Harvesting the Health Potentials of Oat Fibre

Xylanase bioprocessing of arabinoxylan

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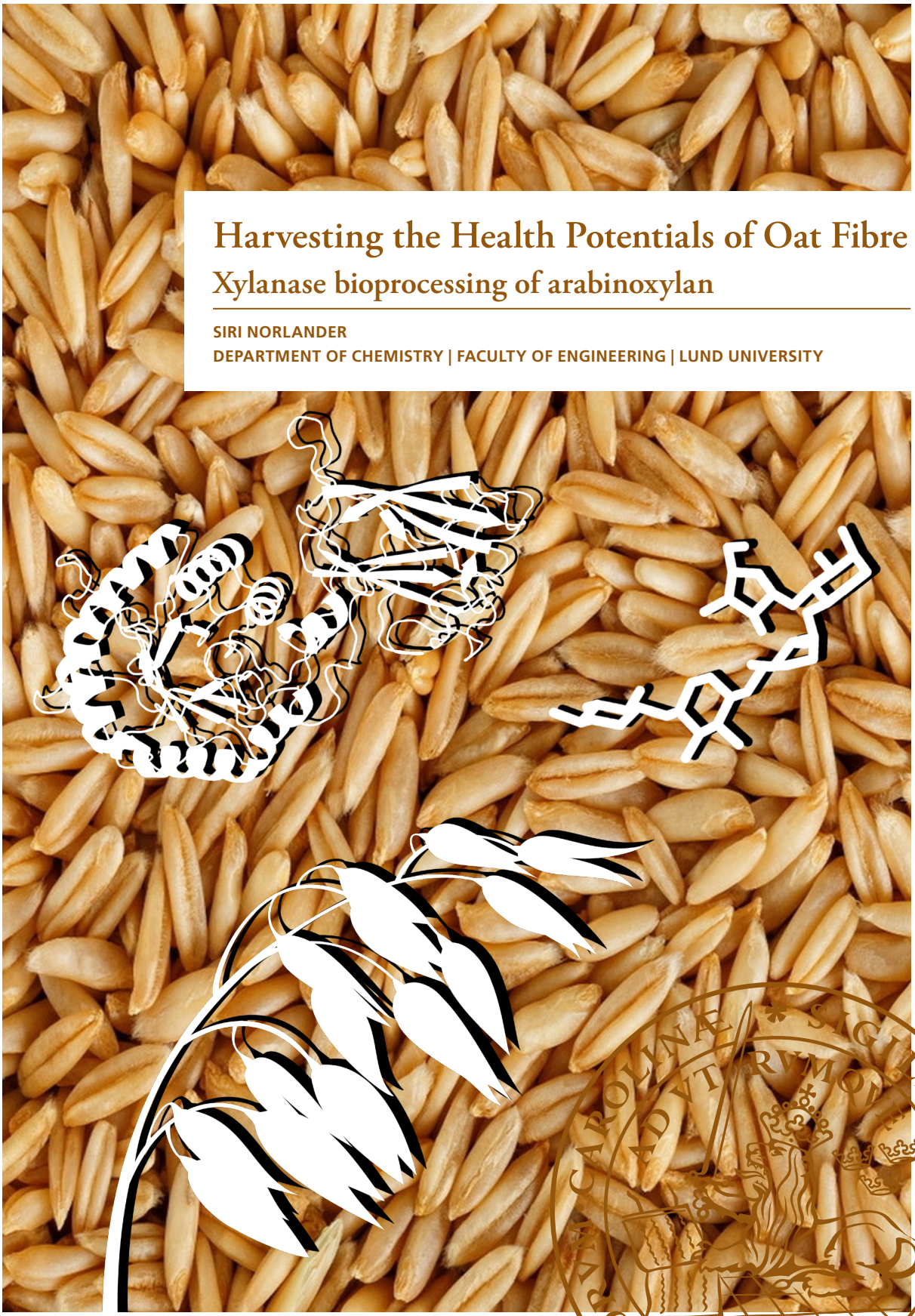

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LUND
UNIVERSITY

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Abstract

Unsustainable food production and unhealthy dietary habits are escalating risks to both the human population and the planet, as they disrupt the ecosystem and contribute to the prevalence of severe diet-related diseases. A global dietary shift towards plant-based, healthy and sustainably produced foods is needed in order to curb this trend. Switching to plant-based alternatives for animal foods, and eating more dietary fibre is considered two of the most important factors to reduce the risks for obesity and cardiovascular disease, as well as to reduce negative climate impact.

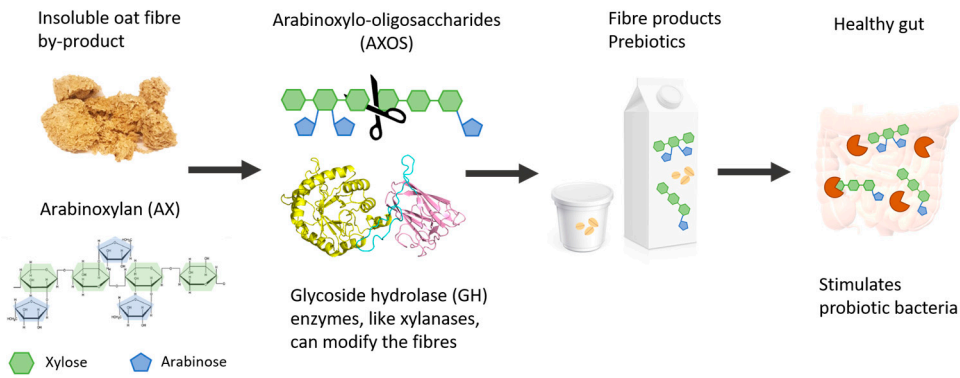
The Swedish oat cereal is rich in dietary fibre, and oat drinks have become a popular substitute for dairy milk, showing its great potential as an ingredient for innovative plant-based food products. The water-insoluble oat fibres are however lost as a by-product in certain types of oat processing, and is sold as animal feed or fuel. This fibre product is rich in the hemicellulose arabinoxylan (AX), which have promising health effects. Shorter, soluble arabinoxylo-oligosaccharides, (A)XOS, can be created by enzymatic hydrolysis of AX and have displayed prebiotic effects, selectively stimulating beneficial bacteria in the human gut and thereby promoting human health. These under-utilized AX fibres could thus be solubilized and valorised into health-promoting foods by employing enzymatic processing, a sustainable approach to maximize the potential of the oat crop and reduce processing waste. However, in order to successfully design such a sustainable bioprocess, maximize yield of desired products and create tailored (A)XOS mixtures, the substrate, enzyme selection and optimal reaction conditions must be carefully investigated.

In this thesis work, enzymatic tools and processes were developed to solubilize AX and create (A)XOS from insoluble oat fibre. Dietary fibre, and specifically AX, showed to be resilient to heat-treatment of oat kernels, but could be extracted from the recalcitrant oat bran matrix using alkali pre-treatment. AX could be further solubilized using combinations of lignocellulose degrading enzymes, in particular xylanases, which hydrolyze the AX backbone. Synergistic effects between enzymes were investigated, to maximize yield of soluble AX, and showed to be highly substrate dependent. We found that ferulic acid esterase and laccase may be needed to solubilize AX in recalcitrant materials, such as insoluble fibres from oats. The introduction of xylanases and fine milling in a liquid oat base production process was additionally studied, and resulted in a substantial increase in soluble AX in the final product, demonstrating the potential of using xylanases to create a high fibre plant-based milk alternative, while simultaneously reducing insoluble fibre waste.

Identifying and characterizing new xylanases for AX valorisation is important in order to develop efficient bioprocesses, for tailored fibre products. In this thesis, a novel glycoside hydrolase family 5 subfamily 34 arabinoxylanase, *Hh*Xyn5A, was studied for potential use in oat AX processing. This enzyme demonstrated

specificity towards arabinose substituted substrates and promising activity on oat AX fibres, without having β-glucan side activity. *Hh*Xyn5A was highly stable during production, storage and processing, which are desired qualities for industrial application. Interestingly, the two-domain *Hh*Xyn5A displayed contrasting (A)XOS oligosaccharide product formation compared to the commercial homologue *Ct*Xyn5A. Swapping of the carbohydrate binding module from family 6 (CBM6) domain and inter-domain linker region, between the two enzymes revealed the importance of CBM6 for substrate specificity as well as the linker region influence on enzyme stability, catalytic activity and oligosaccharide product profile. These insights on enzyme structure-function relationship are important for strategic enzyme engineering to optimize activity and stability, for further process development to create specific (A)XOS products.

By applying specific carbohydrate active enzymes during oat processing, insoluble dietary fibre can be valorized into health-promoting foods, offering consumers more dietary options to enhance their health while promoting a sustainable bioeconomy. This research not only advances the understanding of oat processing but also introduces novel insights into utilizing AX and enzymatic tools to develop sustainable processes and innovative products, in an effort to exploit the full potential of Swedish oat.



