CHARACTERISATION OF THE MICROSTRUCTURE AND ENZYME DIFFUSION IN BRAN USING CLSM AND FRAP

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First Master of Drug Development

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May 28, 2015

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SUMMARY

Cereals are worldwide used as staple foods. The main product derived from cereals is flour, obtained by separation of the starchy endosperm from the grain outer layers and the aleurone during milling. The remaining outer layers and aleurone part is called bran. Today, bran is mostly used as animal feed despite its favourable composition for human’s health. Unfortunately the use of bran as nutrient or its addition to other food products is not as easy as it sounds. Addition causes negative effects on the sensory properties of the food product. Furthermore, microbiological and technological improvements (bioavailability of nutrients) are necessary. To optimise these factors, it is important to know the microstructure of bran. Furthermore, information about enzyme treatment, such as enzyme diffusion, can be used to improve the bioprocessing steps.

By use of light microscopy and confocal laser scanning microscopy, it was possible to investigate the structure of the different layers of bran and the difference between wheat and oat cereals. Additionally, the position of the proteins, starch, β-glucans and arabinoxylans was determined using staining techniques. Microstructural changes after endo-xylanase treatment were visualised, whereby the cereal vary, temperature and enzyme concentration the main influencing factors were. Fluorescence recovery after photobleaching measurements gave us more insight in the diffusion of three diffusion probes (sodium fluorescein, 10 kDA FITC-dextran and an exogenous stained endo-xylanase enzyme) in bran samples, very localised on a 10 micron scale. For the enzyme probe, a significantly higher diffusion rate could be observed after 24 hours possibly due to the partly deconstruction of the bran structure by the active enzyme. In contrast, the two non-enzymatic probes were found to be independent of the soaking time, as expected.

This thesis project provides new insights in the microstructure of bran and it demonstrates the feasibility of the localised determination of enzyme diffusion (rates) in bran flakes, without any further extraction or sample preparation needed. The used protocols can be optimised to investigate other bran samples microscopically or the enzyme diffusion of other types of exogenous enzymes, such as cellulases or glucanases. This approach offers great potential to optimise the refinement of bran and to ultimately utilise more bran in the food industry.
SAMENVATTING

Granen worden wereldwijd als basisvoedingsmiddel gebruikt. Het voornaamste product verkregen uit graan is bloem. Hierbij wordt het zetmeel endosperm tijdens een maalproces gescheiden van de aleurone en de buitenste lagen van graan. Deze overblijvende fractie van buitenste lagen en aleurone wordt zemelen genoemd. Vandaag worden zemelen vooral gebruikt als voedingsmiddel voor dieren, ondanks zijn gunstige samenstelling voor de mens. Het gebruik van zemelen of de toevoeging aan voeding is echter niet zo eenvoudig. Zemelen hebben een negatief effect op de sensorische eigenschappen van het voedingsproduct. Verder zijn microbiologische en technologische verbeteringen (o.a. de beschikbaarheid van nutriënten) noodzakelijk. Om deze eigenschappen te optimaliseren is het belangrijk de microstructuur van zemelen. Ook informatie over enzymbehandeling, zoals enzymbdiffusie, kan gebruikt worden om de bioprocessing technieken te verbeteren.

Met behulp van licht microscopie en confocale laser scanning microscopie was het mogelijk de structuur van de verschillende zemellagen te onderzoeken, alsook de verschillen tussen tarwe- en haverzemelen. Verder kon de positie van de eiwitten, zetmeel, β-glucanen en arabinoxylanen bepaald worden via kleurtechnieken. Microstructurele veranderingen na endo-xylanase behandeling werden in beeld gebracht, waarbij de graansoort, de temperatuur en de enzymconcentratie de voornaamste invloedsfactoren waren. Fluorescentie recovery after photobleaching gaf ons meer inzicht in de lokale diffusie van drie probes (natrium fluorescein, 10 kDa FITC-dextran en een endo-xylanase enzym) in zemelen. Het endo-xylanase enzym diffuseerde significant sneller na 24 uur contact, mogelijks door een gedeeltelijke afbraak van de zemelstructuur veroorzaakt door het actieve enzym. De diffusie van de twee niet-enzymatische probes was zoals verwacht tijdsonafhankelijk.

Dit project geeft nieuwe inzichten in de microstructuur van zemelen en toont de mogelijkheid aan om lokaal de enzymdiffusie (snelheden) in zemelen te bepalen. De gebruikte methodes kunnen geoptimaliseerd worden om andere zemelstalen of de enzymdiffusie van andere enzymtypes, zoals cellulasen of glucanasen, te onderzoeken. Deze benadering biedt de mogelijkheid om het gebruik van zemelen te optimaliseren, met als voornaamste doel de benutbaarheid in de voedingsindustrie te verhogen.
ACKNOWLEDGEMENTS

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Of course, I would like to thank my parents, my boyfriend and my Belgian and international friends for the endless support, the motivating pep talks, the wonderful trips, the joyful after works and the delicious fika breaks and dinners. You all contributed to the best Erasmus time I could have wished for. An experience and so many good memories to never forget!
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<th>Description</th>
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<tr>
<td>AX</td>
<td>Arabinoxylan(s)</td>
</tr>
<tr>
<td>A:X ratio</td>
<td>Arabinose:Xylose ratio</td>
</tr>
<tr>
<td>BFM</td>
<td>Bright field microscopy</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interfering contrast</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium fluorescein</td>
</tr>
<tr>
<td>PLM</td>
<td>Polarised light microscopy</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

Cereals belong to the most important staple foods in many cultures, for example maize in Africa, rice in Asia and wheat in Europe and Northern America. Their worldwide use makes their production an enormous industry, which still tries to improve their efficiency. The utilisation possibilities of each product and by-product and the optimisation of each production step remain some of the main goals of this industry.

Wheat and oat, the two cereals used in this project, are both of great importance worldwide. Grains can be used in total but are mostly processed before they serve as human food. Wheat may give rise to pasta, after it is milled into flour, dried and cooked. Oat is mostly transformed to rolled oats or milled to oat flour. Each production step contributes to the quality and properties of the final product. During the processing steps, parts of the grain are removed or manipulated to obtain a specific product. Other obtained products are called by-products. One of these by-products of the milling process is bran, a product that is today mostly used as animal feed, despite its favourable composition to contribute to human’s health (1–4). Research to define this by-product on chemical, physical, microbiological and technological level is needed to improve its usability.

1.1 CEREALS AND BRAN

Cereals are mostly pre-treated, milled or grinded with the removing of some grain parts to yield flour. Yet, dietary recommendations encourage the intake of whole grains, as it is shown in epidemiological studies that wholegrain intake has an inverse relationship with several chronic diseases (cardiovascular, obesity, diabetes…) and some cancers (2,4–9). The mechanisms of these protective effects are still not totally understood (4).

One important by-product obtained by milling is bran, composed of the different outer layers of the grain. The positive effects of bran can be assigned to different components such as anti-oxidants, dietary fibers, vitamins, sterols and phytochemicals. Anti-oxidants can prevent chronic diseases, while the dietary fibers can contribute to a reduced risk of coronary heart diseases and some cancers (3–6,10). Furthermore, bran is sometimes seen as a prebiotic product. Prebiotic products are non-digestible food products that selectively stimulate the growth and/or activity of one or a limited number of bacteria in
human’s colon, thereby benefiting human’s health (11). Despite these useful properties for nutrition and health, bran is today mostly used as animal feed and less as human food (1–4).

Bran’s use and its addition to food are however not as easy as it sounds. The addition causes negative effects on the sensory properties of the food product such as on the colour (darker), the flavour (bitter) and the taste (marred). These effects are respectively caused by the presence of anti-oxidants, ferulic acids and cellulose (2,5,7,8). Research to optimise bran’s use could contribute to new insights in its food consumption. Processing steps to optimise the sensory properties are heading in the right direction but there is still a long way to go. Additionally, the physical and microbiological properties, such as the moisture content or the presence of microorganisms and mycotoxins, need to be controlled. Bio-processing techniques such as fermentation or addition of enzymes are evaluated (2,7,8).

Ultimately, there has to be attention on the bioavailability of the nutrients, when using bran as a food product. The bran matrix or the lignified cell walls of some bran parts can interfere with the exemption of the nutritive components. Methods such as decreasing the bran particle size or bioprocessing techniques (enzymatic treatments) are described to improve the bioavailability (5–8,12–15). To optimise the use of bran and processing techniques, it is important to know the composition and the (micro)structure of the different bran layers.

1.1.1 The structure and composition of cereals

Botanically seen, cereals are the grains or the fruits of grasses, a common name for plants of the Poaceae family. The grains are known as a caryopsis type of fruits, which means they are single seeded and formed out of one single carpel (1,10,16–19). The composition, the size, the shape and the mass of the grains depend on the cereal varies and variety. Even more variation can be seen in the cell wall thickness, the size of the cells, the protein structure and the starch granule structure. In general, three structural parts can be distinguished in each grain: the germ (2-3%), the endosperm (68-85%) and the outer layers (12-19%) (Figure 1.1) (1–5,16,20).

The germ or the embryo is the fundamental part for the development of the plant, as it contains the genetic information. It is rich in fibers, proteins, lipids, vitamins and phytochemicals (3,5,13,16,17,21). It consists of the embryonic axis and the scutellum; the latter
situated between the embryonic axis and the endosperm. The scutellum transfers the nutrients from the endosperm to the embryo (13,16,17,21).

![Diagram of wheat grain structure](image)

**Figure 1.1**: Structure of wheat grain (3). Bran, starchy endosperm and germ are shown.

The second structural part, the endosperm can be divided into the starchy endosperm (closest to the germ) and the aleurone (closest to the outer layers). The first plays a major role as reserve source of nutrients for the embryo, the latter transports the nutrients from the outer layers to the endosperm. The starchy endosperm constitutes the main part of grains and consists of starch granules embedded in a matrix of proteins (3,5,13,16,21,22). The composition of the cell walls of the endosperm depends on the cereal varies. In wheat, the thin unlignified cell walls contain a high amount of arabinoxylan while in oat β-glucans are the major component (10,16,20,23,24).

The 3th part of cereals consists of the outer layers. It plays a protective and nutritive role during grain development (12,13,16,20,22,25). During milling, the starchy endosperm is separated from the aleurone and the outer layers to yield flour. The remaining aleurone and outer layers part are called bran and attribute for 30% of the whole grain (1–5,11,15,16,21,26).

### 1.1.2 Composition of the bran layers

Bran is the outer layers and aleurone fraction remained after grinding. In total, bran is composed of water, proteins, fat, vitamins, minerals and carbohydrates, more specifically non-starch, starch and lignin. It presents also a high amount of vitamins, minerals and phytochemicals. The specific composition depends on the cereal varies and variety (2–7,10–12,24). Depending on the milling process followed, it is possible that the separation of the bran and the endosperm is not totally successfully completed. The examined bran microstructure can therefore still contain variable amounts of starchy endosperm. Different
milling parameters and processes are possible to yield milling fractions, such as aleurone or pericarp rich-fractions with specific compositions (4,7,13,26).

Bran consists from the outside to the inside of different layers: the pericarp, the testa, the nucellar epidermis and the aleurone layer (Figure 1.2) (1~5,11,15,16,19,21). Each layer has its own composition, thickness, structure and properties (3,11,16,21).

![Figure 1.2: Cross section of wheat bran. P: pericarp, T: testa and A: aleurone(3)](image)

The most outer layer, the pericarp, originates from the carpel wall. It is mostly weakly attached to the underlying testa (1,16,17,26). Table 1.1 gives an overview of the structure and the composition of the pericarp.

