected with an Altra 20 camera (Olympus Corporation, Tokyo, Japan). Samples were investigated using objectives 4x, 10x an 20x magnification. For examination of cell wall structure in bran with LM before and after enzymatic treatment, the samples (on microscopy slides) was stained with Lugols's iodine staining (staining starch and proteins, purple and yellow/brown respectively) and Light Green staining dye (staining proteins, green).

4.2.2 CLSM investigation

CLSM research was performed with a Leica TCS SP2 confocal microscope in combination with Leica Confocal Software (both: Cellab Nordia AB, Sollentuna, Sweden). Samples were investigated vid objectives 10x, 20x and 63x. The CLSM was used a complement to the LM investigation to examine microstructure in bran and also specific for examination of immunolabeled samples. The examination of the immunolabeled samples made it possible to localize AX and BG within the cell wall structure of bran.

4.3 Immunolabeling of AX and BG

In order to visualize the localize of both AX and BG within the wheat bran cell wall, immunolabeling with a monoclonal antibody followed by a fluorescently tagged secondary antibody was used. The immunolabeling was eiother peformed as a single immunolabeling, where only one of the compounds (AX or BG) where labeled and

localized, or as a double immunolabeling where both AX and BG were measured. The immunolabeling process was partly inspired by the protocol used by Dornez et al. (2011), [11], and partly by the protocol used by Johansson et al. (2016), [10]. However, different protocols regarding volume and concentrations of the primary antibodies and the blocking agent (bovine serum albumin, BSA) were performed and evaluated in order to attempt to optimize the method of both single and double immunolabeling of AX and BG. Immunolabeled sections were analyzed with CLSM.

4.3.1 Single immunolabeling

A PBS (Na_2HPO_4, NaH_2PO_4, NaCl, pH 7.2) buffer was prepared for washing and dilution steps performed for the immunolabeled samples. Sections were placed on Thermo Scientific polysine microscope slides (Mezel-Gläser, Braunschweig, Germany) and fixated by incubation with paraformaldehyd (4%). Sections were then washed by dipping into a Coplin staining jar with PBS buffer solution (pH 7.2), this was repeated 3 times, each 5 minutes. The sections were then incubated for 1 hour in PBS buffer solution, (pH 7.2) containing 1% or 4% bovine BSA to prevent non-specific binding. The samples were incubated for 2.0 h with primary antibody solution. Monoclonal antibodies raised against 1->3, 1->4-b-D-glucan (Biosupplies, Parkville, Australia) and AX (LM11 antibody, Plant Probes, Leeds, UK) were diluted to 1:20, 1:50 or 1:100 in PBS buffer solution (pH 7.2). After incubation, the samples were washed identically to previous washing step described. Subsequently, the sections were incubated for 2.0 h in dark with fluorescently labeled secondary antibodies, Alexa Fluor 647®goat anti-rat IgG (AF647) and Alexa Fluor 488®goat anti-mouse IgG (AF488) for AX and BG, respectively. Both secondary antibodies were diluted 1:100 in PBS buffer solution (pH 7.2). All incubation steps were performed in a moisturized chamber in room temperature. A final washing step was performed, identical to the previous washing steps. Before the sections were completely dry, a drop of Aqua-Poly/Mount mounting medium (Polysciences, Inc., Warrington, USA) was placed on top of the sections before placing a cover slip. The mounting medium is, according to Polyscience, Inc., water-soluble, non-fluorescing, non-permanent and aqueous and suitable for immunofluorescent techniques as it enhances and retains fluorescent stain. The samples were kept dark and dry.

Appropriate controls were made for investigation if the secondary antibodies was likely to bind to other proteins within the wheat cells except for their corresponding primary antibodies. Controls were made by replacing the primary antibody solution with PBS (pH 7.2).

After the final washing step, some sections were incubated with an additional staining dye which had to be chosen with respect to the emission spectra of the secondary antibody. The addtitional staining dyes that were used can be seen in Appendix A.1.

4.3.2 Double immunolabeling

For sections that were treated with double immunolabeling, both AX and BG, three different procedures were investigated. The first protocol, similar to the protocol

described above for single immunolabeling, begins with immunolabeling of AX with primary antibody LM11 (1:50) and subsequently secondary antibody AF647 (1:100). After the final washing step, the same section was incubated with primary antibody raised against 1->3, 1->4-b-D-glucan (1:50) followed by incubation with secondary antibody AF488 (1:100). Incubation times and washing steps were identical to the the protocol described for single immunolabeling.

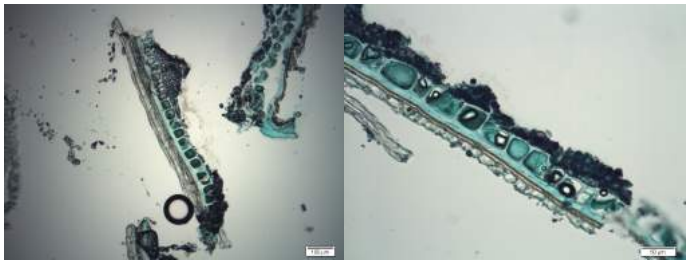
The second protocol was performed with the alteration of starting with the immunolabeling of BG followed by immunolabeling of AX. Incubation times and washing steps were identical to the the protocol described for single immunolabeling. Concentrations of primary and secondary antibodies were identical to the ones described in the first protocol for double immunolabeling.

The last protocol tried for double immunolabeling includes mixing of primary antibodies raised against AX and BG (LM11 and monoclonal antibody raised against BG), incubated at the same time, and mixing of secondary antibodies (AF647 and AF488), incubated at the same time. The incubation times and washing steps were identical to the the protocol described for single immunolabeling. Concentrations of primary and secondary antibodies were identical to the ones described in the first protocol for double immunolabeling.

5
Results and Discussion

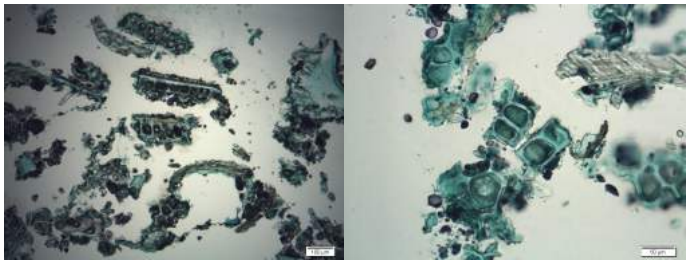
5.1 Bioprocessing of bran

Non-bioprocessed samples of *Kruskakli milled* and *Kruskakli* analyzed with light microscopy are displayed in Figure 5.1. It can be seen that the wheat bran fractions are a lot smaller in *Kruskakli milled* compared to the fractions within the sample for *Kruskakli*. Since the milled form of *Kruskakli* (Kruskakli milled) has identical composition of proteins, fat and carbohydrates, the degradation effect depending on the composition of macromolecules can be neglected. It is therefor not surprising that the results show similar trends. Micrographs from LM of the two non-bioprocessed side stream samples are displayed in Figure 5.1. It can be observed that more pericarp layer has been separated from the aleurone layer within the non-bioprocessed *Kruskakli milled* sample compared to the non-bioprocessed sample for *Kruskakli*.