Populärvetenskaplig sammanfattning

Ohälsosamma kostvanor utgör ett växande hot mot vår globala hälsa genom att öka riskerna för övervikt och sjukdomar relaterade till våra kostvanor, som hjärt-kärlsjukdom, diabetes och cancer. Orsaken till denna ökning verkar vara att vi äter för lite hälsosam mat snarare än för mycket av mat som anses ohälsosam. Vi behöver särskilt äta mer fullkorn och växtbaserade livsmedel som är rika på kostfiber. Kostfibrer är ämnen som finns i växter, till exempel olika typer av kolhydrater, som inte bryts ner i vårt matsmältningssystem. I Europa äter vi mindre än 20 gram kostfiber om dagen. Världshälsoorganisationen (WHO) uppdaterade i juli 2023 sina kostrekommendationer, och rekommenderar att vuxna bör äta minst 25 gram kostfiber varje dag för att förebygga fetma och minska risken för kostrelaterade sjukdomar. Denna avhandling utforskar möjligheter att skapa fiberrika livsmedelsprodukter från havre som kan göra det lättare för konsumenter att öka intaget av kostfiber, och därmed bidra till en hälsosammare livsstil som reducerar risken för kostrelaterade sjukdomar.

Havre är ett tåligt spannmål som är lämpligt att odla i vårt nordiska klimat. Även om havre är ett populärt gryn till frukost och som ingrediens i bröd, så används majoriteten av den havre som odlas i Sverige till djurfoder. Andelen havre som produceras för livsmedelskonsumtion ökar dock stadigt, eftersom havredrycker har blivit ett populärt alternativ till komjölk. Intresset har även ökat för att använda havre som ingrediens i innovativa växtbaserade livsmedelsprodukter, som havregurt och havreprotein. För miljöns skull bör vi minska vårt intag av animaliska livsmedel, vars produktion orsakar större utsläpp av växthusgaser och har större negativ miljöpåverkan jämfört med växtbaserade livsmedel. Havre är dessutom näringsrikt, späckat med hälsosamma fetter och protein, samt kostfiber såsom β-glukan, som minskar risken för hjärt- och kärlsjukdom, sänker blodsockret och minskar nivåerna av det dåliga kolesterolet. Ett annan, mindre känt kostfiber, som finns i havre och har lovande hälsofördelar, är arabinoxylan.

Arabinoxylan (AX) är en vattenolöslig komplex kolhydrat som binder till andra fibermolekyler i den yttre delen av havrekornet, klifraktionen. De vattenolösliga klifibrerna, såsom AX, rensas ofta bort under viss processning av havre, och betraktas som en biprodukt med låg kvalitét, som ofta säljs till foder eller bränsle. För att använda AX på ett mer hållbart sätt och inkludera det i vår kost, behöver vi bryta ner denna molekyl till mindre delar och på så sätt göra AX vattenlösligt och vi får då tillgång till alla dess potentiella hälsofördelar. I forskningsprojekten som beskrivs i denna avhandling uppnåddes detta genom att använda enzymer som en skonsam behandlingsmetod. Enzymer är proteinbaserade biologiska katalysatorer, vilket innebär att de kan användas för att öka hastigheten av en reaktion utan att själv förbrukas, och dessa enzymer används i flera industriella tillämpningar. Inom livsmedelsindustrin används enzymer för att till exempel tillverka ost, laktosfri mjölk och förbättra konsistensen på bröd. Det finns ett oändligt antal enzymer med

olika funktioner; vissa katalyserar specifikt reaktionen för att bryta ner AX och dessa enzymer kallas xylanaser. I denna avhandling utvärderade vi flera typer av xylanaser och andra enzymer, i olika kombinationer, för att förstå hur enzymer med olika verkningssätt kan interagera och lösa upp så mycket AX från havrekli som möjligt. Samarbetet mellan enzymerna visade sig vara beroende av vilken fraktion, till exempel havrekli eller havreskal, som fibrerna kom från. Genom kemisk behandling av havrefibrerna, före enzymbehandlingen, kunde vi bryta ner växtstrukturerna ytterligare och därmed göra AX mer tillgänglig, och på så sätt öka enzymernas kapacitet. Vi utforskade även möjligheten att tillsätta xylanaser för att bryta ner olösliga fibrer i en process för att tillverka havredryck. Vi lyckades skapa en flytande havreprodukt rik på lösligt AX, vilket är fibrer som utan enzymatisk nedbrytning annars skulle blivit en lågkvalitativ biprodukt. Detta är ett steg mot att du i en nära framtid kan köpa en fiber-berikad havredryck i din lokala butik.

När xylanaser klipper och bryter ner AX, bildas olika kortare reaktionsprodukter, fiberkedjor med olika längder och förgreningar. Dessa kortare produkter kallas kollektivt för arabinoxylan-oligosackarider eller (A)XOS, och dess strukturer beror på var enzymet klipper fiberkedjan. Intressant nog har vissa (A)XOS prebiotiska egenskaper. Prebiotika är molekyler som fungerar som näring åt gynnsamma bakterier i vår tjocktarm, och dessa bakterier producerar i sin tur ämnen som främjar en hälsosam tarm. Eftersom endast specifika strukturer och längder av (A)XOS kan utnyttjas av dessa bakterier, är det av intresse att hitta xylanaser som producerar de önskade (A)XOS-typerna i stora mängder. I denna avhandling karakteriserades ett nytt xylanas, *Hh*Xyn5A, med avsikt att skapa (A)XOS från AX. *Hh*Xyn5A visade lovande aktivitet på AX, hög stabilitet och temperaturtolerans, vilket är önskvärda egenskaper hos enzymer avsedda för kommersiellt bruk. Genom att studera relationen mellan *Hh*Xyn5A-enzymets proteinstruktur och dess funktionella egenskaper kom vi fram till att även om två xylanaser kan se mycket lika ut i sin struktur, så påverkar små skillnader i enzymets arkitektur den katalytiska aktiviteten och således också blandningen av resulterande (A)XOS-produkter.

I detta avhandlingsarbete undersökte jag hur enzymatisk processning av havrefibrer kan användas på ett innovativt sätt för att öka mervärdet av AX, som är till nytta för både vår planet och folkhälsan. Vi visade potentialen i att använda specifika xylanaser för att lösa upp AX och skapa prebiotiska (A)XOS, både från mjöl av olösliga havrefibrer och i en process för att göra fiberrika havredrycker. Detta är ett steg mot att skapa nya hälsosammare livsmedelsalternativ för att förbättra vår kost, där havre-(A)XOS kan tillsättas i olika livsmedelsprodukter eller ätas som kosttillskott för att främja en hälsosam tarmflora. På den biotekniska sidan betonar denna forskning vikten av att göra ett noggrant enzymval och att tänka över enzymdesign, baserat på den industriella tillämpning och fiberstrukturen i havrefraktionen, för att maximera utbytet av önskade (A)XOS. Dessutom visade denna forskning värdet av att utforska nya xylanaser med unika verkningssätt och egenskaper, för att utveckla framtidens processning av havre.

Popular science summary

Unhealthy eating habits are a growing threat to global health, as it increases the risk of obesity and diet related diseases such as cardiovascular diseases, diabetes, and cancer. One contributing factor to this is that we are eating too little healthy foods, rather than too much of foods that are considered unhealthy. We specifically need to eat more whole grains and plant foods rich in dietary fibre. In Europe, we are currently eating less than 20 g dietary fibre per day. Dietary fibres are plant substances, for example different types of carbohydrates, which are not broken down by our digestive system. The World Health Organization (WHO) recently updated their dietary recommendations (July 2023), stating that adults should eat at least 25 g of dietary fibre per day, to prevent obesity and reduce risk of diet related disease. This thesis explores ways of creating fibre rich food products from oats, and in that way help consumers increase their dietary fibre intake and adopt a healthier diet to reduce diet related diseases.

Oat is a suitable cereal grain to grow in the Nordic countries, due to its resilience to rain and colder climate. Although oats are a popular breakfast food and bread ingredient, most of the oats grown in Sweden is produced for animal feed. However, the fraction of oats produced for human consumption is steadily growing, as oat drinks have become a popular dairy milk alternative and there is an increased interest in using oats as an ingredient for innovative plant-based food products. For sustainability reasons, we should reduce our intake of animal-based foods, whose production causes larger greenhouse gas emissions and has a greater negative environmental impact compared to plant-based foods. The oat crop has great nutritional value, as it is rich in healthy fats and protein, but also dietary fibre such as β-glucan, which reduces the risk of heart disease, lower blood sugar and reduces levels of bad cholesterol. Another, lesser known, dietary fibre with promising health benefits found in oats is arabinoxylan.