Table 1.1: General overview of the structure and the composition of the pericarp

<table>
<thead>
<tr>
<th>Structure</th>
<th>Composition</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pericarp (6-8% of bran)</td>
<td>Common for outer and inner pericarp:</td>
<td>(1,3,5,6,13,17,22,26)</td>
</tr>
<tr>
<td>- Outer pericarp:</td>
<td>Elongated empty cells // axis</td>
<td>Insoluble dietary fibers</td>
</tr>
<tr>
<td>-</td>
<td>of the embryo cells</td>
<td>(cellulose, lignin and</td>
</tr>
<tr>
<td>- Inner pericarp</td>
<td>Large intercellular spaces</td>
<td>branched heteroxylans)</td>
</tr>
<tr>
<td>-</td>
<td>with empty cells of different cell types*:</td>
<td>- Phenolic acids (bound to</td>
</tr>
<tr>
<td>- Tube cells: // axis embryo</td>
<td></td>
<td>heteroxylans)</td>
</tr>
<tr>
<td>- Cross cells: ( \perp ) axis embryo</td>
<td></td>
<td>- Proteins (minor amount)</td>
</tr>
<tr>
<td>- Other cells</td>
<td></td>
<td>- Lignin (cell walls)</td>
</tr>
</tbody>
</table>

*: different cell types invisible in oat (1,16,17)

The cell walls of the pericarp are very thick and lignified (13,22). The thickness of the outer pericarp layer of wheat cereals is estimated to be around 15 to 30 μm; the inner
pericarp is circa 15 to 25 μm thick (17,21). For oat, absolute numbers are not available, but it is estimated that the pericarp is thinner in oat grains (25).

The adjacent testa can be single layered (oat) or multi-layered (wheat) and derives from the two integuments of the carpel, enclosing during grain development (16,17). The inner pericarp, the testa and the hyaline layer are sometimes called together as the intermediate strip (30% of the bran), the testa together with the hyaline layer is also named ‘the seed coats’ (1,26). The testa layer is circa 5 to 8 μm thick in wheat grains (17,21). Table 1.2 gives an overview of the structure and composition of the testa.

Table 1.2: General overview of the structure and the composition of the testa

<table>
<thead>
<tr>
<th>Structure</th>
<th>Composition</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testa</td>
<td>Thin layer of rectangular cells</td>
<td>- Lignin (cell walls)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Branched heteroxylans</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Alkylresorcinols</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Pigments</td>
</tr>
</tbody>
</table>

The nucellar or the hyaline layer, which lays the closest to the non-starchy endosperm, is a remnant of the tissue in which the embryo and the endosperm developed. The hyaline layer is rich in arabinoxylans. In mature wheat grains, this layer is about 15 to 20 μm thick (15). In mature oat grains, this layer is absent (16).

Table 1.3: General overview of the structure and the composition of the aleurone layer

<table>
<thead>
<tr>
<th>Structure</th>
<th>Composition</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aleurone</td>
<td>Block-like cells (20-75 μm)</td>
<td>Cell walls:</td>
</tr>
<tr>
<td></td>
<td>- Thick un lignified cell walls</td>
<td>- Linear arabinoxylans</td>
</tr>
<tr>
<td></td>
<td>- Large nuclei</td>
<td>- B-glucans</td>
</tr>
<tr>
<td></td>
<td>- Fluorescent granules (1-5μm)</td>
<td>- Proteins (in minor amount)</td>
</tr>
<tr>
<td></td>
<td>- In protein matrix globoid structures with high amount of phytin⁴ and niacin</td>
<td>- Diferulic acids⁴</td>
</tr>
<tr>
<td></td>
<td>Cell content</td>
<td>- Vitamins: B1, B2, B3, B6, B9, E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Minerals: P, K, Mg, Mn, Fe</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Proteins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Phenolic acids⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Phytates⁴</td>
</tr>
</tbody>
</table>

⁴: components responsible for autofluorescence under suitable light conditions
The final layer of the bran is the aleurone layer, named after the presence of a specific protein called aleurone found in protein granules. The layer actually belongs to the endosperm; it surrounds the starch and supports the endosperm in its role as reserve source. In wheat grains, the layer is approximately 40 to 65 μm thick \( (17,21) \). The microstructure of oat cereals has common characteristics with wheat cereals \( (20,25) \). A general overview of the structure and the composition can be found in Table 1.3. Figure 1.3 gives a summary of the different cell layers, with their specific cell structure, for both oat (left) and wheat (right).

![Diagram of bran layers](http://www.grain-gallery.de/en)

The composition and the structure of the cell walls is mostly known in detail. Though, little research focuses on the arrangement of the different components in the cell layers or in the grain in total. Yet, knowledge about the arrangement would be interesting as the structural characteristics influences the bioavailability of the components. Processing to optimise the bioavailability can change the microstructure, which in turn influences the texture, the appearance, the taste, the perception and the final stability of the food product \( (18) \). Microscopic techniques can be used to define the position of some specific components, such as dietary fibers, and changes caused by processing.

1.1.3 Previous microscopic research of bran in literature

Previous microscopic research will be important for the identification of the bran layers later on. A general overview of the intact grain’s structure as seen with an fluorescence microscope is given by Kamal-Eldin A. et al. Acid Fuchsin and Calcofluor were used as dyes to stain the proteins and the β-glucans respectively \( (4) \). The combination of two staining dyes makes the contrast between cell walls and cell content bigger \( (24) \).
By investigating the histological composition of intact wheat grain with confocal laser scanning microscopy (CLSM), Antoine C. et al. distinguished three layers in bran: the outer pericarp, the intermediate (consisting of the inner pericarp, the testa and the hyaline layer) and the aleurone layer. If these terms are used in this project, it refers to this subdivision. As described above, the outer pericarp consists of longitudinal cells parallel to the grain axis (Figure 1.5a.1) and the pericarp is multi-layered (three cell thickness) (Figure 1.5a.2). The intermediate strip is built up of different cell types such as cross cells and tube cells. (Figure 1.5b.2) The aleurone layer on the other hand consists of polygonal cells with fluorescent granular cell inclusions (Figure 1.5c). The distinction of the different layers in oat is more difficult to make and images are not available yet.

Figure 1.4: Microstructure of intact wheat grain section stained with Acid Fuchsin and Calcofluor as seen with an epi-fluorescence microscope. Proteins are coloured red; β-glucans are coloured blue and lignified cell walls appear yellow (4).

Figure 1.5: Fluorescence micrographs of wheat bran layers and cross section; a: outer pericarp, b: intermediate strip, c: aleurone. Images were obtained by superposition of two channels: $\lambda_{exc} = 364$ nm and $\lambda_{em} > 397$ nm for the blue-red emission, $\lambda_{exc} = 488$ nm and $\lambda_{em} > 515$ nm for the green-red emission (1)
Jääskeläinen et al. presented some optical microscopy images of sections (4 µm) of unground wheat grains as well (Figure 1.6) (22). The left image is obtained by staining with Acid Fuchsin and Calcofluor. Proteins are coloured red; β-glucans are blue as could be seen previously in Figure 1.4. The pericarp does not show any presence of β-glucans as opposed to immunolabeling used to visualise the β-glucans (23). It is possible that the autofluorescence of the phenolic acids and the lignin in the pericarp layer overcome the effect of the Calcofluor (19,24). The middle picture is obtained by staining with Light Green and Lugol’s iodine. Proteins are coloured green; starch is coloured purple to black. The protein concentration appears to be the highest in the aleurone layer. The right picture shows the autofluorescence after UV excitation (emission above 420 nm collected), due to autofluorescence substances such as lignin and (di)ferulic acids (5,22,24).

Dornez et al. showed similar pictures for both wheat and oat stained with Acid Fuchsin and Calcofluor (Figure 1.7). The outer layers part seems clearly thinner in oat grains. Furthermore a difference in aleurone cell structure can be seen. In oat grains, the aleurone cells seem more rectangular instead of the squares seen in wheat grains (24). Assumptions about the β-glucans content cannot be made, as the intensity of the colour as representation of the amount of β-glucans is relative (20,24).

Figure 1.6: Optical microscopy images of wheat grain sections (4 µm). Left: staining of section with Calcofluor and Acid Fuchsin. Middle: staining of section with Light Green and Lugol’s iodine. Right: autofluorescence image with UV excitation (330-380 nm) and emission of > 420 nm (22).

Figure 1.7: Microscopic images of cross sections (4 µm) of wheat (A) and oat (B) grains stained with Acid Fuchsin and Calcofluor. Proteins are coloured red; β-glucans are coloured blue. λ_ex: 400 nm – 410 nm, λ_em: > 455 nm (24).
1.2 NON-STARCH POLYSACCHARIDES IN BRAN

The evidence of the importance of dietary fiber’s consumption is undeniable. Dietary fibers are non-digestible or partially digestible polysaccharides or lignin (lipophilic phenolic polymer) derived from plants. They contribute to human’s health by stimulating the intestinal transit and supporting the fermentation by bacteria of the intestinal flora. Fibers reduce the blood pressure, the total and LDL-cholesterol levels, the blood glucose and insulin levels and the energy density of food products. They ensure furthermore a better saturation after eating, which contributes to reach and maintain a healthy weight (10,19).

Bran contains different components belonging to the dietary fiber group, such as lignin, cellulose, β-glucans and branched heteroxylans. One important heteroxylan example found in bran cell walls is the polysaccharide arabinoxylan (AX). This heteroxylan consists of a linear β-(1-4)-linked xylan backbone (D-xylopyranosyl residues) with α-(1-2) and α-(1-3)-linked L-arabinofuranose units as side residues (Figure 1.8). Different substitution patterns of arabinoses (position of substitution) and different arabinose to xylose ratios (A:X ratio, degree of substitution) are possible, which can influence the water extractability and determine the conformation and the physicochemical properties of the cereal. It is known that phenolic acids, such as ferulic acids, can be covalently linked by an ester bond with C- (O)-5 of the arabinofuranose residues. They can participate in the cross-linking of the bran matrix. Also xylose, galactose and glucuronic acids can be found as side residues in smaller amounts (4,5,11,12,15,18,19,23,24,27–29). The molecular structure can therefore be quiet complex, which will influence the action of xylanase enzymes (15,18).

![Figure 1.8: Structure of arabinoxylan chain. A: substitution of ferulic acid to C-(O)-5 of arabinose. B: β-(1-4) – linked xylan backbone. C: α-(1-2)-linked L-arabinofuranose (29)](image)

The molecular weight of the arabinoxylans chains depends on cereal varies and variety, the substitution pattern and on the method used. It can be estimated to range between 65 kDa and 1 000 kDa for wheat arabinoxylans. In cereals, AX plays an important
role as maintainer of the structural network of the different layers, by providing elasticity, allowing transport of nutrients and inhibiting the formation of ice crystals during winter (26).

The position of AX is more challenging to define compared to proteins, starch or β-glucans. Direct staining methods are not available (19,22,24). Several other techniques are used instead. Firstly, immunolabeling was tested to visualise AX, whereby a specific antibody is formed against AX (14,19,20,23,24). The disadvantage of this technique is the production of the antibodies; it is time consuming and requires difficult preparing steps to create a well-defined useful method. Furthermore, the visualisation is not only depending on the suitability of the antibody, but also on the number of potential binding positions for the antibody (19,24). Recent approaches are trying to optimise the use of immunolabeling (14).

Other studies focused indirectly on the position of AX. They compared the fluorescent signal of stained β-glucans before and after xylanase treatment. AX is known to be located close to the β-glucans in the cell walls. Increased fluorescent signal after xylanase treatment was suggested being the result of the disappearance of AX (11,19,24). Another technique determined the AX position by defining the autofluorescence signal of ferulic acids, which are known to be covalently linked to AX. Yet, these techniques are just suggesting the position of AX, without 100% confirming it (19,24). Therefore, a new technique was developed, based on an inactive xylanase enzyme.