(a) Non-bioprocessed *Kruskakli*

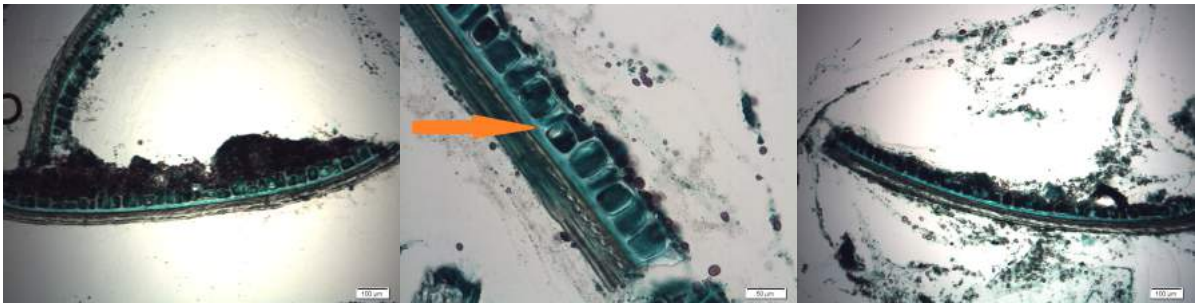
(b) Non-bioprocessed *Kruskakli*



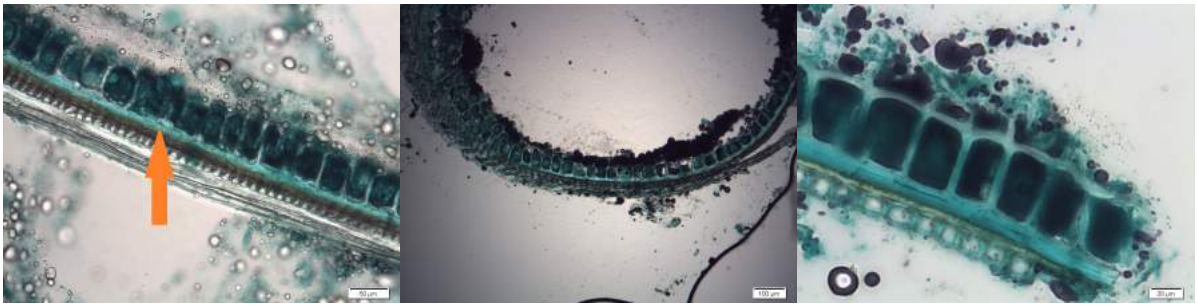
(c) Non-bioprocessed *Kruskakli milled*

(d) Non-bioprocessed *Kruskakli milled*

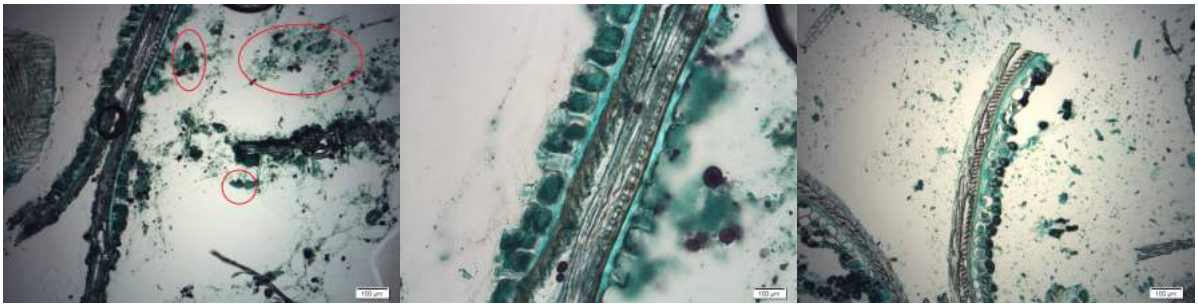
Figure 5.1: Cross sections of non-bioprocessed *Kruskakli* and *Kruskakli milled* analyzed with light microscopy. Scale bars: 100 μm (a and c) and 50 μm (b and d)



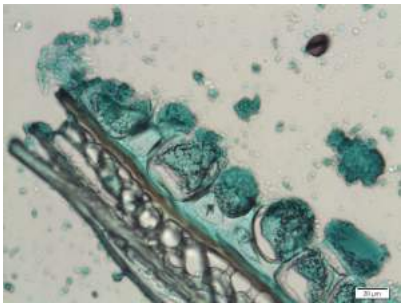
(a) 4h 30°C control **(b)** 4h 30°C control **(c)** 4h 30°C E1



(d) 4h 30°C E1 **(e)** 4h 45°C control **(f)** 4h 45°C control



(g) 4h 45°C E1 **(h)** 4h 45°C E1 **(i)** 4h 55°C E1



(j) 4h 55°C E1

Figure 5.2: Cross sections of *Kruskakli* analyzed with light microscopy of samples incubated during 4 h with the different temperatures 30°C, 45°C and 55°C. All sections stained with Light Green and Lugol's staining solution, staining proteins (green) and starch (purple), respectively. Cell walls and parts of pericarp displayed as lighter green. Testa line displayed as brown/light brown. Control: bran incubated with water, (ratio 30:70, w/v). E1 : bran incubated with distilled water (pH 7) and dissolved exogenous enzyme E1 (ratio 30:70, w/v) at dosage of 500nkat. Sacle bars: 100μm (a, e, i), 50μm (b, d, h) and 20μm (f, j).

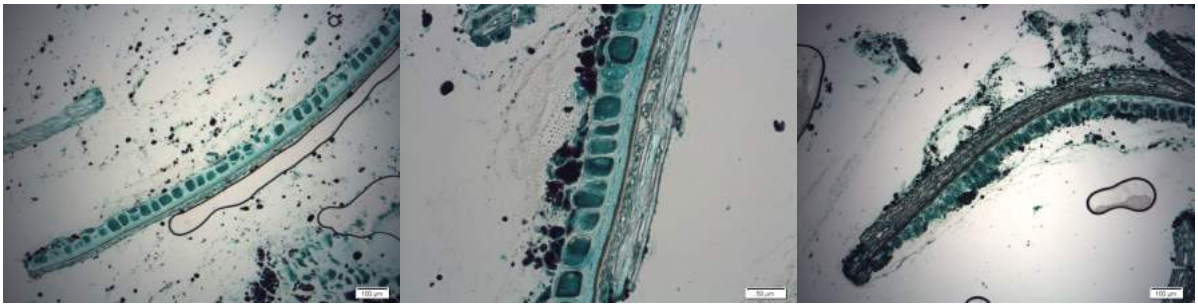
From incubation of 4h the degradation of the aleurone layer and the cell walls of the aleurone cells it was clear that the enzyme E1 had the highest efficiency at 55°C. The temperature 55°C was only tested for 4h and not for the other two incubation times, 8h and 24h. The reason for this was that a lot of the cell walls were completley degraded at 55°C/4h and it was therefor more interesting to compare lower temperatures at different incubation times where it was possible to still destiguish cell walls. In Figure 5.2 controls and enzymatic treated samples at 4h are displayed.