The arabinoxylan (AX) fibre is a long-chained water-insoluble carbohydrate which bind to other fibre molecules in the outer layer of the oat grain, the bran layer. The water-insoluble bran fibres, such as AX, are often removed during certain types of oat processing, and is considered a low value by-product, which is sold as animal feed or fuel. To use AX in a more sustainable way and incorporate it into our diet, we can break this molecule down into smaller pieces which solubilizes it, to access all the potential health benefits. In this thesis, this was achieved by using enzymes as a mild and gentle processing method. Enzymes are protein-based biological catalysts, meaning they can be used to speed up the rate of a reaction without being consumed themselves, and are employed in several industrial applications. For instance, in food industry enzymes are commonly used in the process of making cheese, lactose free milk, and to improve the texture of bread. There are an endless number of enzymes with different specific functions; some specifically catalyse the reaction to break down AX and those are called xylanases. In one study, we

evaluated the synergistic effects in combining several types of xylanases and other enzymes, in combination, in order to understand how enzymes with different activities can cooperate and solubilize as much AX from oat bran as possible. Cooperation between enzymes was shown to be dependent on the oat fibre source, for example oat bran or oat hull. Additionally, using chemical treatment of the oat fibres before enzymatic treatment could increase the activity of the enzymes, by breaking up the plant structures further and making the arabinoxylan more accessible. We also explored the possibility of adding xylanases in an oat drink manufacturing process, to break down insoluble fibre. We were able to create a liquid oat product rich in soluble arabinoxylan, fibres which without enzymatic solubilization would end up as a low value by-product. This is one step towards a future where you can pick up a high-fibre oat drink from your local store.

When the xylanase enzymes cut and break down AX, diverse shorter products with various lengths and branches are formed. These shorter products are collectively called arabinoxylan-oligosaccharides or (A)XOS, and their structures depend on where the enzyme cuts the fibre chain. Interestingly, some of these oligosaccharides have prebiotic properties. Prebiotics are molecules that are food for beneficial bacteria in our large intestine and these bacteria produce compounds which stimulate a healthy gut. Since only specific structures and lengths of (A)XOS can be utilized by these bacteria, it can be of interest to find xylanases that produce only the desired (A)XOS types in high amounts. In this thesis a new xylanase, *Hh*Xyn5A, was characterized for the intended use to create (A)XOS from oat AX. *Hh*Xyn5A showed high stability, temperature tolerance and promising activity, which are all desired qualities of enzymes intended for industrial use. By studying the relationship between *Hh*Xyn5A enzyme structure and its functional properties, we found that even though two xylanases can be very similar in structure, small differences or changes in enzyme architecture influence the catalytic activity and the resulting mixture of (A)XOS products.

This thesis investigated how enzymatic processing and oat fibres can be utilized in an innovative way to increase the value of oat AX, benefiting not only the planet but also human health. We demonstrated the potential of using specific xylanaser to solubilize oat AX and create prebiotic (A)XOS, both from insoluble oat fibre flours and in a process to make fibre-rich oat drinks. This is one step towards creating new healthier food options to improve our diet, where oat (A)XOS could be added to different kinds of food products, or eaten as supplements to stimulate a healthy gut. On the technical side, this research emphasises the importance of careful enzyme selection and design, based on the application and fibre structure, to maximize yield of desired products. In addition, this research showed that exploring new xylanases, with unique activities and characteristics, facilitates the discovery of suitable candidates for industrial oat processing.

List of Papers

I. **Effect of kilning on the macronutrient composition of three Swedish oat varieties**
S. Norlander, L. Dahlgren, R. Sardari, S. Marmon, C. Tullberg, E. Nordberg Karlsson, C. Grey. *2023, Submitted (Cereal Chemistry)*

II. **Lignocellulose degradation for the bioeconomy: The potential of enzyme synergies between xylanases, ferulic acid esterase and laccase for the production of arabinoxylo-oligosaccharides**
E. Schmitz, S. Leontakianakou, S. Norlander, E. Nordberg Karlsson and P. Adlercreutz. *2022, Bioresource Technology 343: 126114*

III. **Novel thermostable GH5_34 arabinoxylanase with an atypical CBM6, displays activity on oat fibre xylan for prebiotic production**
S. Norlander, A. Jasilionis, Z. G. K. Ara, C. Grey, P. Adlercreutz and E. Nordberg Karlsson. *2022, Glycobiology 33(6):490-502*

IV. **Inter domain linker region affects properties of CBM6 in GH5_34 arabinoxylanases and alters oligosaccharide product profile**
S. Norlander, A. Jasilionis, C. Wennerberg, C. Grey, P. Adlercreutz and E. Nordberg Karlsson. *2023, Submitted (Glycobiology)*

V. **Xylanases and high-degree wet milling improve soluble dietary fibre content in liquid oat base**
M. Mohammadi, S. Norlander, M. Hedström, P. Adlercreutz, C. Grey. *2023, Submitted (Food Chemistry)*

Author's contribution to the papers

All of the work described in this thesis was performed under the supervision of Dr. Carl Grey, Prof. Patrick Adlercreutz and Dr. Andrius Jasilionis at the Division of Biotechnology.

I. I performed part of the composition analysis and conducted the experiments regarding arabinoxylan and heat treatment effects on fibres, and wrote the corresponding parts of the manuscript. I participated in editing and revision of the manuscript.

II. I planned and designed the study together with my co-authors. I performed the enzyme synergy experiments on alkali pretreated oat bran as well as insoluble oat bran, and analyzed the corresponding data. I evaluated and discussed the article data together with my co-authors and took part in editing and review of the manuscript.

III. I planned and designed the study together with my co-authors. I performed the production and purification of the enzyme, as well as all characterization experiments and in silico modelling. I wrote the first draft of the manuscript and revised the manuscript together with my co-authors.

IV. I planned and designed the study together with my co-authors. I performed the production and purification of the enzymes, as well as all experiments and in silico modelling. I wrote the first draft of the manuscript and revised the manuscript together with my co-authors.

V. Together with the co-authors, I planned and designed the study. I assisted in conducting the enzymatic reactions, sample analysis and fraction collection with HPAEC-PAD, as well as data analysis. Together with M.M, I wrote the first draft of the manuscript and revised the manuscript together with my co-authors.

Abbreviations

Abf	arabinofuranosidase
Ara*f*	α-L-arabinofuranosyl
AX	arabinoxylan
(A)XOS	arabinoxylan-oligosaccharides
AXOS	arabinoxylo-oligosaccharides
CAZy	carbohydrate active enzyme database
CBM	carbohydrate binding module
DNS	dinitrosalicylic acid
DP	degree of polymerization
DS	degree of synergy
FAE	ferulic acid esterase
GH	glycoside hydrolase
GRAS	generally recognized as safe
HPAEC-PAD	high performance anion exchange chromatography with pulsed amperometric detector
Lac	laccase
LOB	liquid oat base
NMR	nuclear magnetic resonance spectroscopy
MeGlcA	methylglucuronic acid
MS	mass spectroscopy
XOS	xylo-oligosaccharides
Xyl*p*	D-xylopyranosyl

1 Introduction

As a result of poor eating habits, obesity and diet related diseases such as cardiovascular disease and diabetes are increasing in the world and this increase is seen as one of the major threats to global health [1, 2]. Under-consumption of whole grains and dietary fibre is on top of the list of dietary causes for this development [2] (Figure 1). Dietary fibres are defined as plant food substances or carbohydrates that are resistant to digestion and are not absorbed by the human small intestine [3]. In Europe we are currently eating less than 20 g of dietary fibre per day, and in the UK and the US, the consumption is even lower [4]. WHO released updated dietary guidelines in July 2023, where they strongly recommend adults to increase their dietary fibre intake to at least 25 g per day, to prevent obesity and reduce the risk of diet related diseases [5]. Whole grains and cereals contain a lot of dietary fibre, however, much of these health promoting fibres are lost in processing. Therefore, new processing techniques are needed to create innovative, high fibre food products, which can facilitate for consumers to increase their fibre intake.

Compared to a diet rich in animal foods, switching to plant-based alternatives can substantially reduce greenhouse gas emissions, negative environmental impact and land use [6]. Since global food production is putting large pressures on the planets ecosystem, a shift towards healthier diets abundant in plant-based foods and a reduction in food losses and waste is vital to sustain both human well-being and the environment [7].