Xylanases are enzymes able to break the xylosidic linkages of the xylan backbone. They belong to the glycoside hydrolase family of enzymes and are often commercially used in the paper, food and animal feed industry to modify the AX amount, its bioavailability and its functionality (8,14,18,19,28–30). X-ray crystallography, site-directed mutagenesis and proteins enzymology made it possible to determine the regions where the enzyme binds on the xylan backbone (18). Site-directed mutagenesis and amino acid modifying was used to obtain inactive enzymes, which were able to bind to AX but not able to hydrolyse it. The binding of the enzyme was either visualised with atomic force microscopy or by binding of a fluorescently labelled antibody, meanwhile other studies used directly fluorescently labelled enzymes. With this latter technique, it was possible to visualise the arabinoxylan position in all parts of the bran except in the pericarp due to a too high arabinose:xylose ratio (no binding of enzyme possible) (Figure 1.9) (11,18,19,24).
This project is focused on the further investigation of the microstructure of bran, the position of AX in bran and the influence and properties of enzyme treatment to optimise the use of bran. Microscopic techniques and FRAP measurements are the main techniques used in this project.

1.3 MICROSCOPY

Imaging techniques such as microscopy generate data in form of an image. Various microscopic methods are available, which differ in the method of image production, the resolution and the type of signal detected. Two main techniques will be used in this project: optical light microscopy and confocal laser scanning microscopy.

1.3.1 Light microscopy

Light microscopy (LM) is an useful technique to study the differences in microstructure between several cereal varieties. Microstructural changes caused by processing, can also be investigated (20).

The simplest method of light microscopy is the bright field (light) microscopy, BFM. Light is transmitted through a condenser (focuses the light in one direction) and the sample, before it is collected on an objective. The image received is upside down and reversed. The microscopic tube and the ocular lens magnify the image that eventually can be seen through the tube or the photographic film above the microscope. Contrast dyes can be used to improve the visibility of different chemical components by staining them, for example Lugol’s iodine for the staining of starch or Light Green for the staining of proteins (20,31).

Polarised light microscopy, PLM, is a second form of microscopy that can be used for unstained samples. Crystalline or ordered structures are able to rotate the polarised light,
which can be detected by two crossed polarizers. Other structures that cannot rotate the light remain invisible. Differential interference contrast microscopy, DIC, is the third form of microscopy used for unstained samples in this project. The polarised light beam is split in two orthogonal rays by a prism. Interference is caused as the two rays pass the sample with different speed. The beams are combined again after passing the sample. DIC gives three dimensional relief images of structures corresponding to the variation in optical density. Structures which are normally invisible in unstained samples (above or below the resolution limit) can be observed (32).

1.3.2 Confocal laser scanning microscopy

Confocal laser scanning microscopy, CLSM, is a microscopic technique developed by Marvin Minsky in 1957. Compared to LM, a laser is used as source of energy instead of light and visualisation is based on fluorescence (see more 1.4.1 Fluorescence). Figure 1.10 describes more detailed the working mechanism of CLSM: a laser beam passes an entrance pinhole (a) before it is focused on a small, mostly fluorescent, spot of the sample (b) by a lens. The objective lens collects the fluorescent light even as the scattered and reflected laser light. Finally, a second pinhole (c) focuses the light of the spot and transmits it to a detector. The term confocal is based on the composition of the two pinholes (a/c) and the sample (b); they are in conjugate focus or confocal.

This implicates already one advantage of CLSM. By using a pinhole before the detector, the ‘out-of-focus’ light, fluorescence of the regions above and below the region of interest, is removed. This means that the image is only formed based on signals caused by the region of interest (z-plane), creating a higher resolution and a sharper image (33,34).

A second advantage of the CLSM technique is its possibility to move the focal plane in three dimensions: x, y and z-direction. By moving the focal plane in defined steps (µm-
range), an overall 3D image can be created by taken optical section images at different depths. This is a valuable technique for samples of little thickness, whereby light microscopy gives blurry images due to interference of light above or below the plane of focus. The term scanning implicates that images are taken by scanning the samples several times in the x and y direction, whereby the resulting image is based on different scans \(^{(33,35)}\).

In addition to the conventional setup for CLSM, an epi-illumination setup with only one lens can be used. The incident light and the outgoing light (radiated, scattered or reflected) pass the same objective lens. Furthermore, a dichroic mirror is used to direct only a small amount of the light (with special wavelength) in the direction of the detector, by acting as a beam splitter (Figure 1.11) \(^{(33)}\). In this project, the epi-illumination setup is used.

![Figure 1.11: Epi-illumination setup of CLSM (33). The incident and outgoing light pass the same objective lens.](image)

1.4 FLUORESCENCE RECOVERY AFTER PHOTobleaching

1.4.1 Fluorescence

Fluorescence is the spectroscopic principle in which atoms or molecules absorb and re-emit the energy of electromagnetic radiation. The absorption of the energy causes an electronic transition to a higher energy level, whereby an electron is moved from a bonding or non-bonding orbital to an anti-bonding orbital. By re-emitting the energy, they return to their ground state \(S_0\). When the energy is remitted as light, the process is called photoluminescence. As a part of the energy is lost as internal energy (internal conversion, vibrational conversion or intersystem crossing) during the transition, the emitted light has a lower energy and thus a longer wavelength than the excitation light. This change in wavelength is called the Stokes shift. The relationship between the energy and the wavelength can be found in the Planck-Einstein relation (1.1).
\[ E = h \cdot \frac{c}{\lambda} \]  

(1.1)

Where: 
- \( E \): energy (J)
- \( h \): constant of Planck (J.s)
- \( c \): speed of light (m/s)
- \( \lambda \): wavelength (1/m)

Two types of photoluminescence can be distinguished: fluorescence and phosphorence. In the fluorescence principle, the emission light derives from singlet excited states. The electron in the excited state is paired to the electron in the ground state and they have an opposite spin. The emission rate of fluorescence is approximately \( 10^8 \) s\(^{-1}\). In phosphorence, the emitted light is derived from triplet excited states. The excited electron has the same spin as the electron in the ground state. The emission rates are therefore much lower, in the range of milliseconds to seconds. The illustrated Jablonski energy diagram presents the photoluminescence process with the first and second singlet excited states (S\(_1\) and S\(_2\)) and the first triplet excited state (T\(_1\)) (Figure 1.12) (34,36,37).

As mentioned before, some molecules present autofluorescence. When absorbing light, they have a natural emission. They are distinguished from non-fluorescent molecules, which need a fluorescent marker (fluorophore) to be seen with fluorescence microscopy. Autofluorescence can sometimes interfere with the detection of specific fluorophores. Several precautions are needed to overcome this interference.

1.4.2 **Fluorescence recovery after photobleaching**

Fluorescence recovery after photobleaching (FRAP) is microscopic quantitative technique used to visualise the mobility of fluorophores. FRAP measurements consist of different steps; in a first step pre-bleaching images of the region of interest (ROI) are taken.
by using a laser with low intensity to determine the initial fluorescence $F_i$. In a second step, a defined area is bleached by increasing the laser to its maximal intensity, this time point is defined as $t_0$. Finally, post-bleaching images are taken to visualise the fluorescence recovery due to the inward diffusion of the fluorophore from the unbleached areas. If all molecules are mobile, the fluorescence recovers totally to $F_i$; if only a part of the molecules are mobile, the fluorescence recovers to $F_\infty$ (less than $F_i$). Figure 1.13 shows the principle of FRAP measurements (30,38–41). FRAP can be used to calculate the diffusion coefficient by analysing the recovery curve, as the fluorescence recovery is determined by the mobility of the fluorophore (41).

![Figure 1.13: Principle of FRAP measurements](image)

Figure 1.13: Principle of FRAP measurements (38). A region of interest is selected of which pre-bleaching images are taken. At $t_0$, the region is bleached by maximizing the laser intensity. In the post-bleaching period, the recovery of the fluorescence in the region of interest is determined.

The mobility of proteins in living cells was earlier investigated by FRAP. Mobility measurements of carbohydrate-enzymes on natural substrates, such as bran, are for the moment limited (30). Yet knowledge would be interesting to optimise bioprocessing of bran.

1.5 DIFFUSION

Diffusion is the process of mass transport whereby particles spontaneously randomly or from a region of higher concentration to a region of lower concentration move until equilibrium is reached. The process was firstly observed by Brown. In 1855, Adolf Fick derived some equations to describe the diffusion process and to calculate the diffusion coefficient. To describe the transport (flux $J$) caused by a concentration gradient between two areas in one dimension, the Fick’s first law of diffusion can be used (1.2).
\[ J = -D \frac{\delta C}{\delta x} \quad (1.2) \]

Where \( J \): flux (mole/m²s)
\( D \): diffusion coefficient (m²s)
\( C \): concentration (mole/m³)
\( x \): position parameter (m)

The negative sign in the equation demonstrates that diffusion occurs from the higher concentrated area to the lower concentrated area (against the concentration gradient). The equation can only be used in steady state conditions and without any external forces except the concentration gradient. The change in concentration in function of the time during diffusion is described with the Fick's second law of diffusion (also known as the diffusion equation) (1.3) (40,42–44).

\[ \frac{\delta C}{\delta t} = D \frac{\delta^2 C}{\delta x^2} \quad (1.3) \]

Where \( C \): concentration (mole/m³)
\( t \): time (s)
\( D \): diffusion coefficient (m²s)
\( x \): position parameter (m)

The diffusion coefficient can be described with the Stokes-Einstein equation (1.4). This can be used for spherical particles in infinitely diluted solutions. As can be seen in the equation, the diffusion coefficient is influenced by the temperature, the viscosity of the solvent and the size/shape of the particle (40,44).

\[ D = \frac{k_B T}{6\pi \eta r} \quad (1.4) \]

Where \( D \): diffusion coefficient (m²/s)
\( k_B \): Boltzmann constant (1.38e-23 m².kg/(K.s))
\( T \): temperature (K)
\( \eta \): viscosity (kg /m)
\( r \): hydrodynamic radius of sample (m)
2 OBJECTIVES

Bran as by-product of grain milling is nowadays mostly used as animal feed. Yet it contains some components such as dietary fibres, vitamins and minerals, all of which may contribute to human’s health. However, addition of bran can cause negative effects on the sensory properties of the food product as taste, colour and flavour may change. The bran structure can also limit the bioavailability of the different components. To optimise the use of bran, it would be interesting to gain knowledge about the properties on chemical, physical and technological level.

In a first part of this project, we will try to visualise the microstructure of three different bran samples, two wheat bran samples and one oat bran sample. Light microscopy will be used to investigate the different bran layers and their composition. Light Green and Lugol’s iodine staining will be used to visualise the proteins and starch respectively. Furthermore, structural changes after endo-xylanase treatment will be investigated with light microscopy for different enzyme concentrations and different temperature parameters. Endo-xylanase treatment can be used to optimise the bioavailability of different nutrients of bran.

In a second part, we will try to visualise the microstructure of bran with confocal laser scanning microscopy using autofluorescence signals. Additionally, the location of the arabinoxylans will be determined by use of a stained endo-xylanase enzyme. Results obtained by the two different microscopic methods (light microscopy and confocal laser scanning microscopy) will be compared and linked to previous microscopic research in literature.

In a last part, we will try to visualise enzyme diffusion in the bran samples by use of fluorescence recovery after photobleaching. Diffusion rates of three different probes (NaF, 10 kDa FITC-dextran and a stained endo-xylanase enzyme) will be investigated at different time points in absence and presence of bran, to determine possible hold-up of the probe.