After 4h/30°C, incubation with distilled water only, starch is still bound to the aleurone layer and that the nearest environment of the wheat bran does not contain a high number of dissolved starch granules, displayed as purple dots (Fig. 5.2 (a)-(b)). For samples 4h/30°C treated with exogenous enzyme E1, there are more starch and proteins solved in the environment surrounding the wheat bran (Fig. 5.2 (c)), proteins displayed as green and starch displayed as purple. The aleurone layer is still bound to the pericarp layer after incubation with E1, which can be seen when comparing to the control (Fig. 5.2 (a) and (c)). However, looking closer on the cell structure after bioprocessed during 4h/30°C with and without E1 (Fig. 5.2 (b), (d)), it can be seen that E1 has already an effect already after 4h at 30°C. This is assumed after closer examination of the cell walls of the aleurone cells, which are much thinner in the sample treated with E1 (Fig. 5.2 (d)) compared to the control sample (Fig. 5.2 (b)). The cell structure of the control sample (Fig. 5.2 (b)) has a clear cuboidal shape with clearly displayed cells between the aleurone cells, cell walls displayed in lighter green and protein within aleurone cells displayed in darker green. For 4h/30°C with addition of E1 (Fig. 5.2 (d) the cell walls between the cells are much thinner and the cells no longer show a clear cuboidal shape. However the wall between the testa line and aleurone layer, highlighted with orange arrows in Fig. 5.2 (b) and (d), is still present after addition of E1 (Fig. 5.2 (d)), and show a lower degradation compared to the cell walls between the cells.

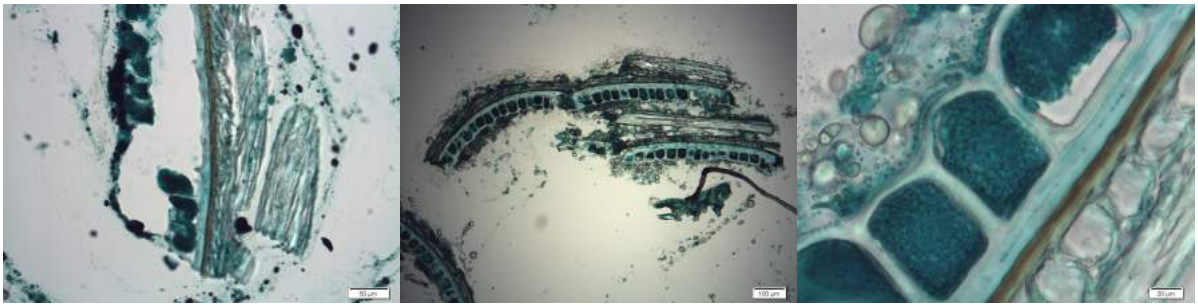
Incubation for 4h at 45°C with E1 (Fig. 5.2, (g)-(h)) showed a higher degradation compared to an incubation time of 30°C (Fig. 5.2, (c)-(d)). Comparing the environment that surrounds the wheat bran fractions after incubation at 4h at 45° for control and after addition of E1 (Fig. 5.2 (e) and (g)), it is clear that more proteins have been released in the surrounding due to a partly degradation by E1 of the aleurone layer. Examples of dissolved proteins and starch granules in the surrounding environment are highlighted in red circles in Figure 5.2 (g), where the proteins are visualized as green and strach as purple/dark purple. Nevertheless, within the samples there were still a number of aleurone layer present that had not been degraded at all by E1 , see (g). Some of the aleurone layers showed a lower degradation where the cell walls were thinner characteristics compared to the control, and some aleurone layers showed no visible degradation. However, the degradation was more abundant in at 45°C than at 30°C , showing that E1 has a higher efficiency at 45°C compared to 30°C.

The observation, that E1 was more efficient at higher temperatures, after comparing 30°C and 45°C at 4h incubation time was confirmed after investigation 4h at 55°C. The samples incubated at 55°C for 4h showed a similar or higher degradation compared to the samples incubated at 45°C for 4h. In Figure 5.2, (i) and (j), a

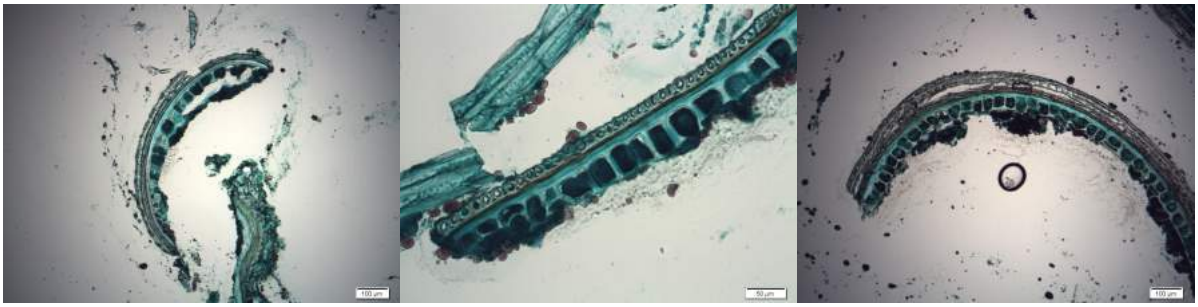
degradation similar to the one displayed in (g) and (h) is displayed. The aleurone layer was still attached to the pericarp layer for both 45°C and 55°C. However, the cell walls of the aleurone layer were partly degraded at more places within the sample in the 55°C sample compared to the 45°C which gives an indication that the enzyme might be even more efficient at 55°C than at 45°C.



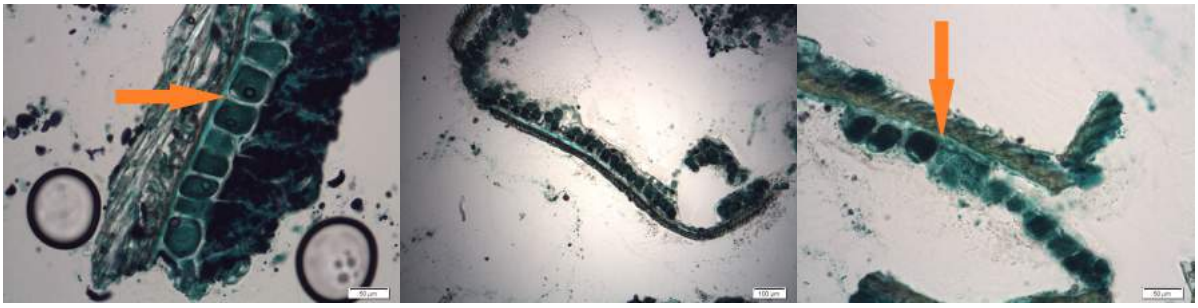
(a) 8h 30°C control **(b)** 8h 30°C control **(c)** 8h 30°C E1



(d) 8h 30°C E1 **(e)** 8h 35°C control **(f)** 8h 35°C control



(g) 8h 35°C E1 **(h)** 8h 35°C E1 **(i)** 8h 45°C control



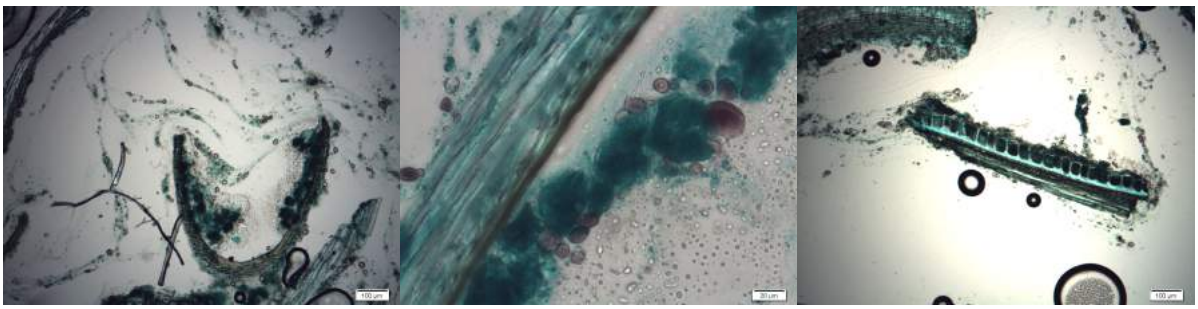
(j) 8h 45°C control **(k)** 8h 45°C E1 **(l)** 8h 45°C E1