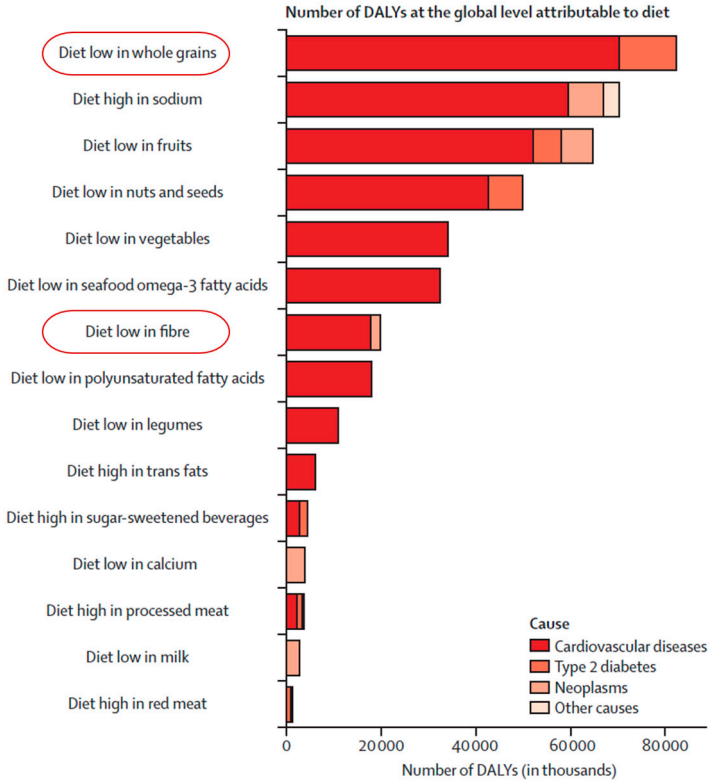


Figure 1. Number of disability-adjusted life years (DALYs) attributed to diet, at the global level in 2017. DALY are the sum of years of life lost due to premature mortality and due to time lived in states of less than full health (WHO). Adapted from Afshin [2].

Oat is a high fibre cereal often eaten at breakfast as oatmeal or müsli, and has also become popular as a base for producing dairy milk alternatives. Although about 85 % of oats grown in Sweden is allocated to animal feed, there is an increased interest in innovative oat food products, not least because of the health and sustainability benefits of reducing consumption of animal-based foods [7, 8]. Oat bran is rich in fibre, but these fibres are currently considered a low value product after certain oat processing and are sold as animal feed or fuel. In addition to the valuable and health promoting high-molecular weight β-glucans, these oat bran fibres contain the lesser known fibre arabinoxylan (AX), a hemicellulose which is part of the cell wall of many plants such as cereals [9].

AX and AX derived oligosaccharides, (A)XOS, have gotten more attention in recent years because of their interesting health properties, such as the prebiotic effects [10]. Prebiotics are dietary fibres that selectively stimulate the growth and activity of healthy probiotic bacteria in the large intestine [11]. The human gut microbiota composition plays a significant role in our health [12] and the structure of the

prebiotic (A)XOS determine which type of bacteria will be accumulated, since their capacity of metabolizing different fibres vary between species [11]. Oat fibre and AX prebiotics can therefore be used for personalized nutrition, to regulate the gut microbiota and reduce the risk of diet related disease.

Valorising the insoluble oat bran fibre side-stream into separate products with higher value is, in addition to the health benefits, a sustainable approach to maximize the potential of the oat crop and reduce processing waste. Enzymes are often employed for these kind of biorefinery purposes, since enzymes are efficient for biomass degradation, specific in their activity and environmentally friendly [13]. In order to develop a cost-efficient enzymatic process for valorising AX, it is imperative to evaluate different enzymes to find the optimal combination and conditions to maximize yield of the desired product [14]. Xylanases, which are enzymes that specifically hydrolyze the backbone of AX fibres, can be used for this purpose to solubilize AX from plant materials and create oligosaccharides with prebiotic activity [15].

Depending on the biomass origin and xylanase specificity, distinctive (A)XOS products can be created. A small group of xylanases called arabinoxylanases are specific to arabinose substitution, and their activity therefore result in a different product profile compared to other types of xylanases. The family of arabinoxylanases is relatively unexplored, with few characterized enzyme members, but has interesting properties for AX solubilization and prebiotic production [15, 16]. Exploring and characterizing new enzymes, such as arabinoxylanases, is needed to design specific and efficient bioprocesses for AX valorisation [14]. In addition, studying the enzyme structure-function relationship can be useful in order to engineer these xylanases, adapting and optimizing them for industrial application.

1.1 Aim and scope of the thesis

This thesis work is part of the industrial research centre ScanOats, which aims to take a world-leading role in oat research and innovation in order to develop and expand the Swedish oat industry. Their research concerns the whole oat value chain, from oat genetics and breeding, to oat processing and the benefits to human health.

The purpose of this thesis is to contribute to the existing knowledge of oat processing, specifically regarding insoluble dietary fibre and the hemicellulose arabinoxylan (AX). The aim was to investigate enzymatic techniques for oat processing and valorisation of various oat fractions, to create novel high fibre foods and prebiotics. By using diverse carbohydrate acting enzymes, in particular xylanases and arabinoxylanases, the possibility of solubilizing AX and create arabinoxylo-oligosaccharides, (A)XOS, was explored. The objectives were to investigate novel enzymes as well as identify important factors related to

bioprocessing of oat fibres and AX, for efficient use of enzymatic tools in an industrial context. The thesis work has been focused on hydrothermal and mechanical oat processing effects on AX (**Paper I** and **Paper V**), solubilization of AX and production of prebiotic oligosaccharides using carbohydrate active enzymes (**Paper II** and **Paper V**), and the investigation of a novel arabinoxylanase for AX modification (**Paper III** and **Paper IV**).

The effects of kilning on the macronutrients, such as dietary fibre and AX solubility, of three different oat varieties were investigated in **Paper I**. In **Paper II**, the synergistic effects of various enzymes for solubilization of AX from hemicellulose rich oat bran, oat hull fibres and corn bran were investigated. Identification and characterization of a novel GH5 arabinoxylanase *Hh*Xyn5A, for potential use in oat AX processing, is described in **Paper III**. This enzyme was further examined in **Paper IV**, where the influence of the carbohydrate binding module (CBM) from family 6 of *Hh*Xyn5A and homologous *Ct*Xyn5A was investigated through domain-swapping between the two enzymes. In **Paper V**, xylanase treatment in combination with fine milling was introduced in a liquid oat base (LOB) process, with the aim of increasing soluble AX in the LOB product, while simultaneously reducing the insoluble fibre waste.

2 The oat grain and its fibres

The common oat (*Avena sativa* (L.)), is ranked 6th of the world cereal crop production [17] and is the third most produced cereal crop in Sweden, after wheat and barley [18]. Sweden is one of the larger producers of oats [19], harvesting 743800 tonnes in 2022 [18], and exporting around 200000 tonnes annually [20]. The large majority of the harvest is being utilized as animal fodder, however, approximately 15 % of the Swedish oat is used in the food industry and the percentage is expected to grow steadily every year, thanks to product innovation and the many health benefits of oats [17, 20].

The edible part of the oat, the dehulled oat kernel or groat (Figure 2A), is made up of a starchy and lipid-rich endosperm (up to 70 % of the groat), which is surrounding the embryonic germ. Additionally, the groat has an outer bran layer, containing much of the oats' micronutrients and fibre [21]. The most abundant macronutrient of the oat cereal is starch (around 47-64 %) [22, 23] and it has a high lipid content (up to 13 %) relative to other cereals [24] and a protein content around 12-24 % [21]. However, this thesis will focus on the underutilized, yet impactful, oat fibres.

2.1 Oat dietary fibre - arabinoxylan

The dietary fibres in oat (10-23 %) consist mainly of the polysaccharides located in the kernel cell wall of the cereal; β-glucan (3-11 %), arabinoxylan (2-6 %), cellulose and lignin [25, 26]. Oat is known for its high content of β-glucan, which has shown to induce many health benefits, including prevention of coronary heart disease and colorectal cancer, lowering of postprandial blood glucose levels, and reduction of LDL cholesterol [27, 28]. In addition to the β-glucans, the hemicellulose arabinoxylan (AX) has shown potential health benefits as well, such as improving glycaemic control, reducing cholesterol uptake and stimulating short-chain fatty acid (SCFA) production of bacteria in the large intestine [10, 11]. AX is a linear polysaccharide with a backbone of β-1,4-linked D-xylopyranosyl (Xyl*p*) units, which in oat can be single- or double substituted with α-L-arabinofuranosyl (Ara*f*) units at position O-2 and O-3 of the Xyl*p* sugar (Figure 2B) [9, 29]. In addition, glucuronic acid and 4-O-methylated glucuronic acid (MeGlcA) substitutions can be found in oat AX [30]. Phenolic acids such as ferulic acid can be attached to the O-5

position of the Ara*f* substituent, providing a connection to other AX chains and to lignin, connecting the plant materials into a recalcitrant matrix, thus making most of the AX water unextractable [9, 31]. The AX in oat has an arabinose to xylose ratio, A/X, around 0.6-0.8 (**Paper 1**) and it has been suggested that the arabinose substituents are differently distributed along the xylan backbone depending on the oat fraction [30]. Additionally, the AX substitution type, pattern and frequency very much depend on the cereal origin [11, 30] and will have implications when it comes to processing, specifically for making tailored AX fibre products, such as xylo-oligosaccharides (XOS) and arabinoxylo-oligosaccharides (AXOS), with potential prebiotic health benefits. Collectively, XOS and AXOS are commonly referred to as (A)XOS.