The overall aim of the project focuses on the structure of bran, linked to enzyme treatments. Industry can possibly use this information for the optimisation of bran as nutrient.
3 MATERIALS AND METHODS

3.1 SAMPLES

Samples were supplied by Kungsörnen (Lantmännen Cerealia, Stockholm, Sweden). The three samples were Kruskakli (wheat bran), Vetekli (wheat bran) and Havrekli (oat bran). Table 3.1 shows the composition of the different samples per 100 g. Information about the milling process and the relative amount of fibres components was unavailable for industrial innovatory reasons or public secret.

Table 3.1: Composition of the three different bran samples per 100 g

<table>
<thead>
<tr>
<th></th>
<th>Kruskakli (wheat)</th>
<th>Vetekli (wheat)</th>
<th>Havrekli (oat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>14 g</td>
<td>16 g</td>
<td>18 g</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>15 g</td>
<td>23 g</td>
<td>41 g</td>
</tr>
<tr>
<td>Fat</td>
<td>6 g</td>
<td>6 g</td>
<td>8,5 g</td>
</tr>
<tr>
<td>Fiber</td>
<td>50 g</td>
<td>40 g</td>
<td>18 g</td>
</tr>
</tbody>
</table>

In this project, tests were only preformed with exogenous enzymes. The endo-xylanase enzyme used for endo-xylanase staining, xylanase treatment of bran and diffusion measurements was produced by fermentation of the fungi Thermomyces lanuginosus (Sigma-Aldrich, St Louis, USA). Optimal conditions for xylanase activity are pH 7.0 and 60-70°C. The molecular weight varies between 22.5-25.5 kDa, depending on the method used to determine. The enzyme is stable for 96 h at 60°C in a pH 5.0-9.0 (28). The enzyme has an activity of ≥ 2500 units/g. One unit is defined as “the amount of xylanase required to release 1 μmol/min of reducing equivalent of xylose from birchwood xylan at 60°C” (12,25).

3.2 PARTICLE THICKNESS

The particle thickness of 20 untreated bran flakes was measured 5 times on 5 different days with a thickness meter, ICD-112CB (Mitutoyo Cooperation, Takatsu-ku, Japan). The precision of the instrument was 0.001 mm. Normal distribution was examined with SPSS.

3.3 LIGHT MICROSCOPIC DETERMINATION OF THE MICROSTRUCTURE

3.3.1 Preparation of the samples

Samples were cryo-sectioned using liquid nitrogen (AGA, Lidingö, Sweden). The bran flakes were arranged separately in the cryo-embedding compound (Pelco Prod No.
27300, Ted Pella Inc, Redding, USA) to obtain separated layers and cut after at least 20 minutes of freezing in the microtome. The samples were cut in sections of 10 μm with a microtome, Leica CM 1900 (Cellab Nordia AB, Sollentuna, Sweden) and a sharp microtome blade (Leica 819, Leica Biosystems, Nussloch, Germany) and transferred to Thermo Scientific polysine microscope slides (Menzel-Gläser, Braunschweig, Germany). The sections were dried for at least 3 hours at room temperature (RT).

3.3.2 Light microscopic investigation

Light microscopy research was done with the Nikon Microphot-Fxa microscope (Bergman Labora AB, Danderyd, Sweden), connected with an Altra 20 camera (Olympus Corporation, Tokyo, Japan) and computer with the CellSens Dimension software (Olympus Corporation, Tokyo, Japan). The sections were examined with BFM, PLM and DIC in different stages (unstained or stained). Each sample was investigated using objectives 4x, 10x and 20x magnification. The DIC form could only be observed using the 20x magnification objective. Each observation was confirmed by investigation of at least 3 cryo-sectioned samples.

3.3.2.1 Light Green staining

The Light Green staining dye can be used to visualise the proteins in the samples by colouring them green. The structure of the Light Green is found in Figure 3.1. The acidic pH of the solution charges the sulphate groups negatively. They can interact with positively charged groups of proteins such as amino groups. To stain the sample with Light Green, the solution was added in minor amount with a dropper on the microscope slide. After 30 sec, the microscope slide was rinsed with water by putting the slide in a water holder for 10 sec. Finally, the microscope slide was dried at 40°C on a metal heating plate Leica multiplate 2208 (LKB, Bromma, Sweden) for 2 minutes.

Figure 3.1: Light Green staining dye. It stains proteins by interaction of negatively charged sulphate groups with positively charged groups.(51): http://www.sigmaaldrich.com/catalog/product/sial/l1886?lang=en&region=SE
3.3.2.2 **Lugol’s iodine staining**

The Lugol’s iodine solution can be used to visualise the starch and proteins, by colouring them purple/black and yellow/brown respectively. A combination of \( I_2 \) and \( \Gamma \) forms \( I_3^- \), which can react in his turn with the coil structure of the polysaccharide starch. The solution should be stored in the dark due to the possible conversion of \( \Gamma \) to \( I^0 \) caused by light.

To stain the starch, two methods were used. In the first method, the Lugol’s iodine solution was added in minor amount with a dropper to the dried microscope slide (unstained or stained with Light Green). The microscope slide was not rinsed afterwards. In the second method, a mixture of 1:1 Light Green and Lugol’s iodine solution was prepared and added in minor amount with a dropper to an unstained sample. The microscopic slide was rinsed with water after 30 sec by putting the slide in a water holder for 10 sec. In both methods, a cover glass (Menzel Gläser, Germany) was used to prevent evaporation of the iodine solution and damage of the microscopic lens. The excess of the solution was removed using a tissue. Finally, the cover glass was attached to the microscopic slide by using nail polish.

### 3.4 STRUCTURAL CHANGES AFTER ENDO-Xylanase TREATEMENT

#### 3.4.1 Sample preparations

The protocol was based on earlier investigated xylanase treatments with some adjustments \(^{[12,15]}\). The treatment was performed with unstained *Thermomyces lanuginosus* enzymes. Tests were taken for 24 hours at room temperature (RT) and at 60°C (Memmert UNB 100 oven (Memmert GmbH & Co, Schwabach, Germany)) in an environment of 25mM sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) (Sigma-Aldrich, St Louis USA or Alfa Aesar, Ward Hill, USA). Three enzyme concentrations were used: 1 Unit/mL, 10 Units/mL and 50 Units/mL. According to a xylanase activity of 2500 Units/g (specifications of enzyme), respectively 12 mg, 120 mg and 600 mg enzyme was dissolved in 30.0 mL HEPES buffer to obtain the desired concentrations. 2 small spoons of bran samples were submerged in 5.0 mL of each enzyme solution and stored at room temperature and at 60°C. Control samples were prepared, containing 2 small spoons of bran samples in 5.0 mL HEPES buffer, and stored at both temperatures. The room temperature samples were covered with a parafilm and protected from light using thin foil. The 60°C samples were covered with thin foil.
3.4.2 Microscopic investigation

The reaction was stopped by cryo-sectioning the samples as prescribed before. The samples were cut in 10 µm sections and dried for at least 3 hours at RT. At least 3 samples were investigated in unstained form with the 10x objective.

3.5 CLSM DETERMINATION OF THE MICROSTRUCTURE OF BRAN

Confocal laser scanning microscopy investigations and FRAP measurements were performed with a Leica TCS SP2 confocal microscope connected to a computer with the Leica Confocal Software (both Cellab Nordia AB, Sollentuna, Sweden). Parameter settings for images were: objective 10 x magnifications, zoom 4.00 or 8.00, scanning speed 400 Hz, mode xyz or xyt, format 1024*1024 pixels and line average 4.

For the autofluorescence, several samples were examined in unstained form using two lasers (Argon and Helium/Neon), both at a laser intensity of 25%. The Argon laser was used at two excitation wavelengths: \( \lambda_{ex} = 458 \text{ nm} \) (\( \lambda_{em} = 465 \text{ nm} - 520 \text{ nm} \)) and \( \lambda_{ex} = 476 \text{ nm} \) (\( \lambda_{em} = 485 \text{ nm} - 540 \text{ nm} \)); the Helium/Neon laser at \( \lambda_{ex} = 543 \text{ nm} \) (\( \lambda_{em} = 550 \text{ nm} - 630 \text{ nm} \)). Images were taken in a sequential mode (each wavelength separately) to prevent influence of autofluorescence emission caused by other wavelengths. No staining was used as literature provides several good images of grain samples stained with Acid Fuchsin and Calcofluor (Introduction 1.3), which visualise the distribution of proteins and β-glucans.

3.6 ENDO-XYLANASE ENZYME STAINING

As mentioned before, an endo-xylanase enzyme is able to break the xylan backbone of heteroxylans. Properties of the enzyme can be found in part 3.1 Samples and Apparatus. The stained enzyme will be used for visualisation of AX in the different bran samples and to determine the enzyme diffusion by FRAP measurements.

3.6.1 Enzyme staining

To visualise AX, an endo-xylanase enzyme from Thermomyces lanuginosus (Sigma-Aldrich, St Louis, USA) was stained with the isothiocyanate derivate of fluorescein (FITC. Sigma-Aldrich, St Louis, USA). FITC and fluorescein are both synthetic fluorescent pigments widely used as fluorophores. The aromatic structure contributes for the fluorescence
phenomenon. Absorption of energy from wavelengths between 475 nm to 495 nm causes a maximal emission between 510 nm and 520 nm. Fluorescein is photosensitive; each fluorescein solution is therefore protected from the light with thin foil (48). FITC can link to the amine groups of proteins with the formation of a thioamide-linkage.

9 mg FITC was dissolved in a 9.0 mL mixture of 6 mL dimethyl sulfoxide (Merck, Darmstadt, Germany) and 3 mL distilled water. 35 mg endo-xylanase enzyme was dissolved in 1.75 mL 25mM HEPES.

![Figure 3.2: Structure of Fluorescein isothiocyanate](image)

Both solutions were filtered with a 0.45 μm filter (CODAN, Lensahn, Germany). 450 μL of the filtered endo-xylanase solution were dissolved in 9.0 mL FITC-solution and stirred for 5 h at 4°C. The mixture was then dialysed in a dialysis tube (SpectrumLabs, Rancho Dominguez, USA) over 4 days against ultrapure water followed by 3 days against 25 mM sodium HEPES buffer. To stain the sample, 1 drop of the enzyme solution was added to a cryo-section sample. A cover glass was attached to the microscopic slide with nail polish to cover the sample. Investigation of the samples was performed using the same lasers with the same excitation and emissions spectra as for the determination of the autofluorescence.

3.6.2 Control of enzyme activity

Due to the staining protocol, the enzyme was stored for more than 96 h at 7°C-8°C. It is known that the enzyme loses his activity after 96 h at 60°C, but knowledge about storage at fridge temperatures was not available. Therefore, it was necessary to control if the enzyme maintained its activity. Hence 5 Kruskakli flakes were embedded in 300 μL of the stained enzyme solution in a small aluminium cup (volume 350 μL) and stored for 24 h at 60°C. Evaporation was prevented by using a thin foil envelope. After 24 hours, the samples were cryo-sectioned to stop the possible enzyme activity. Light microscopic investigation (10x objective) was used to visualise any degradation caused by the enzyme. Results were linked with the results of the structural changes after endo-xylanase treatment.
3.7 **FRAP MEASUREMENTS**

For FRAP measurements, an Argon laser of 488 nm was used whereby wavelengths between 500 nm and 550 nm were detected. The laser intensity before and after bleaching was set as low as possible (4.5%), to avoid bleaching during scanning periods. For bleaching, the intensity of the 488 nm laser was increased to its maximum. The 476 nm and 496 nm lasers were equally set maximally to boost the bleaching process. Measurements for bleaching were taken in ROI circle of 30 μm diameter. The circle was bleached for 4 seconds. Other parameter settings for FRAP measurements were: water objective 63x, zoom 2.00, scanning speed 800 Hz, beam expander 1 and format 256*256 pixels. Images were taken every 0.5 seconds, with a pixel size of 0.465030 μm and stored as 12 bit images.