Figure 5.3: Cross sections of *Kruskakli* analyzed with light microscopy of samples incubated during 8 h with the different temperatures 30°C, 35°C and 45°C. All sections stained with Light Green and Lugol's staining solution, staining proteins (green) and starch (purple), respectively. Cell walls and parts of pericarp displayed as lighter green. Testa line displayed as brown/light brown. Control: bran incubated with distilled water, (ratio 30:70, w/v). E1 : bran incubated with water and dissolved exogenous enzyme E1 (ratio 30:70, w/v) at dosage of 500nkat. Scale bars: 100μm (a, c, e, g, i, k), 50μm (b, d, h, j, l) and 20μm (f).

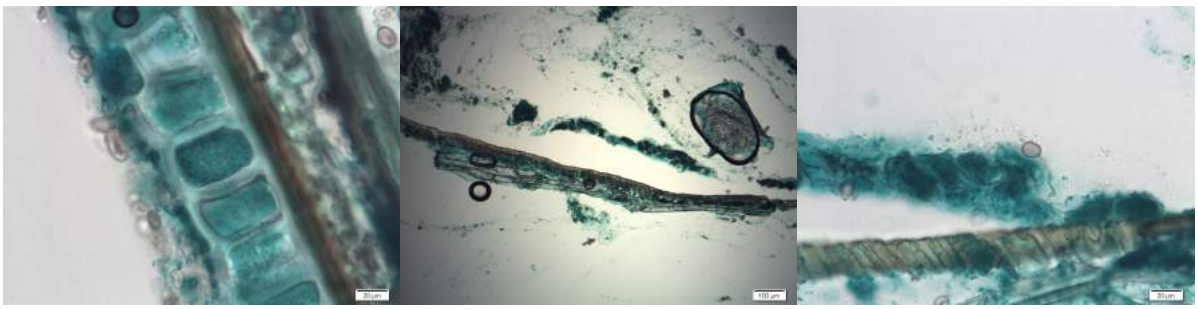
Further results from bioprocessing at 8h with the temperatures at 30°C, 35°C and 45°C are presented in Figure 5.3. Comparing the controls for 30°C and 45°C for 8h (Fig. 5.3 (a), (i)) and 4h (Fig. 5.2 (a), (e)) it is shown that more starch is dissolved in the surrounding environment after a longer incubation time, 8h. In Figure 5.3 (a) and (i), single starch granules are present in the surrounding environment. However, this was not the case for all the cross sections within the examined samples. The control sample for 8h at 45°C (Fig. 5.3 (i)) still contained starch that was bound to the aleurone layer but the degree of starch dissolved within the surrounding environment was still higher in total in the controls for 8h compared to the controls for 4h. Comparing the samples bioprocessed with E1 for 8h for at 30°C, 35°C and 45°C (Fig. 5.3 (d), (h) and (l), respectively), the highest degradation was observed in the sample bioprocessed at 45°C. For incubation during 8h/45°C (Fig. 5.3 (l),) the cell walls between the aleurone cells within the aleurone layer are thinner compared to the control sample (Fig. 5.3 (j)). Also, it can also be shown that the wall between the testa line and the aleurone layer has almost been fully degraded for 8h/45°C, the aleurone layer has partly become separated from the pericarp layer and testa line (Fig. 5.3 (l)). The orange arrows in 8h/45°C control (Fig. 5.3 (j)) and in 8h/45°C E1 (Fig. 5.3 (l)) highlights differences in thickness of the wall between the testa line and aleurone layer for control and with the addition of E1 . *Kruskakli* incubated for 8h at 35°C showed less degradation than *Kruskakli* incubated for 8h at 30°C, which based on previous observations and assumption that E1 has a higher efficiency at higher temperatures is an unexpected result. Possible error sources could be that the 8h at 30°C sample was, after incubation, stored in the fridge for some time before it was possible to stop incubation entirely with frozen nitrogen. This due to lack of equipment. If E1 is active at low temperatures, it is possible that the incubation time became longer than 8h for the enzyme and that this might have affected the degradation in a positive way. Looking at both 4h and 24h (Fig. **??** and 5.4) for the samples that have bioprocessed with E1 , there is a clear trend that highest degradation of the aleurone layer is observed in the samples treated with the highest temperature. This makes is unlikely that incubation for 8h would show a different result and that it is possible that one or more source of error have effected the result. Another observation that can indicate that the *Kruskakli* bioprocessed for 8h at 30°C is that the degradation is very similar to the one observed for *Kruskakli* incubated for 8h at 45°C. This also deviates from the results received for both 4h and 24h, where the highest degradation of the aleurone layer is observed in the samples treated with the highest temperature.

(a) 24h 30°C control **(b)** 24h 30°C control **(c)** 24h 30°C E1

(d) 24h 30°C E1 **(e)** 24h35Ccontrol **(f)** 24h 35°C control



(g) 24h 35°C E1 **(h)** 24h 35°C E1 **(i)** 24h 45°C control

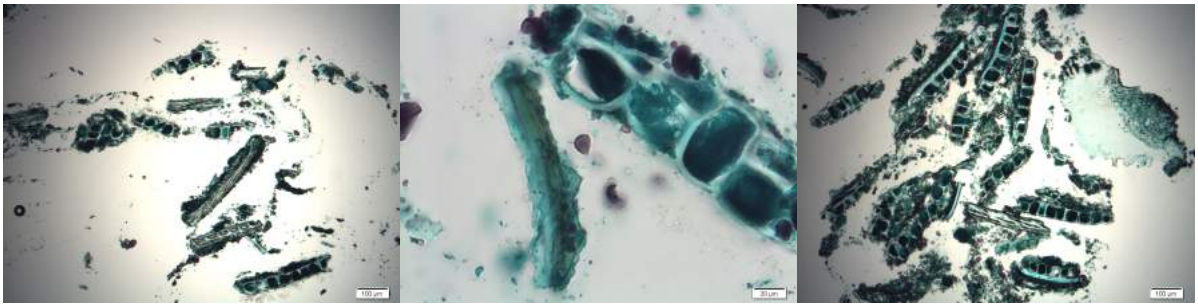


(j) 24h 45°C control **(k)** 24h 45°C E1 **(l)** 24h 45°C E1

Figure 5.4: Cross sections of *Kruskakli* analyzed with light microscopy of samples incubated during 24 h with the different temperatures 30°C, 35°C and 45°C. All sections stained with Light Green and Lugol's staining solution, staining proteins (green) and starch (purple), respectively. Cell walls and parts of pericarp displayed as lighter green. Testa line displayed as brown/light brown. Control: bran incubated with distilled water, (ratio 30:70, w/v). E1 : bran incubated with water and dissolved exogenous enzyme E1 (ratio 30:70, w/v) at dosage of 500nkat. Scale bars: 100μm (a, c, e, g, i, k), 50μm (b, d, f) and 20μm (h, j, l).