The nomenclature that is often used, and will be used in this thesis, to describe (A)XOS molecular structure is based on the form X_n, where *n* describes the number of xylose (X) units in the (A)XOS backbone, and A^m, where *m* is 2 or 3 depending on the Ara*f* + Xyl*p* (A) residue linkage and position (O-2-linkage or O-3-linkage). The nomenclature describes the structure from non-reducing end to the reducing end of the oligosaccharide. For example, xylotriose will be written X_3 in short form and α-1,3-arabinofuranosyl-β-1,4-linked D-xylopyranosyl- β-1,4-linked D-xylopyranosyl will be written A^3X.

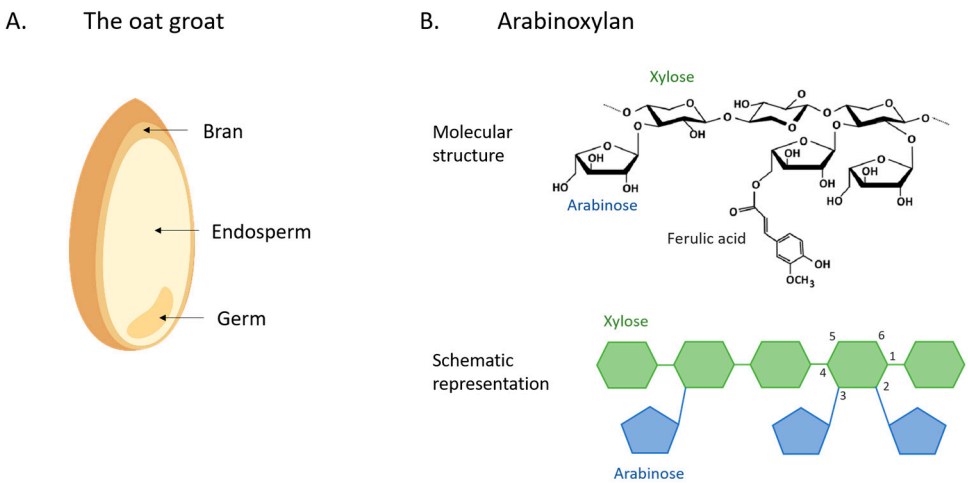


Figure 2. A. Schematic view of the parts of the oat groat. **B.** The molecular structure of arabinoxylan. A schematic representation of arabinoxylan will be used in the thesis, where xylose is represented as a green hexagon and arabinose as a blue pentagon.

2.2 Health benefits of arabinoxylan derived (A)XOS

While cereal AX has a number of health benefits as mentioned above, AX derived oligosaccharides (A)XOS have shown prebiotic properties [32]. These compounds are not digested or absorbed in our small intestine, but instead selectively stimulate the growth of probiotic bacteria (typically increasing *Bifidobacterium* and *Lactobacillus* species) and promote a healthy microbiota in our large intestine [11, 32]. The fermentation of prebiotics by these colonic bacteria produces beneficial SCFA, such as acetate, butyrate, lactate and propionate. For instance, these SCFA lower intestinal pH, prevent proliferation of harmful bacteria and have anti-cancerous properties [11].

The health effects and prebiotic properties of AX and (A)XOS depend on their structure. Results from *in vitro* human faecal fermentation studies suggests that (A)XOS with low (2-11) degree of polymerisation (DP) have greater prebiotic potential, stimulating *Bifidobacterium* and SCFA production at a greater extent, compared to AX with high DP and high degree of arabinose substitution (A/X > 1.0), which seems to be more difficult to degrade [11, 33]. *Bifidobacterium* species can grow on both XOS and AXOS, although preferring short X_2 and X_3, while *Lactobacillus* species seem to only utilize undecorated XOS [11, 34]. Feruloylated cereal AX and (A)XOS have shown to have strong antioxidative properties as well as prebiotic properties in the colon [11, 35], although high levels of feruloylation can unfortunately make the (A)XOS more difficult for some probiotic bacteria to metabolize [36]. However, the ferulic acid linkages are often damaged and removed by pretreatment of the recalcitrant lignocellulosic matrix and extraction of AX [37], for example when using alkali pretreatment to extract oat AX as in **Paper II**.

It is evident that the bacterial composition of the gut microbiome can be regulated by various types of prebiotics, for example (A)XOS [11, 12]. Although not investigated in this thesis work, the identification of new probiotic bacterial species, along with exploring their various metabolic preferences, is an interesting and important field of study. This type of research aims to uncover potential synbiotic effects between individual bacteria and various types of fibres, leading to the development of personalized nutrition strategies for regulating and optimizing the gut microbiome [11, 12, 32]. In addition, it is essential to develop methods for creating diverse and bioavailable (A)XOS products, for example using an enzymatic approach [15]. When it comes to oats, there is a need to enhance existing processing methods to fully unlock the potential of AX, create customized (A)XOS and high fibre oat products, with least possible damage to beneficial high molecular weight β-glucan. This includes utilizing oat side-streams rich in AX, refining the extraction of AX and employing specific enzymatic hydrolysis activities to produce specific (A)XOS. In **Paper II, III** and **V**, these aspects were investigated.

3 Processing of oat

3.1 General oat processing

The oat grain is processed post-harvest, generally initiated with dehulling followed by heat treatment, or kilning, of the grains to avoid rancidity and increase shelf life. Depending on the food application, the oat grains can then for example be pressed into flaked or rolled oats, milled into flour or undergo more advanced processing such as becoming liquid oat base (LOB) or fractionated to separate starch, bran, protein, oils and β-glucan.

3.1.1 Kilning

Kilning is the heat- and steam treatment that the oat grain undergoes to inactivate endogenous lipases [38, 39]. This is an important step, since the high lipase activity occurring after processing otherwise will lead to lipolysis and oxidation of the lipids in the oil-rich oat, creating undesired, off-smelling and off-tasting reaction products. Additionally, the kilning destroys contaminating bacteria and fungi, can facilitate post-kilning dehulling and helps with flavour development [38]. In a general kilning procedure with a total retention time of 90-120 min, the oat is heated up to 90-100 °C with applied steam, at an oat moisture content no less than 12 % [38].

Notably, kilning has shown to have adverse effects on the nutritional profile of oat, as well as processing properties [39-45]. For example, milling and flour properties are effected depending on kilning temperature [44], and increased starch damage and changes in pasting properties has been observed when comparing heat-treated with non-heat-treated samples after milling [40]. The ratio of soluble and insoluble dietary fibre has shown to be affected by heat treatment, in turn affecting further processing and functional properties of the oat [40-42]. Lipid oxidation [43], antioxidants [45] and protein solubility [46] can also be affected by severity in heat treatment and kilning. Therefore, it is important to assess alternative processing methods, such as employing milder kilning, to prevent rancidity without compromising other important quality factors of oat products. In addition, the tolerance of oat to heat treatment may be influenced by the specific oat variety used and its phenotypic traits, which, in turn, can have various effects on its nutritional and functional aspects [40, 44].

In **Paper I**, we investigated the effect of kilning (industrial and a milder version) on the macronutrient profile of three different oat varieties. The study showed that even milder kilning could inactivate lipases effectively, however antioxidative capacity was reduced compared to untreated oats and the soluble protein fraction was reduced with kilning. For the fibres, we found that dietary fibre solubility as well as AX content and composition in different oat varieties was marginally affected by the industrial heat treatment.

3.1.2 Fractionation and extraction of fibres

When the oat grain is processed into flour and fractionated for extraction of various valuable compounds, such as β-glucan and protein, the insoluble fibres from the bran layer will be separated. This side-stream fraction is of low value and is mostly sold as animal feed or biofuel. The dried insoluble oat fibre fraction used throughout this thesis is rich in AX (14 %) but also contains around 25 % starch and 30 % protein, indicating its potential to be valorised by further extraction. To access the AX from the bran matrix, pre-treatment approaches can be applied, such as hydrothermal or acid pre-treatment [13]. Initial destarching, using starch degrading enzymes amylase and amyloglucosidase, can be necessary if the fraction still contains a significant amount of starch, which could gelatinize during further treatment and negatively affect the efficiency of certain extraction methods. Alkali pre-treatment is another common method [13, 47], which was used in **Paper II** in order to solubilize AX from the insoluble oat fibre fraction. The alkaline solution, such as sodium hydroxide, disrupts the linkages within the lignocellulosic matrix; the hydrogen bonds binding AX to cellulose and the ester bonds binding AX to lignin. The pre-treated fibre solution is then neutralized, the AX fibres are precipitated using alcohol (ethanol) and finally washed with water. Extracting the AX from the fibre matrix using harsher methods like alkali pre-treatment can be of advantage when producing (A)XOS, since it provides easier access for xylan specific enzymes in further enzymatic treatments. Although this thesis did not investigate pre-treatment in depth, the severity of pre-treatment should be adapted to the oat fraction used, in order to limit formation of undesired reaction products or unnecessary damage of material, and to conserve resources. For instance, for extracting AX from oat bran fibres, a milder alkali pre-treatment might prove more suitable compared to what is needed using other oat waste streams, like recalcitrant oat hulls [47].