3.7.1 **Reference measurements of the diffusion probe solutions**

In a first step, the diffusion coefficients of a sodium fluorescein probe solution (NaF), a 10 kDa FITC-dextran probe solution and the stained endo-xylanase enzyme ([protocol 3.3.3.2](#)) were calculated as reference measurements at different time points (0 hour, 1 hour and 24 hours) with storage at 40°C. FITC-dextran or fluorescein isothiocyanate dextran (Life Technologies, Carlsbad, USA) is as the name explains a derivate of fluorescein isothiocyanate. The dextran molecule is labeled with FITC. Dextran consists of α-1,6 linked glucans with α-1,3 branched glucans (branch not more than 3 residues) ([Figure 3.3](#)). Depending on the chain length of the linkage, the molecular weight can vary. In this protocol, a 10 kDa FITC-dextran was used as the molecular weight was situated between the NaF buffer (376.27 Da) and the endo-xylanase enzyme (22.5-25.5 kDa). The linkage (succinimidyl coupling) between FITC and dextran can happen between a random hydroxyl group of a glucan residue of the dextran chain and the isothiocyanate group of FITC ([49](http://www.sigmaaldrich.com/technical-documents/protocols/biology/fluorescein-isothiocyanate-dextran.htm)).

![Dextran and FITC](http://www.sigmaaldrich.com/technical-documents/protocols/biology/fluorescein-isothiocyanate-dextran.htm)

*Figure 3.3 FITC dextran ([49](http://www.sigmaaldrich.com/technical-documents/protocols/biology/fluorescein-isothiocyanate-dextran.htm))*
To obtain the 150 ppm NaF probe solution, 37.5 mg NaF salt (Sigma Aldrich, St Louis, USA.) was dissolved in 250 mL distilled water. To obtain the 150 ppm 10 kDa FITC-dextran probe solution, 3 mg 10 kDa FITC-dextran was dissolved in 20.0 mL distilled water.

For the determination of the reference measurements, two protocols were used. In the first one, 1 drop of the either NaF probe solution, the 10 kDa FITC-dextran probe solution or the stained endo-xylanase enzyme was investigated with FRAP in an adhesive spacer of 9 mm diameter and 0.12 mm depth (Secure-Seal™, Life Technologies, Carlsbad, USA) between two cover glasses, attached to each other with nail polish. Measurements for each solution were taken immediately after preparation at RT. For measurements after 1 h or 24 h storage at 40°C, 700 µL NaF probe solution, 10 kDa FITC-dextran probe solution or stained endo-xylanase enzyme was stored in closed glass vials of 5 mL (Hecht-Assistent, Sondheim, Germany) to avoid evaporation. After the desired time, samples were cooled down to RT and 1 drop of the solution was investigated in an adhesive spacer between two cover glasses, attached to each other with nail polish. For each sample, the average diffusion coefficient and the standard deviation were determined using the FRAP model for data evaluation as stated in the article of Jonasson et al (41).

### 3.7.2 Diffusion in bran

Diffusion coefficients of the NaF or 10 kDa FITC-dextran probe solutions in bran samples were determined immediately after contact, after storage for 1 h at 40°C and after 24 h at 40°C. For the immediate measurement, one flake was wetted either with NaF or 10 kDa FITC-dextran probe solution and investigated in an adhesive spacer between two attached cover glasses. For the longer time measurements, five flakes were wetted in a covered cup with 700 µL of the solutions and stored at 40°C. After the desired time, samples were cooled down to RT before one flake and one drop of the solution were investigated in an adhesive spacer between two cover glasses. To determine the enzyme diffusion in bran, the same protocols for the test samples were used as for the NaF or 10 kDa FITC-dextran probe solution diffusion in bran samples. The NaF or 10 kDa FITC-dextran probe solution was replaced by 700 µL fluorescently labelled endo-xylanase enzyme. Diffusion coefficients and standard deviations were calculated using the same protocol as for the reference measurements. SPSS was used for statistical analysis.
4 RESULTS

4.1 PARTICLE THICKNESS

Table 4.1a and b shows an overview of the average particle thickness of the samples Kruskakli (wheat) and Havrekli (oat). The Vetekli samples (wheat) were too thin (< 60 μm) to measure correctly. The Kruskakli particles had an average thickness of 139 μm with a standard deviation of 32 μm (Table 4.1a). The Havrekli had an average thickness of 488 μm with a standard deviation of 131 μm (Table 4.1b). Based on the analysis of the skewness with SPSS, the particle thickness of the Kruskakli sample did not have a normal distribution (skewness = 1.001, SE = 0.241). For the Havrekli sample, it could be assumed that the particle thickness was normally distributed (skewness = 0.141, SE = 0.241). Based on the 95% confidence intervals (calculated assumed that the samples were normally distributed), the particle thickness of the Kruskakli sample (95% interval: 130 μm; 147 μm) was significant different from the particle thickness of the Havrekli sample (95% interval: 462 μm; 515 μm).

### Table 4.1a: Particle thickness (μm) of Kruskakli sample (wheat); M: measurement. SD: standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
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<td>142</td>
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<tr>
<td>SD</td>
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<td>39</td>
<td>32</td>
</tr>
</tbody>
</table>

### Table 4.1b: Particle thickness (μm) of Havrekli sample (oat); M: measurement. SD: standard deviation.

<table>
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<td>148</td>
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4.2 LIGHT MICROSCOPIC DETERMINATION OF THE MICROSTRUCTURE

Pictures were taken for every bran layer in each examined stage (BFM, PLM and DIC unstained, BFM Light Green and/or Light Green/Lugol). Figures 4.1A – C give an overview of the cross section of the three different unstained samples seen with BFM, PLM and DIC: A for Kruskakli, B for Vetekli and C for Havrekli. Images selected were taken with 20x objective. The similarity in the general structure for both wheat samples (Kruskakli and Vetekli) could be seen immediately. The oat sample (Havrekli) on the other hand had obviously another general structure and contained a lot of starchy endosperm, marked on the PLM picture with the letter X. The block-like cells of each sample could be identified as the aleurone layer. The
intermediate layer and the pericarp were clearly visible in the wheat bran samples. In the oat sample, this part was either very thin or absent. In the polarised light form, it was mainly the cell walls that gave the signal. The DIC pictures are taken in for completeness, even if they did not give new information.

It was possible to distinguish the three different layers (pericarp, intermediate and aleurone) separately in the Kruskakli bran sample. The distinction was based on literature’s information about the cell structures (16). For the other samples, it was more difficult to find separated pericarp or intermediate layers. Figure 4.2A shows the pericarp layer of the Kruskakli in BFM, PLM and DIC form. Figure 4.2B shows the intermediate layer in the same way. The pericarp shows the expected elongated cells. Images are recorded perpendicular to the cross sections.

The different cell types of the intermediate layer (inner pericarp, testa or hyaline layer cells) could not be distinguished clearly as the thin layers could not be separated in most cases. Though, different cell structures could be seen in some samples, as in Figure 4.2B. Especially in the PLM image, there could be made a distinction between two cell types.

Figure 4.1: Cross section of Kruskakli (A), Vetekli (B) and Havrekli (C) bran samples seen with BFM, PLM and DIC respectively. X: endosperm of Havrekli sample
lying almost in right angle to each other (diagonally down from left upper corner and diagonally up from left lower corner).

Figure 4.2: A: Pericarp layer of Kruskakli sample in BFM, PLM and DIC form. B: Intermediate layer of Kruskakli sample in BFM, PLM and DIC form.

Figure 4.3 shows the aleurone layer of the different bran samples (A = Kruskakli, B = Vetekli, C = Havrekli). The images are taken perpendicular to the cross sections. In general, the cell structure of the three samples was quite similar in size (± 45 μm) and shape. In polarised light form, only the cell walls gave signal. Cell content could be seen in some cells.

Figure 4.3: Aleurone layer of Kruskakli (A), Vetekli (B) and Havrekli (C) in respectively BFM, PLM and DIC form.
For the further description of the light microscopic investigation after staining, we mainly focused on the cross sections and the aleurone layer. Staining of the pericarp and the intermediate layer with Light Green and Lugol’s iodine solution did not give remarkable results. Light Green and Lugol’s iodine staining pictures of the cross sections can be found in Figure 4.4 for respectively Kruskakli (A), Vetekli (B) and Havrekli (C). The proteins are coloured green; the starch is coloured purple to black. Picture C confirms another time the presence of a high quantity of endosperm in the Havrekli sample.

![Figure 4.4: Cross sections of Kruskakli (A), Vetekli (B) and Havrekli (C) after staining with Light Green and Lugol’s iodine. Proteins are coloured green; starch is coloured purple to black.](image)

Figure 4.5A shows the aleurone layer of the Kruskakli sample after staining with Light Green. Proteins are coloured green. Figure 4.5B presents the aleurone layer of the Kruskakli sample after staining with Lugol’s iodine. Starch is coloured black; proteins are coloured yellow to brown. Figure 4.5C shows the aleurone layer of the Kruskakli sample after staining with Light Green and Lugol’s iodine. Proteins are coloured green (as Light Green overcomes the binding of Lugol’s iodine), starch is coloured black.

![Figure 4.5: Aleurone layer Kruskakli after staining with respectively Light green (A), Lugol’s iodine (B) and Light Green/Lugol’s iodine (C). Proteins are coloured green with light green, yellow to brown with Lugol’s iodine. Starch is coloured black with Lugol’s iodine.](image)

4.3 **STRUCTURAL CHANGES AFTER ENDO-XYLANASE TREATMENT**

For interpreting the results, a distinction has to be made between the wheat and the oat bran samples. For wheat bran, pictures are shown for the Vetekli sample, as Kruskakli bran gave similar results. Estimations are based on cross sections, as separated
layers could not be found. Figure 4.6 shows the cross sections of the two control samples (A: RT; C: 60°C) and the two bran samples treated with the lowest endo-xylanase concentration of 1 Unit/mL (B: RT; D: 60°C). No structural changes could be observed between the control and test samples after 24 hours of treatment. In each sample, the aleurone layer was preserved almost totally.

Figure 4.6: Cross sections of Vetekli as seen in BFM after 24 hours of treatment (left: control, right: 1 Unit/ml). A: control sample at RT; B: test sample at RT; C: control sample at 60°C and D: test sample at 60°C.

Figure 4.7 shows the samples after 24 hours of endo-xylanase treatment with a concentration of 10 Units/mL (A and C) and 50 Units/mL (B and D) at room temperature and 60°C. For the samples treated with 10 Units/mL endo-xylanase enzymes, the aleurone layer is mostly partly degraded or absent.

Figure 4.7: Cross sections of the Vetekli as seen in BFM after 24 hours of endo-xylanase treatment (left: 10 Units/mL , right: 50 Units/mL). A: test sample 10 Units/mL at RT; B: test sample 50 Units/mL at RT; C: test sample 10 Units/mL at 60°C and D: test sample 50 Units/mL at 60°C.
The degradation at 60°C is mainly further progressed, as it is especially in these samples that the aleurone layer is completely disappeared (Figure 4.7C). For the highest enzyme concentration of 50 Units/mL, the aleurone layer cannot be found anymore and is completely degraded (Figure 4.7B and D). Additionally, the pericarp layer can be detached from the testa and the hyaline layer (Figure 4.7D).

To interpret the oat bran samples after 24 hours of endo-xylanase treatment, a distinction has to be made between room temperature and 60°C storage. As regards the room temperature samples, no structural changes could be seen between the untreated control samples (A1) and the test samples treated with 1 Unit/mL (A2) or 10 Units/mL enzymes (A3). No cross sections could be found in the samples treated with 50 Units/mL enzymes, suggesting a total degradation. Storage at 60°C visually changed the endosperm, which looked less dense (Figure 4.8B). Degradation of cross sections could be seen for the samples treated with 10 Units/mL (B3) and 50 Units/mL endo-xylanase enzymes. The latter one suggested even total degradation. As the pericarp layer is very thin or absent and difficult to identify, no statements can be made about the presence of this layer.