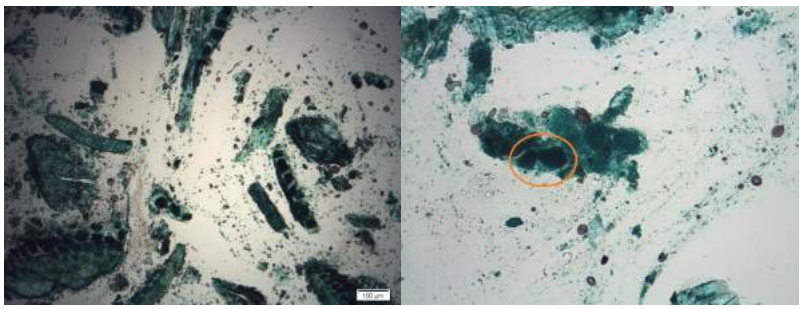
The incubation time of 24h shows, as for 4h and 8h, the highest degradation at the highest temperature, that is at 45°C. For the control samples, (Fig. 5.4 (a)/(b), (e)/(f) and (i)/(j)), there were no obvious difference that could be observed even though the temperatures varied. The aleurone cells are intact, with a clear cuboidal shape with distinct cell walls between them. Also, the aleurone layer is intact with the testa line and pericarp layer at the majority of the cross sections. For the area highlighted with a red circle in 24h 30°C (Fig. 5.4 (b)), some of the aleurone cells shows an indication that they have been degraded. However, looking at the aleurone cells highlighted with a blue circle it is clear that the aleurone cells still have an intact structure. The cells within the red circle are therefor most likely damaged by the cryo sectioning or the preparation of the sectioning. Based on observations on the aleurone cells and the aleurone layer, the controls for 24h, shows similar results as the controls for 4h (Fig. 5.2) and 8h (Fig. 5.3). However, some differences regarding how much starch bound to the aleurone layer could be observed between the controls of the different incubation times. For the longer incubation times, 8h and 24h, more starch in the surrounding environment could be observed than for shorter incubation time at 4h. When comparing controls for 24h/30°C (Fig. 5.4 (a)) with 4h (Figure 5.2 (a)) it can be observed that less starch is bound to the aleurone layer after 24h than after 4h were there is almost no visible starch granules within the surrounding environment of the wheat bran. From this observation, it is possible to assume that the incubation time is important for the purpose of separating starch from aleurone layer. Nevertheless, there were no obvious difference between the controls for 24h and 8h regarding the amount of starch that had been separated from the aleurone layer. For both 8h (Fig. 5.3) and 24h (Fig. 5.4), there are still starch bound to the aleurone layer but in a lesser extent compared to 4h (Fig.5.2 (a)). // There was already an observed effect of E1 at 30°C after 24h of incubation. In Figure 5.4 (c)-(d) it is displayed that the aleurone layer has been cut loose, as an effect of the added enzyme E1, from the pericarp layer. Also, the cell walls between the aleurone are not as distinct as i the corresponding control samples. For 24h/30^{o}C (Fig. 5.4 (d)) it can be observed that the aleurone cells don't show a cuboidal structure anymore, but even though they are separated from the pericarp layer, they are still arranged next to each other as for in the control sample (Fig. 5.4 (b)). The aleurone layer has been cut loose from the pericarp for 24h/30^{o}C (Fig. 5.4 (c)). Also, it can be observed that there are proteins, highlighted with a red circle in (c), within the surrounding environment which indicates that proteins have been degraded from the aleurone layer. For 24h/35°C there are also obvious observations that E1 have had an effect, see (g)-(h). As for 24h/30°C, the aleurone layer has been separated from the pericarip and the cells walls between the aleurone cells are almost not visible. Comparison of the degradation of the aleurone layer at 8h/35°C (Figure 5.3 (h)) to 24h/35°C (Fig. 5.4 (h)) it can be seen that the degradation for 24h is to a higher extent. This indicates that E1 still has an effect at 35°C but the rate of degradation is dependent on the incubation time. The highest degradation was found for 24h/45°C, (Fig. 5.4 (k)-(l)). In this sample, almost all aleurone layers were separated from the pericarp layer with no distinct cell walls between the cell. For the aleurone layers that still were bound to the pericarp layer, no cells walls between the cells were visible and the wall between the testa line/pericarp and the

aleurone layer was a lot thinner compared to the control. This showed that E1 had had an effect also on the aleurone layers that were still attached to the pericarp layer.
An interesting observation for both 24h/35°C and 24h/45°C (Fig. 5.4 (h),(l)) was that even though there were no visible cell walls between the aleurone cells, and even though are not arranged next to each other anymore, it seems like the proteins are still enclosed as they appear enclosed in small "protein packages". This observation is also was also observed for *Kruskakli milled* 24h/45^{o}C (Fig. 5.5).
In order to get an indication whether the degradation was more dependent on the time or the temperature the observations for 4h/55°C was compared to the observations for 24h/30°C. Comparing 4h/55°C (Fig. 5.2 (i)-(j)) with 24h/45°C (Fig. 5.4 (c)-(d)), it was observed that some aleurone layers have been separated from the pericarp layer after 24h/30°C and not after 4h/55°C. However, the cell walls between the aleurone cells were degraded to some extent in both cases. Also, released proteins could be observed in the surrounding environment for both conditions. Nevertheless, the fact that some aleurone layers had been separated from the pericarp at 24h/30°C and not at 4h/55°C implied that the degradation performed by E1 might depend more on time than on temperature. Yet, this requires further investigation, where a quantitative measurement of released proteins would be one suggestion.



(a) 4h 30°C control **(b)** 4h 30°C control **(c)** 4h 30°C E1

(d) 4h 30°C E1 **(e)** 24h 45°C control **(f)** 24h 45°C control



(g) 24h 45°C E1 **(h)** 24h 45°C E1

Figure 5.5: Cross sections of *Kruskakli milled*. 45 and 30 degress at 4h at 24 h. Scale bars: 100μm (a, c, e, g) and 20μm (b, d, f, h).

Kruskakli milled showed very similar results as *Kruskakli* thus the sample showing the highest degradation was the sample incubate for 24h and 45°C and the lowest degradation was found in the sample incubated for 4h at 30°C. The two conditions (4h/30°C and 24h/45°C) are displayed in Figure 5.5 and when comparing the results in 5.5 to the results displayed in 5.2 for the same incubation time, they are very similar. In Figure 5.2 (c) and in Figure 5.1 (c), both conditions for 4h 30°C bioprocessed with E1 , the surrounding environment contains more dissovled proteins and starch granules compared to their respective controls. Also both samples showed low degradation at 4h 30°C with intact aleurone cells with a clear cubdoial shape surrounded by non-degraded, thick cell walls. Neither *Kruskakli* nor *Kruskakli milled* showed an obvious differences from the control samples at 4h and 30°C. This similarity between the samples can be observed when comparing Figure 5.1 (a)-(d) to 5.2 (a)-(d).