3.1.3 Liquid oat base production

Liquid oat base (LOB) is used as a component for making dairy milk alternatives such as oat drinks, yoghurts and ice creams. A general process of making LOB includes an initial wet milling step, which can be performed using oat flour or whole

oat grains, with 14-17 % total solids. The oat starch needs to be depolymerized in order to, primarily, decrease viscosity of the LOB [48]. For this purpose, starch degrading enzymes are employed, commonly amylases and amyloglucosidases, which hydrolyse the starch into oligosaccharides, maltose and glucose. Depending on the desired sweetness of the final product, reaction time and enzyme composition can be modified, as this will determine how much of the starch is hydrolysed down to glucose. Heating of the LOB in a stepwise process to various temperatures around 55-90 °C is required to gelatinize the oat starch and to reach optimal activity for each enzyme. When desired consistency and sweetness of the LOB product is reached, the enzymatic process is terminated by a pasteurization step, heating the LOB to high temperatures to inactivate the enzymes and to increase shelf life. Lastly, to create a liquid product, a decanting step is included to remove solid components such as insoluble fibres, which otherwise may contribute to an undesired taste and mouthfeel of the final food product.

The resulting insoluble fibre cake from production of LOB is, like insoluble oat fibre from fractionation, underutilized but contain valuable nutrients [49]. In an attempt to reduce the insoluble fibre waste and simultaneously increase the soluble AX content in the final LOB product, various xylanase enzymes were evaluated in combination with increased milling in **Paper V.** The oat grains were in this case wet milled in a pilot scale plant, using three consecutive mills of different type, where the disk gap space of the corundum mill was adjusted in order to create LOB with different particle sizes; 1, 0.1 and 0.05 mm (Figure 3.1). Finer milling showed to increase the suspended AX content of the final product. Additionally, LOB produced from a mixture of wholemeal oat and oat bran as demonstrated in **Paper V**, or exclusively from oat bran as shown recently by Patra [50], show promising features such as increased dietary fibre (AX and β-glucan) and protein, as well as increased viscosity and separation stability at neutral pH. As with oat processing in general, the variety used and its phenotypic traits can influence the LOB properties significantly, and should additionally be taken into consideration when developing new oat products. Ultimately, consumer acceptance of the final oat drink product is of highest priority, and will determine the amount of AX fibre that can be introduced in LOB without compromising the sensory properties [51].

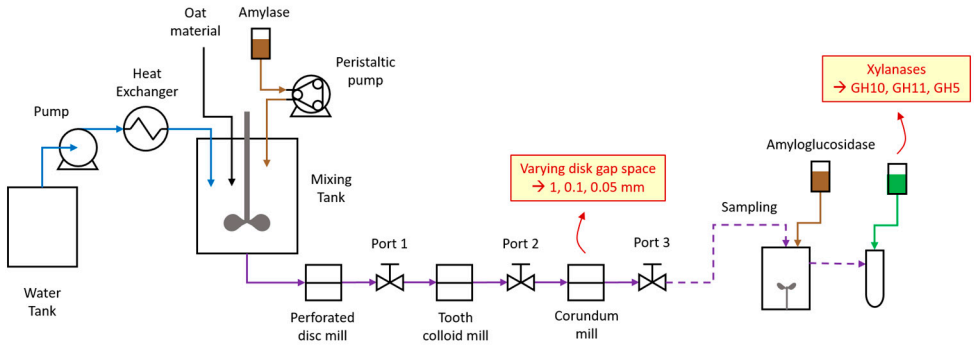


Figure 3.1 Process scheme of the pilot plant used for manufacturing liquid oat base (LOB). The disk gap space of the corundum mill was varied between 1, 0.1 and 0.05 mm to produce LOB samples of different milling degree.

3.2 Enzymatic processing of AX

Most of the cereal AX is regarded insoluble and water unextractable (>80 % for oat AX) [26, 29], since AX is often cross-linked together with lignin, and also interacting with β-glucan and cellulose, creating a recalcitrant structure [9]. To access AX, mechanical and chemical processing is therefore often required as a first step, for example by milling and alkali extraction [52]. Enzymatic processing is in addition a useful way of elucidating AX, further lowering the degree of polymerization and create specific (A)XOS. Commonly employed enzymes for this purpose include glucoside hydrolases such as cellulases, xylanases and arabinoxylanases, or ferulic acid esterase and laccase [14, 15, 53]. While the utilization of certain enzymes can lead to higher processing costs, employing enzymatic processing offers several advantages over chemical processing such as reduced by-product formation, less environmental impact, and the ability to achieve more precise hydrolysis [13]. Some form of pre-treatment before enzymatic treatment is still often necessary in order to break up the structure, particularly separate lignin, which presents a physical barrier but also inhibit enzyme activity [53, 54]. On the other hand, pre-treatment of lignocellulosic material can produce unwanted by-products, such as furfural or phenolic compounds, which in turn can be inhibitory to enzymes [52, 54]. The pre-treatment method should therefore be carefully selected depending on the nature of the biomass, for an efficient subsequent enzymatic treatment.

Suitable enzymes and reaction conditions for efficient solubilization of AX and (A)XOS production will depend on the nature of the substrate, specificity and stability of the enzymes, desired final fibre composition and characteristics of the final product

[53]. Some aspects of enzymatic processing of oat AX that were investigated in this thesis work are discussed further in detail below; synergistic effects using several enzymes, the use of enzymes in a LOB process, and considerations for evaluating the reaction products from processing of oat and AX hydrolysis.

3.2.1 Enzymatic synergy

Recalcitrant biomass often require the cooperation of several enzymes, specific for each bond connecting the different structures, to efficiently hydrolyze the substrate [53]. Using these enzymes together can increase the hydrolysis rate or the product yield, compared to using each enzyme alone, and thus creating enzyme synergy. For example, cellulases and laccases can be added to break down cellulose and lignin in order to enable xylanase enzymes, which hydrolyze the AX backbone, to access the AX substrate. The synergistic effect of enzymes can be measured as degree of synergy (DS), defined as the ratio between the rate or yield of products formed by the enzymes when used together, and the sum of the rates or yields when the same enzymes are used separately [53]. A DS > 1 indicates that there are synergistic effects between the enzymes, while a DS around 1 suggests that there is little synergy. However, this does not necessarily mean that an enzyme is redundant, but instead suggests that the enzymes can work independently and do not benefit from cooperation. A DS < 1 suggests that there are inhibitory effects between the enzymes, sometimes also referred to as anti-synergy [53]. The DS can be a helpful measure when determining if an enzyme is contributing to the degradation of the substrate, by assisting the activity of other enzymes. Enzyme synergy can also be explained as homosynergy or heterosynergy. Homosynergy is the term for synergy between enzymes with similar activity, hydrolysing the main chain of the substrate, while heterosynergy refers to synergy between enzymes with different actions, for example between a main-chain cleaving enzyme and a debranching enzyme removing substituents [53]. The enzyme synergy concept can be used as a tool to find the minimal combination of key enzymes that is needed to achieve optimal yield of desired products [55], in this case soluble AX and (A)XOS.

In **Paper II**, enzyme synergy was evaluated for the solubilization of AX from insoluble oat bran fibre, as well as oat hull and corn bran. The enzymes were evaluated both on destarched alkali pre-treated oat fibre and directly on the insoluble oat fibres for comparison. The enzyme selection was based on an initial screening process, evaluating which enzyme types had the greatest influence on solubilizing AX. The selected enzymes included xylanases from glycoside hydrolase (GH) family 11, 10 and 5 (termed GH11(P), GH11(F), GH10 and GH5), ferulic acid esterase (FAE) and laccase (Lac). Xylanases cut the β-1,4-linkages between Xyl*p* units in the AX backbone, while FAE specifically cleave the ferulic acid ester bond

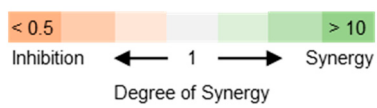
linking AX strands together and linking AX to lignin [53], and Lac can oxidize lignin and phenolics present in the substrate [56]. Cellulases and enzymes removing specific side-residues can also be employed for efficient hydrolysis of lignocellulosic biomass, but for the specific cereal substrates used in **Paper II,** these enzymes were not the main contributing enzymes for AX solubilization and were therefore excluded.