![Figure 4.8: Cross sections of Havrekli control sample (1) and test samples treated with 1 Unit/mL (2) and with 10 Units/mL (3) endo-xylanase enzyme as seen in BFM after 24 hours of treatment at room temperature (A) and 60 °C (B).](image)

4.4 CLSM DETERMINATION OF THE MICROSTRUCTURE OF BRAN

Autofluorescence of the three different samples (Kruskakli = A, Vetekli = B and Havrekli = C) was recorded for the cross sections (Figure 4.9). Emission (465 nm - 520 nm) recorded after excitation with $\lambda_{exc} = 458$ nm was shown in green, in red after $\lambda_{exc} = 476$ nm
(emission 485 nm - 540 nm) and in yellow after excitation $\lambda_{exc} = 543$ nm (emission 550 nm - 650 nm). The emission spectra were overlaid as they were separately obtained.

![CLSM Image](image)

Figure 4.9: Autofluorescence of Kruskakli (A), Vetekli (B) and Havrekli (C) cross sections. Green: $\lambda_{exc} = 458$ nm, $\lambda_{em} = 465$ nm - 520 nm. Red: $\lambda_{exc} = 476$ nm, $\lambda_{em} = 485$ nm - 540 nm. Yellow: $\lambda_{exc} = 543$ nm, $\lambda_{em} = 550$ nm - 650 nm.

The difference in cross section structure between the wheat bran samples and the oat bran sample (thinner or absent pericarp) observed with BFM can be visualised again in CLSM. In Figure 4.9B, the glue seems to give a fluorescence signal. On the other hand, this observation cannot be seen in the other pictures. Experimental investigation suggested that the glue was autofluorescent upon excitation with a 488 nm laser and emission uptake > 500 nm, an unexpected observation.

4.5 ENDO-XYLANASE ENZYME STAINING

4.5.1 Enzyme staining

The signal caused by attachment of the endo-xylanase enzyme to the arabinoxylans could not be distinguished from the autofluorescence of the bran samples. Figure 4.10 gives the fluorescence of the Kruskakli sample after endo-xylanase staining. Same excitation and emission wavelength areas were used as for the autofluorescence investigation. As autofluorescence is present over the whole wavelength spectrum between 465 nm and 600 nm, it was not possible to find a wavelength where the endo-xylanase signal was distinguishable.

![CLSM Image](image)

Figure 4.10: Endo-xylanase staining of Kruskakli sample Green: $\lambda_{exc} = 458$ nm, $\lambda_{em} = 465$ nm - 520 nm. Red: $\lambda_{exc} = 476$ nm, $\lambda_{em} = 485$ nm - 540 nm. Yellow: $\lambda_{exc} = 543$ nm, $\lambda_{em} = 550$ nm - 650 nm.
4.5.2 Control of the enzyme activity

After 24 hours of treatment with the stained endo-xylanase enzyme, it was possible to find unaffected (A), partly degraded (B) and totally degraded (C) aleurone layers in the cross sections of the Kruskakli bran sample (Figure 4.11). It can be suggested that the enzyme is still active.

![Figure 4.11: Unaffected (A), partly degraded (B) and totally degraded aleurone layers (C) in cross sections of the Kruskakli sample after 24 hours of treatment with the stained endo-xylanase enzyme.]

4.6 FRAP MEASUREMENTS

FRAP measurements were carried out on three typical diffusion probes, one of them being an endo-xylanase enzyme. The endo-xylanase enzyme was chosen to show the feasibility on a typical enzyme; the two other probes (NaF and 10 kDa FITC-dextran) were chosen in order to correlate the measurements on the enzyme with non-enzymatic probes.

Figure 4.12 shows images of the FRAP measurement process on sodium fluorescein in absence of bran at different times. The orange sphere (left under) in the curves is an artefact of the lens system, which was confirmed to have no influence on the measurement.

![Figure 4.12: FRAP measurement of sodium fluorescein in absence of bran. A: prebleaching image. B: image immediately after bleaching. C: image 5 seconds after bleaching. D: image 20 seconds after bleaching. The fluorescence recovers totally.]

Figure 4.13 shows the diffusion coefficients in absence of bran for the three probe solution: NaF (blue), 10 kDa FITC-dextran (orange) and endo-xylanase enzyme (red). These diffusion coefficients values will be used as reference measurements.
Figure 4.1: Diffusion coefficients of three probe solutions NaF (blue), 10 kDa FITC-dextran (orange) and the stained endo-xylanase enzyme (red) in absence of bran. Error bars are displaying the standard deviation.

Average diffusion coefficients for the NaF probe, the 10 kDa FITC-dextran probe and the endo-xylanase enzyme over the different times were respectively 3.61e-10 m²/s (SD: 1.10e-11 m²/s), 7.04e-11 m²/s (SD: 3.49e-12m²/s) and 2.83e-10 m²/s (SD: 2.87e-11m²/s). For statistical analysis, the Wilcoxon signed-rank test was preformed, as the samples were paired and not normally distributed (too less samples). The diffusion coefficient for NaF immediately after sample preparation was significantly different from the NaF diffusion coefficients of the other time parameters based on a rejection value of p < 0.05 (1 h: p = 0.002; 24 h: p = 0.039). Diffusion rates after 1 hour and after 24 hour were not significantly different for the NaF probe (p = 0.972). For the 10 kDa-FITC dextran, the diffusion coefficients of each time parameter were significantly different from the other times (0 h-1 h: p = 0.002; 0 h-24 h: p = 0.001; 1 h-24 h: p = 0.001). Diffusion rates for the endo-xylanase enzyme were not significantly differing over time (0 h-1 h: p = 0.799; 0 h-24 h: 0.386; 1 h-24 h: 0.087). An explanation for the possible differences over time is given in the discussion.

Based on the average diffusion coefficients over the different times, it was possible to calculate the hydrodynamic radius by using the Stokes-Einstein relation (equation 1.4) for a room temperature of 20°C (Figure 4.14). To use the equation, we need to assume that our probes are spherical particles. As expected on molecular weight basis, NaF gives the smallest radius (0.6 nm). The 10 kDa FITC-dextran shows the highest radius (2.8 nm) despite is lower molecular weight compared to the endo-xylanase enzyme. This latter has a molecular weight between 22.5 kDa and 25.5 kDa and an estimated hydrodynamic radius of 0.7 nm.
Figure 4.14: Hydrodynamic radius of NaF, 10 kDa FITC-dextran and endo-xylanase enzyme calculated with the Stokes-Einstein equation 20°C as room temperature.

Further, FRAP measurements of the three different probes were carried out in bran flakes. The absolute values for the diffusion coefficients of the three probes are presented in Figure 4.15 (NaF probe = blue, 10 kDa FITC-dextran = orange, enzyme = red). Results for diffusion coefficients in bran are based on measurements with the Kruskakli sample only as measurements in bran were challenging and gave too much variation in the other samples (RSD: >80). Relatively high standard variations were in the Kruskakli sample only found for the measurements of NaF diffusion in bran, as can be seen in the graph.

Figure 4.15: Diffusion coefficients of three probe solutions NaF (blue), 10 kDa FITC-dextran (orange) and the stained endo-xylanase enzyme (red) in bran (Kruskakli sample). Insert: Enlarged results for endo-xylanase enzyme diffusion in bran. Error bars are displaying the standard deviation.

As regards the diffusion of NaF in bran, the diffusion rate immediately after sample preparation is approximately 24.8% of the reference measurement. After 1 hour or 24 hours,
the diffusion rates are respectively 6.2% and 13.2%. The coefficients are significantly different based on the Wilcoxon signed-rank test (0 h-1 h: p = 0.001; 0 h-24 h: p = 0.012; 1 h-24 h: p = 0.003). Based on the recovery curves, it could be suggested that a fraction of the probe solution was immobilised after 1 hour or 24 hours of contact as the fluorescence intensity does not recover totally (Figure 4.16 left). This observation is taken in to discussion later on. Other measurements revealed total recovery (Figure 4.16 right).

For the diffusion of the 10 kDa FITC-dextran, it can be estimated that the hindrance immediately after sample preparation, after 1 hour or after 24 hours is comparable as diffusion rates in bran are respectively 45.3%, 50.5% and 53.7% of the diffusion rates in absence of bran. The Wilcoxon test confirms that the diffusion coefficients are not significantly different (0 h-1 h: p = 0.169; 0 h-24 h: p = 0.062; 1 h-24 h: p = 0.386). The recovery curves suggested no immobile fractions, as the fluorescence intensity recovered almost completely (Figure 4.17).

Figure 4.16: Example of the fluorescence recovery curves of NaF in bran after 1 hour contact. Similar curves were found for measurements after 24 hours of contact. Left: partly immobile NaF; right: mobile NaF.

Figure 4.17: Example of the fluorescence recovery curves of 10 kDa FITC-dextran in bran immediately after sample preparation. Similar curves were found for measurements after 1 hour and after 24 hours of contact.

The diffusion rates of the endo-xylanase enzyme in bran are lowered to respectively 1.1%, 0.8% and 2.6% of the diffusion rates in absence of bran (Figure 4.15). Based on the Wilcoxon test, diffusion coefficients immediately after sample preparation and after 1 hour differed not significantly (p-value = 0.139). After 24 hours of storage at 40°C, the enzyme moved significantly faster compared to the other time parameters (0 hours: p-value =0.008 and 1 hour: p-value = 0.003), as visually could be suggested based on the insert in Figure
4.14. Considering the fluorescence recovery curves of the enzyme probe, it was suggested that the enzyme was partly bound to the bran structure (immobile fraction, left curve) and partly free moving (mobile fraction, right curve)(Figure 4.18).

To evaluate the retardation of the three probes in bran, we compared the mobility in bran to the mobility in absence of bran. The normalised diffusion coefficients $D/D_0$ are shown in Figure 4.19. $D/D_0$ values were calculated based on the individual diffusion coefficients of Figure 4.13 and Figure 4.15. Error bars are not shown as the variation on the results is shown in the not normalised graphs.

Figure 4.18: Example of the fluorescence recovery curves of the stained endo-xylanase enzyme in bran after 1 hour. Similar curves were found for measurements immediately after sample preparation and after 24 hours of contact. Left = immobilised enzyme; right: mobile enzyme.

Figure 4.19: Relative diffusion coefficients ($D/D_0$) for NaF (blue), 10 kDa FITC-dextran (orange) and the stained endo-xylanase enzyme (red). Error bars are represented in Figure 4.13 and Figure 4.15.

In general, it can be stated that the diffusion in bran is slower compared to the diffusion in absence of bran. This observation was expected, as the presence of (ordered) structures hinders the probes’ mobility. It can be stated that the enzyme is hindered the most by the bran compared to the other probes. Comparing the retardation of the NaF probe and the 10 kDa FITC-dextran probe, an unexpected observation is found when thinking of the probe radius. 10 kDa FITC-dextran is less hindered despite its higher radius. Possible explanations are taken into the discussion.
5 DISCUSSION

At first, it needs to be mentioned that we consciously choose to not pre-treat our samples before analysis (except cryo-sectioning), as some pre-treatment procedures can change the composition of the sample. As example, we can mention the destarching of bran which is used in some papers (1,12,15). Destarching would improve the investigations of some parts of the project, especially for the oat sample. Nevertheless, we opted to analyse the sample as rough as they were, to hold the difference between research and interpretation of the results for daily use as small as possible.