Even though it was not an objective for this report, the investigation of the

relationship between incubation time, temperature and size of the wheat bran fractions is of great importance for the industry. A high temperature at a shorter incubation time might result in a higher degradation compared to a lower temperature at the same incubation time. Still, this might not be an ideal condition from an industrial point of view. It is necessary for the industry to consider the back draws of using higher temperatures, such as the costs and environmental impact of energy resources of a constant heating to higher temperatures. In this case, it might be more beneficial with a lower temperature for a longer incubation time. However, this raises other issues such as storage room for a longer time and that it might take longer time to process the wheat bran into the final product. A lower throughput of final product might in some cases be more economically beneficial for the industry, since it might require less process costs for heating, storage room etc. The most ideal particle size of the wheat bran to be enzymatically degraded is also important for the industry, since a more milled formed of wheat bran requires more processing steps before bio-processing with enzymes. In this project, no visuable differences between *Kruskakli* and *Kruskakli milled* could be observed. Hence, the degradation of aleurone cell walls seems not to depend on particle size. However, this requires further investigation, where a quantitative measurement of released proteins is suggested.

5.2 Bioprocessing with acidic conditions

Previous literature (Arte *et al.* 2016 and Arte *et al.* 2014) have published data for enzymatic activites at slightly acidic pH, [7], which implies that E1 might be more efficient at a lower pH compared to the neutral pH of destilled H_2O that was used throughout the project. In an earlier published study by Arte *et el.* (2015), the authors used acidic conditions in order to activate the endogenous enzymes in wheat bran, based on a previous study on sourdough performed by Loponen *et al.* 2007, [8]. The authors found that the effect of pH was crucial for activation of endogenous enzymes within wheat bran and that sole endogenous enzymes were more effective in solubilizing proteins than a combination of active endogenous enzymes and exogenous enzymes with xylanase effect. Thus, it was interesting to investigate if bioprocessing at acidic pH would give rise to a difference in degradation of cell walls in wheat bran.
The acidic conditions of pH 4.0 and 2.9 where examined with LM only. No obvious observed differences regarding cell wall degradation could be observed with LM for neither pH 4.0 nor 2.9, hence no correlation with the previous founding in Arte *et al.* (2015) could be confirmed. However, it is important to mention that the samples of *Kruskakli* and *Kruskakli milled* are from side streams in May 2016 and this might have effected the activity or presence of endogenous enzymes. Regarding the activity of E1 , it is necessary to perform complementary analysis with CLSM or quantitative measurements of solubilized proteins in order to confirm a possible difference in degradation. Subsequently, these results would have to be compared to the results for incubation with distilled H_2O (pH 7).

5.3 Immunolabeling of AX and BG

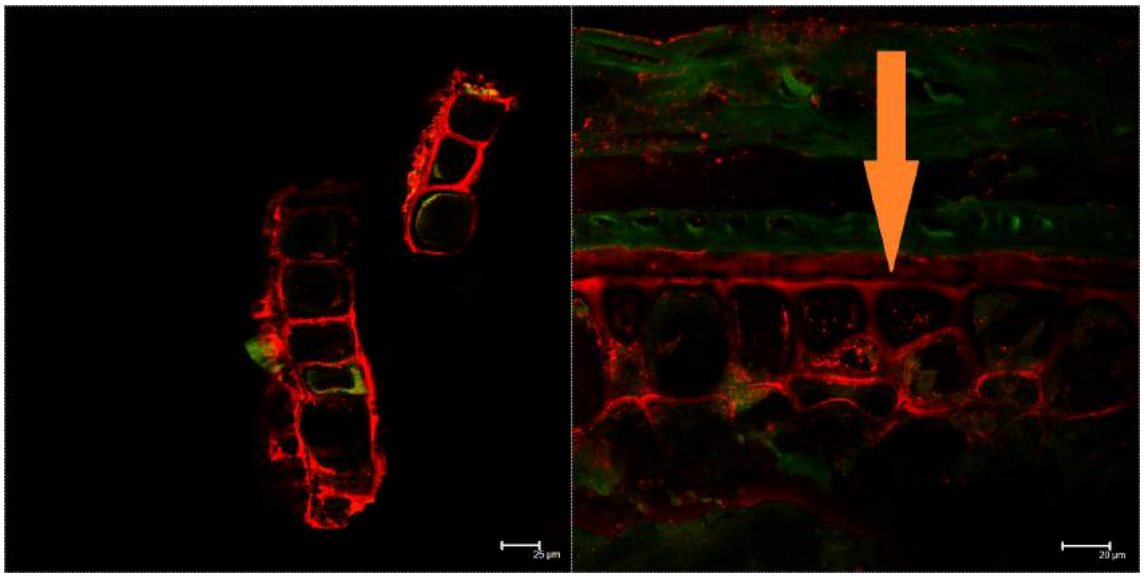


Figure 5.6: Cross sections of *Kruskakli milled* (left) and *Kruskakli* (right) labeled with AF647, fluorescently labeled antibody labeling AX. Samples analyzed with confocal laser scanning microscopy. AX visualized as red. Autofluorescence visualized as green. Junction zone between aleurone cells and testa line/pericarp in *Kruskakli* highlighted with an orange arrow. Scale bars: 25μm (left) and 20μm (right).

Single direct-immunolabeling of AX for both untreated *Kruskakli milled* and *Kruskakli* is displayed in Figure 5.6. It can be observed that AX (red) is present within the cell walls of the aleurone cells for both samples. AX is also present in the junction zone between the aleurone layer and the testa line/pericarp, highlighted with an orange arrow in the right image. Due to autofluorescence within wheat bran, it is possible to see the pericarp layer (green). Due to that *Kruskakli milled* contains smaller fractions of wheat bran where the aleurone layer have been separated from the pericarp already in the milling process, there are no visible pericarp layers in the left image in Figure 5.6. This corresponds well to the light microscopy analysis of the untreated samples, displayed in Figure 5.1.

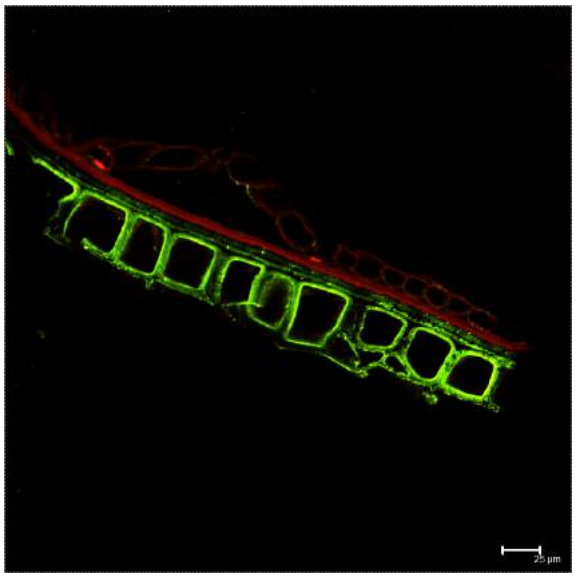


Figure 5.7: Cross sections of *Kruskakli* incubated for 4h at 30°C labeled with AF488, secondary fluorescently labeled antibodies labeling BG. Samples analyzed with confocal laser scanning microscopy. BG visualized as green, auto fluorescence as red. Pericarp is visual due to autofluorescence. Scale bar: 25μm.

Single direct-immunolabeling of BG (green) in a *Kruskakli milled* sampled incubated for 4h at 30°C is displayed in Figure 5.7. Pericarp and testa line is visible due to autofluorescence (red). BG is present within the cell walls of the aleurone cells, as for AX that is illustrated in Figure 5.6. It appears from Figure 5.7 that BG is less present in the junction zone between the aleurone layer and the testa line/pericarp compared to AX in Figure 5.6. From Figures 5.7 and 5.6 Also, it appears like BG is located closer to the cells and AX further out from the cells.