The results of **Paper II** demonstrate how substrate source, composition and pre-treatment play a crucial role in enzyme synergy. For example, FAE in combination with a xylanase resulted in synergy for both pre-treated oat fibre and insoluble oat fibre, while Lac resulted in anti-synergy when used on pre-treated oat fibre, but could result in synergy when used on insoluble oat fibre (Figure 3.2). The alkali pre-treatment showed sufficient to remove blocking or inhibiting lignin, leaving further treatment with Lac redundant and even inhibiting. In contrast, Lac could contribute to increased solubilization of AX in the insoluble oat fibres, which still contain significant amount of lignin. Few homosynergies between xylanases could be seen for the oat fibre substrates, but were seen for corn bran and oat hull fibres, although resulting in quite low DS. This is possibly due to variations in AX structure and side-decorations between the various substrates. Even though xylanases are hydrolysing the same bond in the main chain of AX, the specificities between xylanases from different families vary greatly, which is why homosynergies may occur. Xylanases from family GH11, GH10 and GH5, commonly employed for AX processing, are further discussed in Chapter 4.

One of the main objectives in **Paper II** was to maximize yield of soluble AX, including (A)XOS and monosugar arabinose and xylose. However, for a high yield of prebiotic (A)XOS, the production of non-prebiotic monosugar xylose should be minimized [13]. It is therefore important to modify calculation of DS to consider only the desired product yield, to make it a relevant measure when evaluating enzyme synergy and efficiency [53]. In addition, a high DS does not necessarily translate into the most optimal choice of enzymes for achieving the highest yield, as can be seen in Figure 3.2. Adding FAE and a xylanase together on pre-treated oat fibre could result in synergy, compared to using the two enzymes separately. However, the highest yield was achieved by simply using one xylanase (GH10). When evaluating enzymes for AX solubilization or (A)XOS production, it is imperative to also consider the enzyme amount and time dependence, sequential or simultaneous addition of the enzymes, enzyme ratio, temperature and pH for the reaction [53, 57]. For example, the time factor can considerably change the composition of the final (A)XOS mixture, since longer XOS and AXOS could be further hydrolysed in a prolonged reaction. Therefore, finding the optimal enzyme mix and parameters for maximal yield of a desired product from a specific substrate requires much research effort.

	Enzyme Combination	Synergy (DS) OF	Synergy (DS) IOF	Yield (%) OF	Yield (%) IOF
Individual enzyme	GH11(P)	-	-	30.5	17.8
	GH11(F)	-	-	27.2	36.1
	GH10	-	-	66.5	30.5
	GH5	-	-	0.46	10.7
	FAE	-	-	0.89	0.12
	Lac	-	-	16.3	7.2
Xylanases	GH11(P) + GH11(F)	0.51	0.56	29.2	29.9
	GH11(P) + GH10	0.43	0.58	42.0	28.2
	GH11(F) + GH10	0.52	0.58	49.2	38.5
	GH11(P) + GH5	0.61	0.42	18.9	11.9
	GH11(F) + GH5	1.02	0.55	28.1	25.6
	GH10 + GH5	0.61	0.76	40.9	31.2
	GH11(P) + GH11(F) + GH10	0.31	0.44	37.9	36.9
	GH11(P) + GH11(F) + GH5	0.34	0.41	16.2	26.6
	GH11(P) + GH10 + GH5	0.28	0.52	32.8	30.6
	GH11(F) + GH10 + GH5	0.37	0.41	34.8	31.9
	GH11(P) + GH11(F) + GH10 + GH5		0.47		30.5
Ferulic acid esterase Xylanases	FAE + GH11(P)	0.90	0.91	28.4	16.3
	FAE + GH11(F)	1.24	0.75	34.9	27.1
	FAE + GH10	0.94	1.35	63.5	41.4
	FAE + GH5	0.80	0.03	1.1	0.27
	FAE + GH11(P) + GH11(F)		0.44		23.7
	FAE + GH11(P) + GH10		0.93		45.1
	FAE + GH11(F) + GH10		0.56		37.5
	FAE + GH11(P) + GH5		0.54		15.4
	FAE + GH11(F) + GH5		0.43		20.0
	FAE + GH10 + GH5		0.68		27.9
	FAE + GH11(P) + GH10 + GH5		0.67		32.6
	FAE + GH11(F) + GH10 + GH5				
	FAE + GH11(P) + GH11(F) + GH5		0.23		15.2
Laccase Ferulic acid esterase Xylanases	Lac + GH11(P)	0.72	1.58	33.5	39.5
	Lac + GH11(F)	0.79	0.60	34.3	25.9
	Lac + GH10	0.70	0.85	58.0	31.8
	Lac + GH5	0.72	1.92	12.1	34.3
	Lac + FAE	0.81	0.51	13.9	3.7
	Lac + GH11(P) + GH11(F)	0.43	0.65	32.0	39.6
	Lac + GH11(P) + GH10	0.36	1.048	40.6	58.1
	Lac + GH11(F) + GH10	0.40	0.66	44.2	48.8
	Lac + GH11(P) + GH5	0.51	0.90	24.0	32.0
	Lac + GH11(F) + GH5	0.78	0.66	34.3	35.8
	Lac + GH10 + GH5	0.53	0.68	44.1	33.0
	Lac + FAE + GH11(P)		0.76		19.2
	Lac + FAE + GH11(F)		0.66		28.8
	Lac + FAE + GH10		0.63		23.7
	Lac + FAE + GH5		0.36		6.4
	Lac + GH11(P) + GH11(F) + GH10		0.52		28.6
	Lac + GH11(P) + GH10 + GH5		0.52		29.0
	Lac + GH11(F) + GH10 + GH5		0.42		30.7
	Lac + GH11(P) + GH11(F) + GH5		0.49		17.3
	Lac + FAE + GH11(P) + GH11(F)		0.40		17.3
	Lac + FAE + GH11(P) + GH10		0.49		27.3
	Lac + FAE + GH11(F) + GH10		0.38		27.9
	Lac + FAE + GH11(P) + GH5		0.34		12.2
	Lac + FAE + GH11(F) + GH5		0.39		16.9

Figure 3.2. Enzyme synergy on alkali pre-treated oat bran fibre (OF) and insoluble oat bran fibre (IOF). Green highlight indicates synergy or high yield, whereas red highlight indicates anti-synergy or low yield.



3.2.2 Enzymes in a liquid oat base process

The addition of starch degrading enzymes is vital to create a liquid oat base (LOB) product of desired consistency. Enzymes such as amylase and amyloglucosidase must be added to break down the starch, decrease viscosity and also add sweetness to the final product. However, there is little published research on the addition of xylanases to an LOB process. A recent study using xylanase treatment, in combination with cellulase, on the by-product press cake from LOB processing, resulted in extraction of bound protein and a decrease in the anti-nutrient phytic acid [49], but the AX release was not investigated. Additionally, a patent describing the use of xylanase in LOB processing focused on particle size reduction of oat bran and physical stability of the resulting oat drink [58], however, did not include the implications of using xylanases from different families or production of (A)XOS. In an attempt to increase the soluble AX fibre content and create (A)XOS in a LOB process, the addition of xylanases from various families were explored in **Paper V**. With xylanase treatment, more AX was solubilized into the LOB, up to 100 % of the available AX depending on the xylanase used (Figure 3.3). The most efficient xylanase, E-XYNBS from glycoside hydrolase family 10, produced short XOS such as X_2, but also longer AXOS with DP5-7. This proof-of-concept study demonstrates the potential of xylanase processing for high fibre LOB products and prebiotic oat drinks on the future market.

For food application, it is important that the enzymes used are produced using GRAS (generally recognized as safe) organisms and are food graded, especially if the enzymes will remain in the final consumer product [59]. This was not the case for all enzymes employed in this thesis work. Thus, for further scale-up studies and for human trials investigating health effects of oat (A)XOS and high fibre oat products, the enzymes applied should be produced in GRAS organisms and approved for food application.

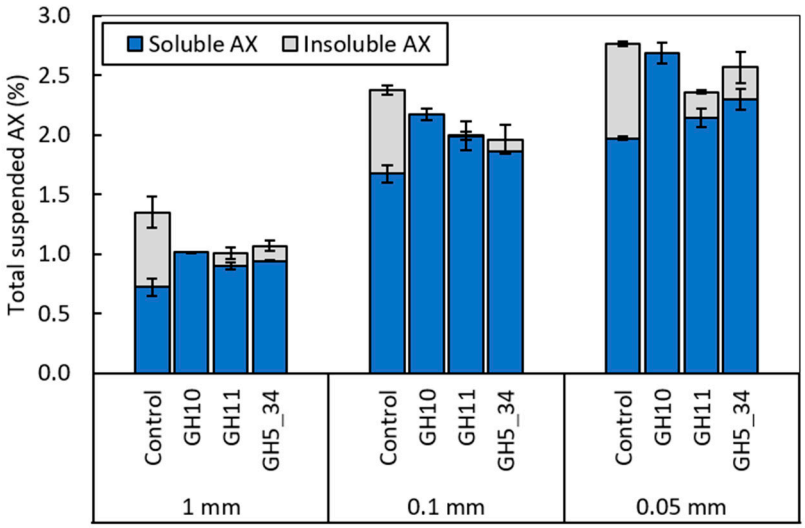


Figure 3.3. Solubilization of AX using xylanases in a liquid oat base process. Three particle sized (1, 0.1 and 0.05 mm) were evaluated in combination with three xylanases from family GH10, GH11 and GH5_34. Control is LOB without added xylanase.