5.1 PARTICLE THICKNESS

It needs to be mentioned that we investigated the particle thickness as measurement of the height of the sample and not of the width, as we are particularly interested vertically in the different layers of our samples. In literature, the particle size is often determined by sieving (4,7,26) or with laser scattering particle size analysers (3,6,8,19,26). These techniques measure the size uncontrollable in both directions. Furthermore, sieving can reduce the particles thickness and the structures, especially of the pericarp layer, can be affected by the sieving movement. Cell walls can be broken open (5).

Based on literature, we would suggest that the oat bran samples are thinner than wheat bran samples as the pericarp is estimated to be thinner, the testa is single layered instead of multi-layered and the hyaline layer is absent in oat (16,24,25). Yet, our oat bran samples are significantly thicker than the wheat bran samples. The difference in the particle thickness can be explained in several ways. As first, there are different cereal varies and varieties, which estimate commonly different properties (16,20). This can maybe explain the difference between the two wheat bran samples.

A second possible explanation is the milling process; different milling process parameters can give rise to different particle thicknesses (4,26). The separation between the endosperm and the bran fraction can be influenced and limited. Thicker particles can suggest the presence of more endosperm. Microscopy suggested even more that the Havrekli samples contained more endosperm compared to the wheat bran samples. Additionally, the composition of the bran samples showed that the Havrekli sample contained remarkably more carbohydrates (41 g/100 g vs 15 g - 23g /100 g for the wheat...
samples, Table 3.1) and a lower concentration of fibers (18 g/100 g vs 40 g – 50 g/100 g). If we assume that the carbohydrate fraction consists of starch and dietary fibers, we would suggest a higher amount of starch in the oat sample. This would confirm our hypothesis about a less efficient separation between starchy endosperm and bran considering the oat sample. A third explanation is the presence of curved samples, who give wrongly the impression of thicker samples. Especially for the oat samples, curved samples were present.

As mentioned before, the Vetekli samples were too thin to measure correctly. It was practically impossible to separate one flake. The particle thickness of such a small samples can be determined by the use of laser scattering particle size analysers as this technique will give the most accurate results.

5.2 LIGHT MICROSCOPIC DETERMINATION OF THE MICROSTRUCTURE OF BRAN

The determination of the microstructure was mainly based on literature, by comparing the obtained micrographs with the prescribed composition of bran. The pericarp cells were elongated (1,16); the aleurone cells had a block-like structure which are more rectangular in wheat and elongated in oat (22). Some reflections can be made regarding the LM results. Firstly, when investigating the images of the aleurone layer, we notice that some aleurone cells still contain cell content. The empty category is possibly caused by the milling process. Furthermore we can see that only the (aleurone) cell walls give a signal in PLM. This can be explained by the presence of highly ordered structures in the cell walls, such as AX. The presence or absence of cell content has no influence on the signal, suggesting the lack of ordered structures as cell content. The pericarp was absent or very thin in oat, caused by different milling parameters and pretreatment whereby the pericarp is removed.

Additionally, the distinction between the different cell types in the intermediate layer as mentioned in literature could not be made (1,16). Sometimes it was possible to see different cell structures (Figure 4.2B PLM) but in most cases, distinctions were unrealisable. The impossibility to prepare sections of the different inner pericarp cells can explain this observation, even though our sections were thinner than the estimated thickness of the inner pericarp layers. The reason therefore remains unclear, but uneven flakes are the most likely explanation. Handpeeling can overcome this problem (see further). For oat samples, it
was likewise not possible to distinguish the different cell types and the different outer layers of bran as stated in literature (16).

Thirdly, there are some remarks considering the Light Green and Lugol’s iodine staining. As mentioned, staining of the pericarp and intermediate layers did not give remarkable results. This can be explained by the expected absence of starchy endosperm and the smaller amount of proteins present (5,13,22,26). As regards the aleurone layer, a difference between the cell content and the cell walls was distinguished. The Light Green signal in the cell was stronger than in the cell walls, suggesting a higher amount of proteins as cell content (Figure 4.4). This observation is analogous as prescribed in literature (14,20,22). Both Figure 4.5B and 4.5C confirm this phenomenon. As there is no difference between the cell content and cell walls in Figure 4.5A, it is suggested that the cells are empty and the excess of the Light Green is not enough washed away.

5.3 STRUCTURAL CHANGES AFTER ENDO-XylanASE TREATMENT

Firstly, it has to be mentioned that due to the water environment for the xylanase treatment, the bran samples were swollen. Additionally, it was not possible to select separated flakes to make sections as the wet bran samples formed a mush. Results were therefore based on cross sections as it was not possible to find separated aleurone, intermediate or pericarp layers after sectioning.

As mentioned before, the protocol for the endo-xylanase treatment was based on papers of Benamrouche et al. and Beaugrand et al where an enzyme concentration of 10 Units/mL and a temperature of 60 °C were used. The same parameters were confirmed despite the differences in the followed protocol, such as stirring and treatment stop by centrifugation (12,15). In our experiment, an enzyme concentration of 10 Units/mL delivers the best results for further investigation as well. Furthermore, the temperature parameter was confirmed: samples treated at 60°C were more destroyed compared to room temperature samples. This can be explained by the properties of our enzyme, which has an optimal temperature for activity between 60°C and 70°C (28).

Comparing the results with earlier endo-xylanase treatment, some analogy and difference in results can be noticed. A total disorganisation of the aleurone layer after enzyme treatment as mentioned by Benamrouche et al could be partly confirmed as it was
still possible to find some aleurone layers that were only partly degraded (12). Van Craeyveld et al. noticed a separation between the aleurone layer and the nucellus layer after xylanase treatment and a preservation of the aleurone layer (11). This latter observation was not seen by Benamrouche et al, but Van Craeyveld et al. explained this difference by different detection methods. They used Calcofluor staining to visualise β-glucan in the cell walls instead of the autofluorescence of the ferulic acids-carbohydrate complex used by Benamrouche et al. As we used LM investigation for analysing, these difference in detection will not affected our conclusions.

A possible explanation for the difference with Benamrouche et al. can be found in the different protocol used. Benamrouche et al. samples were stirred during treatment which made the contact between the enzyme and the bran sample more optimal. It is suggested that stirring in our protocol would provide analogous results as earlier investigations. A possible explanation for the difference with Van Craeyveld et al. can maybe be found in the use of different cereal varieties.

In opposition, analogous results were found for the pericarp. It remained unaffected after treatment with an enzyme concentration of 10 Units/mL (12). An explanation of the unaffected pericarp, despite the presence of branched heteroxylans (1,3,5,6,13,17,22), can be found in the Arabinose:Xylose ratio. The A:X ratio of the pericarp is remarkably higher than in the aleurone (5,11–13,19,21,23,24). This means that the amount of arabinose related to the xylose residues is higher, causing more steric hinder for the enzyme to move. Furthermore, the enzyme is possibly not able to bind to the xylose residues as the amount of non-substituted residues is too low. Benamrouche et al. suggested as well the necessity of a high A:X ratio for endo-xylanase treatment (12). Pre-treatment with arabinofuranosidase can maybe influence the A:X ratio positively (18).

When combining the use of a xylanase and esterase enzyme, more degradation is suggested. The esterase enzyme is able to break the linkage between phenolic acids and arabinoxylan as well as the linkage between two arabinoxylan chains. Consequently, the xylanase enzyme has more possibilities to breakdown the xylose backbone (14).

Additionally, it would be interesting to focus a bit more on the difference between the wheat and the oat samples. As previously mentioned, oat storage at 60°C visually
changed the endosperm. During heat treatment in a water environment, the process of starch gelatinisation takes place. In this process, the crystalline structure of starch is disordered as the amylopectin chains start to take up water and the structure swells (47). It can be stated that at 60°C the process is ongoing as start temperatures for starch gelatinisation are assumed to be 45°C for both wheat and oat cereals (45,46). As visualised before, our oat sample contained more endosperm compared to the wheat samples. The change in endosperm structure is therefore suggested to be more visually noticeable. A comparison with the paper of Benamrouche et al. or Beaugrand et al. cannot be made as they used starch depleted bran (12,15).

5.4 CLSM DETERMINATION OF THE MICROSTRUCTURE OF BRAN

As regards the CLSM results, some considerations can be taken in. Firstly, the existence of autofluorescence can be explained by the presence of different autofluorescent molecules: ferulic acids, lignin and phenolic acids. The first is present in all the different bran layers, the latter ones are present in the pericarp and the intermediate layer (5,12,13,20,22,24,26). As different signals are detected for different excitation wavelengths, it can be suggested that this is caused by different ferulic acids. The autofluorescence signal of the aleurone layer is suggested to be caused by (di)ferulic acids, present in the aleurone cell walls, or by autofluorescence granules present in the protein matrix of the cell content (5,16,20,22,24).

Compared to the autofluorescence pictures of the different cell layers presented by Antoine C. et al, it was not possible to distinguish the three-cell thickness of the pericarp in wheat bran samples (1). Possible explanation is the difference in wheat cultivars used for investigation or the impossibility to create separated pericarp layers in cross section position. Additionally, it was not possible to detect the difference in autofluorescence between the anticlinal (between two aleurone cells) and periclinal (between aleurone and endosperm) cell walls of the aleurone layer, as described by Jääskeläinen et al (22).

A few improvements can be suggested to optimise the CLSM determination. Firstly, the autofluorescence of the glue remains a problem that we could not solve. The reason therefore is still unclear and literature research did not give any explanations. It is suggested to use a different glue or sample preparation (for example fixation steps) to solve this problem. The cryo-sectioning part of the sample preparation can be tested with more
parameters to find out if this can explain the glue autofluorescence and even more important if it is possible to avoid it. Additionally it seems that hand peeled separation and manual dissection of the different layers promotes a more complete investigation of the samples (1,3,5,12,21,25,26) as the influence of milling and/or different milling parameters is avoided. There is also a better control over the amount of endosperm present in the sample. Especially for the Havrekli sample, this would be an enormous improvement.

5.5 ENDO-XYLANASE ENZYME STAINING

5.5.1 Enzyme staining

As mentioned, it was not possible to distinguish the autofluorescence and the fluorescence caused by the stained endo-xylanase enzyme. Several explanations are possible. Firstly, the protocol followed was based on the article of Videcoq P. et al, where they stained pectin methylesterases (47). Not only the type of enzyme, but also the enzyme concentration (1 mg/mL instead of 15 mg/mL) was an adjustment made in our protocol. Possibly, the signal from the fluorescent enzyme was weaker than the autofluorescence signal. This phenomenon was also seen by Dornez et al and Jääskeläinen et al, whereby the autofluorescence of the pericarp the Acid Fuchsin/Calcofluor staining overcame (19,22).

Compared to the articles using endo-xylanase staining, a different sample preparation and staining technique could explain the difference in result (19,24). In the papers, they milled the samples themselves, which provide a better control over the milling process. Furthermore, it is not known whether or not the used fixations steps (paraformaldehyde, glutaraldehyde and agar), the Historesin Embedding kit (Leica Microsystems, Bensheim, Germany) and the formation of bran pellets influence the sample preparation positively. Possibly it affects the autofluorescence signal of the sample, but this hypothesis could not be confirmed as it was not possible to obtain bran pellets ourselves.

Additionally, we need to think about the different fluorescently label used to stain the enzyme. Our enzyme was stained with FITC instead of the Alexa Fluor488 C₅ maleimide dye used in the articles of Dornez et al. (Invitrogen Carlsbad, CA) (19,24). It is believed that this dye exhibit more intense fluorescence signal, which can reduce the interference of the autofluorescence. Furthermore, there is the difference of using an inactive endo-xylanase enzyme (papers) or an active endo-xylanase enzyme (4.5.2 Control of the enzyme activity).
Dornez et al opted to use the inactive form as it gave a stronger signal after staining for 24 hours due to the fact that the active was able to break down the AX and thereby reducing its own substrate. As our staining time was much shorter (couple of minutes), the influence of the enzyme activity is limited. At last, we may not forget to notice that it was impossible to define the binding position (i.e. the amount of xylose residues needed to bind) of our endo-xylanase enzyme. This factor strongly influences the success rate of the method.