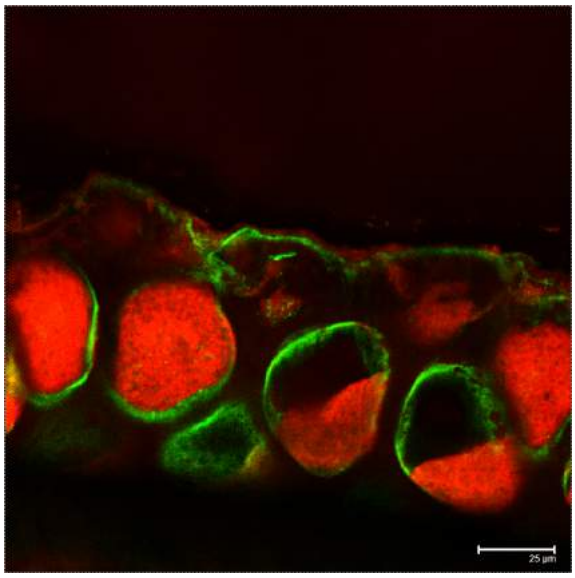


Figure 5.8: Cross sections of *Kruskakli* incubated for 4h at 30°C labeled with AF488, secondary fluorescently labeled antibodies labeling BG, counter stained with Nileblue, staining proteins. Samples analyzed with confocal laser scanning microscopy. BG visualized as green, proteins as red. Scale bar: 25μm.

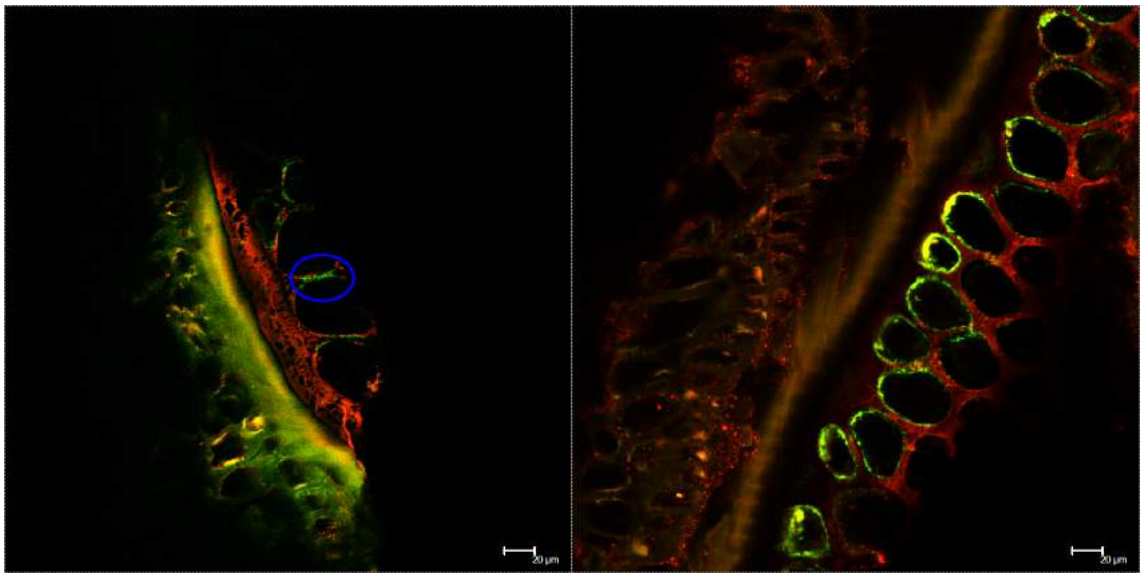


Figure 5.9: Cross sections of *Kruskakli* incubated for 4h at 30°C labeled with AF647 and AF488, secondary fluorescently labeled antibodies labeling AX and BG, respectively. Samples analyzed with confocal laser scanning microscopy. Arabinoxylan visualized as red, BG as green. Pericarp is visible due to autofluorescence, as green in the left picture and as red in the right picture. Scale bars: 20μm.

In order to further investigate the microstructure of cell walls within *Kruskakli* and *Kruskakli milled*, samples where direct-immunolabeled of BG and counter stained with a dye staining proteins. In Figure 5.8, BG is visible in green and proteins in red, it can be observed that BG (green) is located in the inner part of the cell walls, close to the cell lumen, proteins within cells visuzalised as red. Double immunolabeling of AX and BG also implied results that suggested that BG was located in the inner cell wall, and AX located around it, which is illustrated in Figure 5.9. In the left image, the BG is highlighted with a blue circle, and in both images in Figure 5.9 it is observed that the BG is located in the inner part of the cell wall closest to the aluerone cell lumen and that AX (red) is located further out in the cell wall and makes up the space between the cells. Similar observation was found in a publication by Guillon *et al.* (2004), where the authors immunolabelled AX and BG to investigate cell-wall structure in endosperm of wheat by light and electron microscopy.The authors found that (1-3)(1-4)β-glucan was absent at the junction zones between aleurone cells at the seed coat face whereas regions of the wall close to the cell lumen were heavily labelled, [48]. This corresponds well to the findings in this project, where β-glucan appeared to be present closer to the cell lumen and less abundant at the junction zone between the aleruone layer and the testa line/pericarp (Fig.5.7 and 5.8). Guillon *et al.* (2004) also found that the region between aleurone cells at the testa line exhibited the highest labelling, whereas the labelling of the wall zone close to the cell lumen was less intense than the outer wall. This also corresponds to what was found in this project. AX was found in the outer part of the cell walls (Fig. 5.6) and the junction zone between the aleurone cells and the testa line was richer in AX than in BG (Fig. 5.9 (left)).

The observation that BG and AX are possible located at different places within the cell walls gave rise to the hypothesis that the aleurone proteins are still visible

as "protein packages" (Fig. 5.5 (d)) which don't appear as fully degraded due to the fact that E1 does not degrade BG and only AX. Previous literature states that E1 has both xylanase and β-glucanase effect which would disprove the hypothesis (Arte *et al.* 2016). However, the article presents a table of hydrolytic activities for different enzymes at pH 6 which is a lower pH than the distilled H_2O used in this project (pH 7), hence the hydrolytic activities for E1 in this project might differ from previous published data.

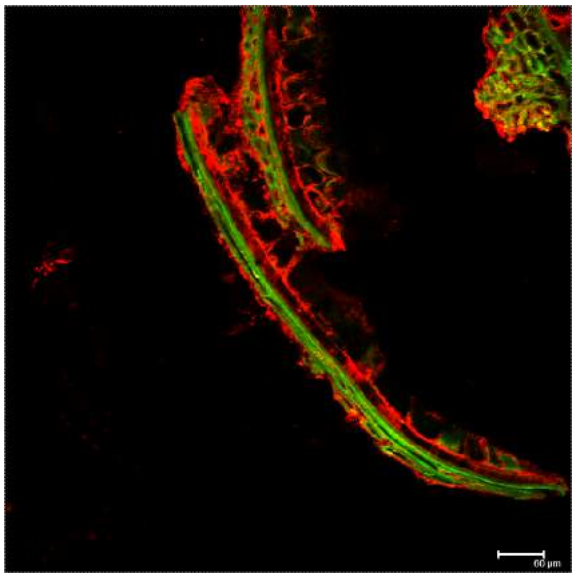


Figure 5.10: Cross sections of *Kruskakli* incubated for 8h at 35°C, with addition of E1 labeled with AF488, secondary fluorescently labeled antibody labeling AX. Samples analyzed with confocal laser scanning microscopy. Arabinoxylan visualized as red. Pericarp (green) is visual due to autofluorescence. Scale bar: 60μm.