Several improvements can be suggested, based on knowledge about the binding of the enzyme to AX and on the possibilities to banish the autofluorescence signals. Unfortunately we were not able to investigate them due to a lack of time and materials. In ideal conditions, it would be the best to control the production of the endo-xylanase enzyme by a defined bacteria type ourselves, whereby knowledge of the enzyme properties and binding capacity is available. One possibility is the use of the *Bacillus subtilis* xylanase (XBS), the enzyme used by Dornez et al (19,24). In addition, the different staining methods need to be tested and the sample preparation needs to be optimised to lose the autofluorescence signal. The influence of the fixation steps and the milling process can be investigated. Different staining parameters (time and concentration) as suggested in the papers by Dornez et al. can be tested (19,24). Finally, the possibility to avoid the glue autofluorescence would improve the investigation.

### 5.5.2 Control of the enzyme activity

To compare the results of the control of the enzyme activity test with the results of the structural changes after endo-xylanase treatment (4.3), a first notice needs to be made concerning the enzyme concentration. It was calculated that the enzyme concentration of our stained enzyme is $\geq 2.5$ Units/mL. In opposition, the concentrations used for the endo-xylanase treatment were 1 Unit/mL, 10 Units/mL and 50 Units/mL. The results are therefore expected between no degradation (1 Unit/mL) and partial degradation (10 Units/mL).

As we found a combination of non-degraded, partly degraded and totally degraded samples, we conclude our enzyme is still active. Observations are not caused by cryo-sectioning as similar structures were not found in untreated samples (4.2 Light Microscopic determination of the microstructure of bran). The difference in degree of degradation could be explained by the amount of enzyme available. As only 300 μL (0.75 Units) of enzyme was
used instead of 5 mL (5-50 Units) for endo-xylanase treatment, the amount of enzyme available per flake is lesser in this protocol. This influences probably the contact between the enzyme and the structure. Some (places in the) flakes were less in contact with the enzyme causing no degradation, while others were totally degraded after full contact.

5.6 FRAP MEASUREMENTS

Firstly, it needs to be explained why the samples were stored at 40°C. It is known that the enzyme is the most active between 60°C and 70°C (28). But storage at 60°C can induce starch gelatinisation which makes investigations more difficult. Consequently, we decide to store at 40°C as it suggested that starch gelatinisation would have no influence (starch gelatinisation starts at 45°C (45,46)) and the enzyme activity would be higher than at room temperature.

As mentioned before, results are only shown for the Kruskakli sample as measurements on the other samples were practically very demanding and yielded data of high variation. The high standard deviation of the Vetekli sample can be explained by the small particle size in width. Vetekli particles had a width < 1 mm (compared to the approximately 4 mm wide Kruskakli and Havrekli samples) and were very heterogeneous in size and shape. This made measurements in the sample practically impossible, explaining a very high standard deviation. Regarding the oat samples, particle swelling impeded good sample preparations as the particles were too thick to fit properly between the two cover glasses. Possible explanation for the more pronounced swelling of the oat samples could be the higher amount of starchy endosperm present. As it was not possible to destarch the bran or to control the milling process in order to obtain a higher particle width, these suggestions could not be examined. Absolute conclusions can therefore not be made regarding these two samples. Further assumptions about the diffusion in bran are thus based on measurements in present of the Kruskakli sample.

In absence of bran (Figure 4.13), we would have expected no differences in the diffusion coefficients over time, except of measurement variation. Yet, we saw that for the sodium fluorescein and the 10 kDa FITC-dextran probe, diffusion coefficients sometimes differed significantly. The only reason here for can be found in temperature influence, as other environmental factors of our samples did not change. As each sample was treated in
the same way, we suggest that some samples after 1 hour or 24 hours of storage did not cool down enough before the FRAP measurement. This can suggest a faster movement of the probes. It is therefore recommended to retake the measurements several times more with more attention for the temperature of the sample before measuring.

When comparing the diffusion coefficients of the different probes in absence of bran, we need to take in the molecular weight and the molecular structure/the hydrodynamic radius of the probes (Figure 4.14). NaF has the lowest molecular weight, the smallest radius and the highest diffusion coefficient, which was an expected observation. More interesting is it to compare the 10 kDa FITC-dextran and the endo-xylanase enzyme. As the enzyme has almost a double weight, it would be expected that it moves slower (lower diffusion coefficient). Yet, the 10 kDa FITC-dextran was the slowest probe and had consequently the highest radius. Therefore, the difference between the 10 kDa FITC-dextran and the endo-xylanase enzyme cannot be found in the difference in molecular weight but in a difference in molecular structure. The 10 kDa FITC-dextran is a branched polymer and has additionally a more spread out structure (higher radius) compared to the enzyme that is suggested to be more compact into a global structure. Information about the radius of the endo-xylanase enzyme was not available in literature.

Regarding the measured diffusion coefficients in bran (Figure 4.15), it is necessary to discuss some factors that can influence the variation in results. As stated for the reference measurements, temperature can play an influence. Further control is recommended. Additionally, the specific place in the bran sample where the measurement is taken can influence the diffusion coefficient. It is suggested that measurements closer to the border of the sample give higher diffusion coefficients as the inward diffusion of the fluorophore is less limited by the bran structure. Furthermore, the probe can be influenced differently (i.e. enzyme binding) depending on the place in the sample. Specific measurements in the bran sample could confirm this hypothesis but further optimisation of the measurement is necessary before drawing conclusions.

No changes of mobility for both the 10 kDa FITC-dextran and the NaF probes in bran would be expected, as no degradation of the bran structure takes place. Yet, it was seen that the diffusion rates of the NaF changed significantly over time. Furthermore, we expected
based on the hydrodynamic radius (Figure 4.14) that the NaF probe is the least hindered in the bran sample, as smaller molecules are believed to find easier their way in ordered structure. Though, the retardation of the NaF probe in bran is relatively higher compared to the retardation of the 10 kDa FITC-dextran probe (Figure 4.19).

Some hypothesis to explain these observations are set up. Firstly, it is suggested that the system needs some time (max 1 h) to equilibrate, for the NaF being well penetrated and dispersed in the bran. Diffusion coefficients immediately after sample preparation can therefore be higher than after 1 or 24 hours. Additionally, the negative charge of the NaF probe can have an influence. The negative charge can be found in the O-atom, bound with an ionic binding to Na+. Repulsion caused by other negative charges in the bran sample is possible as well. These interaction slows down the probe and/or creates an immobile fraction, seen in recovery curve Figure 4.16. The 10 kDa FITC-dextran is expected to have smaller charge which limits the mobility less. At last, temperature influences can create variation.

For the enzyme diffusion in bran, a significant difference can be found between diffusion immediately after sample preparation or after 1 hour and diffusion after 24 hours. This latter type is significantly faster. A possible explanation for the limited diffusion in the first hours, can be found in the ability of the enzyme to attach to the bran structure, thereby limiting its own movement and possibly the movement of surrounding enzymes. To explain the difference between 1 hour and 24 hours, two suggestions are possible. Firstly, it is suggested that the enzyme detaches again of the arabinoxylan backbone after a certain time, which makes diffusion possible again and results in a higher diffusion rate.

Furthermore, as our enzyme was still active (4.5.2 Control of the enzyme activity), it is feasible that the bran matrix is broken open by degradation of AX in the aleurone cell walls. This makes enzyme diffusion easier as the molecule is less hindered by a now more open structure. Cuyvers et al. (30) suggested this hypothesis as well, by comparing enzyme diffusion of an inactive and an active endo-xylanase enzyme. A second explanation is also given by Cuyvers et al. As almost every binding site is occupied, the molecule cannot attach to the arabinoxylan backbone and needs to move further, however it is unlikely that all available binding sites did not get occupied within the first hour. Instead it is more likely that
even less binding sites are available, due to the breakdown of the structure and hence a higher mobility is due to a change towards a more open structure. The enzyme binding could be elaborated in more detail with a FRAP and binding model, as used by Cuyvers et al, but this goes beyond the scope of this work.

5.7 FURTHER PROSPECTIVES

Research about bran, the position of arabinoxylans and enzymatic treatments to optimise the bioavailability is today incontestable. Some further prospectives about these topics are summarised.

Firstly, the arabinoxylans remain an interesting compound with recent research about enzymatic treatment to increase the bioavailability (5-8,12-15). Furthermore research to define the position of AX is still ongoing with for example the use of inactive or mutant fluorescently labelled enzymes for localization by Dornez et al (19,24). Additionally, the use of immunolabeling is recently further developed by Hell et al (14). Influence of autofluorescence was limited as the used antibody had a very high emission spectrum (excitation $\lambda = 633$ nm; emission $\lambda = 650$ nm – 750 nm) and sample preparations are optimised.

Secondly, more investigations about the enzyme diffusion (rate or localisation) in bran will be done. Information about enzyme diffusion linked to chemical analysis can help to find the best enzyme for bran degradation and optimisation of the bioavailability of different nutrient, as well as the necessary time parameters for treatment. Recent information about enzyme diffusion in AX was giving by Cuyvers et al (30). With this project, we had the aim to demonstrate the feasibility of developing a protocol for the localised determination of enzyme diffusion (rates) usable on bran samples/flakes without any extraction or sample preparation needed.

The protocols used in this project can be optimised to investigate other bran samples microscopically or the enzyme diffusion of other types of enzymes, such as cellulases or glucanases. This approach offers great potential to optimise the refinement of bran and to ultimately utilise more bran in the food industry.
6 CONCLUSION

In this project, we investigated the microstructure and the diffusion properties of two wheat and one oat bran sample, with the aim to understand and optimise the enzymatic deconstruction of bran. It was possible to determine the particle thickness of the Kruskakli and the Havrekli sample. Vetekli samples were too thin to measure with a particle meter, probably laser scattering particle size analysers need to be used for correct determination. Control over the milling process would be able to influence the particle thickness in such way that results are better corresponding with literature.

By use of light microscopy, it was possible to determine the microstructure of the different bran layers. Furthermore, the position of the proteins and starch could be determined. Additionally, structural changes after endo-xylanase treatment were investigated, whereby a degradation of the aleurone layer could be observed. The pericarp remained unaffected. The best treatment conditions were an enzyme concentration of 10 Units/mL and storage at 60°C. CLSM confirmed the microstructure of bran as seen with LM. The position of the proteins and β-glucans could be found in literature. Further optimisation of an endo-xylanase staining technique to determine the localisation of the arabinoxylans is necessary to distinguish the staining and the autofluorescence signal.

FRAP measurements gave us more insight in the localised diffusion rates of three probes (sodium fluorescein, 10 kDa FITC-dextran and endo-xylanase enzyme) in bran flakes. The hydrodynamic radius of the three probes was determined based on the average diffusion coefficients in absence of bran. Furthermore, it could be suggested that the endo-xylanase enzyme attaches to the bran structure in the first hour after contact, thereby slowing down the diffusion rate. After 24 hour, a detachment of the enzyme and a higher diffusion rate could be observed probably caused by degradation of the bran structure (especially the aleurone layer). Probably, further investigation of different times can help to determine the time parameters needed for optimal enzyme treatment.

This project characterised the microstructure of bran. Additionally, protocols for localised diffusion measurements of an endo-xylanase enzyme in bran flake were tested and its feasibility was shown. It is suggested that the enzyme diffusion of other types of enzymes can be evaluated in the same manner.
7 REFERENCES