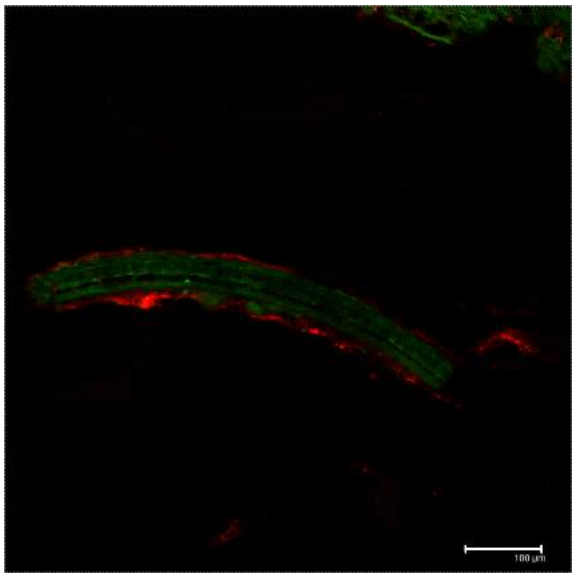


Figure 5.11: Cross sections of *Kruskakli* incubated for 24h at 35°C, with addition of E1, labeled with AF488, secondary fluorescently labeled antibody labeling AX. Samples analyzed with confocal laser scanning microscopy. Arabinoxylan visualized as red. Pericarp (green) is visual due to autofluorescence. Scale bar: 100μm.

Further investigation of how the microstructure in bran changes after enzymaztic treatment with E1 was performed and in Figure 5.10 and 5.11, degradation of AX is displayed. For 8h/35C°C (Fig. 5.10) AX was partly degraded. There is still AX located between the cells and at the wall between the aleurone layer and the testa line/pericarp but compared to the untreated sample (Fig. 5.6) it AX is more degraded after 8H/35C°C (Fig. 5.10). Compared to the light microscopy analysis (Fig. 5.3 (g)-(h)) where the cell walls still could be detected the partly degradation of AX implies that the cell walls observed with LM might be composed of other structures than AX, since the CLSM analysis showed that this is partly degraded (Fig. 5.10).

Further CLSM analysis of degradation of AX at longer incubation time implied that AX was still present closest to the testa line/pericarp but degraded between the aleruone cells (Fig. 5.11). However, the CLSM analysis of degradation of AX after enzymatic treatment with E1 would be complemented with analysis of immunolabeled samples counter stained with a stain that enables visualization of proteins.

6
Conclusions and outlook

6.1 Conclusion

The present work describes microscopic, LM and CLSM, analysis of changes in cell wall structure in wheat bran after enzymatic treatment with an industrial produced enzyme. By comparing LM results from different incubation times and temperatures it was confirmed that the highest tested temperatures (45C°C and 55°C) in combination with the longest tested incubation time (24h) were favorable for the activity of the enzyme. No obvious difference regarding degradation as an effect of the enzyme between the two investigated side stream samples, *Kruskakli* and *Kruskakli milled*, could be observed. LM analysis showed that even though cell walls were fully degraded after enzymatic treatment at certain temperatures and incubation times, the proteins from the aleurone cells still appeared as enclosed "protein packages". It was not possible, from LM analysis only, to observe an obvious difference in degradation from samples bioprocessed at acidic conditions (pH 4.0 and 2.9) compared to samples bioprocessed at a neutral pH 7, hence no obvious effect of endogenous enzymes could be observed. However, it was unknown if endogenous enzymes were present or active due to the age of the samples. Also, experiments regarding bio-processing at acidic conditions where only tested at 35°C for 35h, compared to appropriate controls.

From CLSM analysis it was possible to localize BG and AX within the cell walls of the aleurone cells present in the aleurone layer of the wheat bran. Results from immunolabellig with fluorescently labeled antibodies indicated that BG where located closer to the cell lumen compared to AX, which was located in further away from the cell lumen. Another observation based on CLSM analysis of immunolabelling was that BG was less abundant than AX at the junction zones between the aleurone cells and the testa line. Immunolabelling of AX after enzymatic treatment showed that degradation of AX increased with longer incubation time. Degradation of BG after enzymatic treatment has unfortunately not been investigated in this project, due to lack of time.

6.2 Outlook

In order to complement the work done throughout this project and to give a more thorough understanding of the micro structure of wheat bran and how components within cell walls in wheat bran are affected by enzymatic treatment, further experiments are needed.

The variables that were investigated throughout this project were temperature and time. Other variables could be varied in order to achieve optimized conditions for the enzyme E1. It is suggested that pH could be optimized as well as the dosage of the enzyme together with optimized conditions for temperature and time. It is desirable for the industry to optimize these suggested conditions in a way that both could lower cost and environmental impact. The optimization of pH could preferably include examination of fresh samples, in order to investigate a possible effect of endogenous enzymes at an acidic conditions. In this project, older samples were used and the presence and possible activity of endogenous enzymes were therefor uncertain.

Further CLSM analysis of immunolabeling of BG after enzymatic degradation is necessary in order to evaluate micro structure in wheat bran after enzymatic degradation more thorough. One approach would be to immunolabel samples where the aleurone layer had been fully or partly degraded and where "protein packages" were observed. Double immunolabelling of AX and BG after enzymatic treatment would also give rise to more knowledge about how the cell walls are degraded by E1.

A more thorough investigation of enclosed "protein packages" that appeared after enzymatic degradation with E1 at higher temperatures would possibly increase the understanding of how to bio-process wheat bran into a product rich in protein. This investigation could include separation steps of "proteins packages" from the remaining starch, pericarp and cell wall material after enzymatic treatment. A labeling of AX and BG of the "protein packages" would perhaps explain if any of these polysaccharides still remain and if this might be a reason for that the proteins still tend to stick together. Also, further investigations of the aleurone proteins is suggested in order to understand more on how and why they might tend to stick together even after bio-processing with enzymes.

In order to increase the understanding of the micro structure within wheat bran it is suggested to look for other targets within cell walls for enzymes. In this project, the targets have been AX and BG, but it is possible that other polysaccharides would be conceivable targets for enzymes for cell wall degradation.

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A

A.1 Staining dyes for CLSM

This appendix shows what additional staining dyes that has been used throughout the project as a complement to CLSM analysis except for staning with fluoroscent antibodies. The usage of different staining dyes was a part of the method optimization of how to visualize micro structures of wheat bran with immunolabeling in combination with additional stain. Only Sirius red and Nileblue was chosen colors after attempts.

1. The following staining dyes were used for CLSM analysis.

Stain	**used to visualize**	**Emission**
Sirius red	protein	550 nm
Nile blue	protein	670 nm
Achsid fuchsin	protein	630 nm
Fluorescein (FITC)	protein	520 nm
Calcofluor white	starch/cell walls	415 nm
Acridin orange (H_2O)	proteins	530-650 nm
Kongo red	starch/cell walls	520 nm