3.2.3 Analysis and evaluation of reaction products

To analyse fibre in cereal biomass, or to identify and evaluate carbohydrate products from enzymatic reactions, many different methods and techniques can be used. An appropriate method is often chosen based on availability, but most importantly the level of detail and specificity that is needed to adequately answer the research question. For quantification of soluble and insoluble dietary fibre, practical kits can be used that are based on AOAC (Association of Official Analytical Chemists) and AACC (American Association of Cereal Chemists) approved methods. Usually, these methods are gravimetrical or chromatography based. However, results obtained by different fibre quantification methods may vary, depending on the substrate matrix composition, the technique sensitivity or adaptions of the method, and as the definition of dietary fibre changes over the years [60, 61]. For example, some AX fibres can be defined or quantified as water- , alkali- or alcohol extractable/soluble. Therefore, it is important to consider the method suitability and the relevance of the solubility measure, when analysing AX fibre solubility. In **Paper V,** soluble AX was quantified by measuring the AX left in the water-based supernatant after centrifugation, as this would be the relevant portion of AX retained in the LOB product and available for consumption.

A simple technique that is often used for analysis of enzymatic reactions using carbohydrate based substrates is the dinitrosalicylic acid (DNS) assay, a method quantifying reducing ends of sugars and oligosaccharides [13, 62]. The DNS compound is reduced by the reducing ketone or aldehyde group of the reducing sugar, and is converted to the red coloured 3-amino, 5-nitrosalicylic acid, which is then detected spectrophotometrically by measuring absorbance at 540 nm. The hydrolytic enzyme activity can therefore be monitored over time, since every cleavage point give rise to a new reducing end. The DNS assay has some advantages over other time-consuming chromatography methods, such as being fast and requiring little resources. However, the DNS method has many drawbacks, such as being unspecific to the sugar type and length, interference by other compounds like furans and amino acids, and over-oxidation of longer oligosaccharides [13, 63, 64]. This method may therefore not be suitable for quantifying AX hydrolysis in a starch-rich sample, because the prevalence of reducing ends from maltooligosaccharides would overshadow any changes in AX composition. On the other hand, the DNS method can be efficiently used when comparing enzyme activities on destarched or pure AX substrates, which was done in **Paper III**.

High performance anion exchange chromatography with pulsed amperometric detector (HPAEC-PAD) is one of the most used techniques for glycan analysis, for example to quantify AX and identify (A)XOS [13]. The separation of components is based on the sugar or oligosaccharide charge, and is affected by molecular structure and DP. With a strong alkaline eluent (pH >13), the hydroxyl groups of the sugars will be deprotonated and become anionic, and can therefore be separated [65]. The low limit of detection and high resolution are two of the advantages with HPAEC-PAD, however the detector is sensitive for contamination, which effects retention and response of the analytes, and the non-linear response to oligosaccharides due to conformational differences makes the technique dependent on available standards for analyte identification [66]. For example, XA^2XX and XA^3XX which only differ in the arabinose position can be baseline separated, but the prediction of their elution position in relation to each other and other AXOS may be difficult without standards.

Quantification of AX is performed by first applying acid hydrolysis to break down the AX to its xylose and arabinose monosugars, which are thereafter quantified by HPAEC-PAD. For identifying oligosaccharide (A)XOS, the retention times of sample components are commonly compared to commercially available standards, however standards with arabinose substitution are limited. To address the limitations of HPAEC-PAD, specific peaks or components that are of interest can be collected after separation and further analysed by nuclear magnetic resonance spectroscopy (NMR) or mass spectroscopy (MS) [65]. In **Paper V**, oligosaccharide products were collected after separation with HPAEC-PAD, then desalted and neutralized in order to be compatible with further MS analysis. With MS analysis, the DP and structure can be determined to some degree, for example if the

oligosaccharide is made up of pentose (e.g xylose or arabinose) or hexose (e.g glucose) sugars. Enzymatic approaches can also be used to elucidate the identity of some oligosaccharides separated by HPAEC-PAD. Arabinofuranosidases (Abfs), enzymes which specifically hydrolyse the bond between Ara*f* residues and the xylan backbone, can be used to identify AXOS [67]. By comparing the HPAEC-PAD resulting product chromatograms before and after hydrolysis with specific Abfs, compounds containing single or double substitution of Ara*f* can be identified as these peaks will diminish and there will be an increase in arabinose and XOS peak area. By applying this simple Abf assay in **Paper V**, presumed AXOS components could be confirmed and additional unknown peaks could be identified as arabinose substituted AXOS (Figure 3.4).

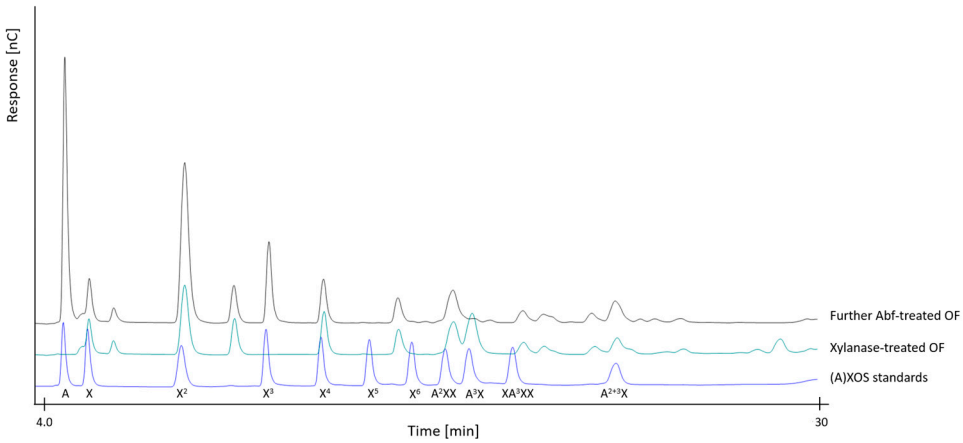


Figure 3.4 HPAEC-PAD chromatogram of xylanase treated oat bran fibre (OF) and further treatment with arabinofuranosidase (Abf). As a result of Abf treatment, peaks which corresponded to AXOS products, for example A^3X and other unidentified product peaks, disappears. The removal of arabinose substituents can also be seen by the increase in arabinose (A), X^2 and X^3.

4 Glycoside hydrolases and carbohydrate binding modules

4.1 Glycoside hydrolases

To degrade lignocellulosic materials, glycoside hydrolase (GH) enzymes can be employed, since these enzymes can hydrolyze glycosidic bonds within various polysaccharide chains, as well as bonds between carbohydrates and other components [68]. The GH enzymes (EC.3.2.1.-) are divided into different families depending on the amino acid sequence, but this feature also reflects similarity in fold and catalytic mechanism. Information about GH subfamilies and their mechanisms, as well as GenBank entries of defined enzymes, is compiled in a database for carbohydrate active enzymes, CAZy [69].

Hydrolytic activity of GH enzymes occur in the active site by two amino acids; one acting as a proton donor and the other as a nucleophile/base [70]. Depending on the exact mechanism, the GH enzymes can be classified as either retaining or inverting. In practice, the mode of action determines whether the configuration of the anomeric carbon (α or β) at the cleavage site will be changed to the opposite configuration compared to the substrate (inverted; β to α) or remain unchanged (retained; β to β) in the resulting hydrolytic products [71]. In this thesis, exclusively retaining GH enzymes were employed. GH enzymes can additionally be classified as endo- or exo-acting, depending on whether hydrolysis occurs internally or at the end terminals of the substrate.

The active site of GH enzymes has several binding subsites, which are used to describe how the sugars in the substrate are binding to the active site and subsequently cleaved [72]. These subsites are numbered from the point of cleavage, occurring between the -1 and +1 subsite, expanding to +*n* subsites in the reducing end direction of the ligand, and to -*n* subsites in the opposite direction, towards the non-reducing end (Figure 4.1). The –*n* subsites are commonly termed the glycone subsites and the +*n* subsites are called the aglycone subsites. The active site amino acid residues that comprise these subsites and determine the surface topology in the area, play a paramount role in shaping the enzyme's substrate specificity, mechanism and hydrolysis products.

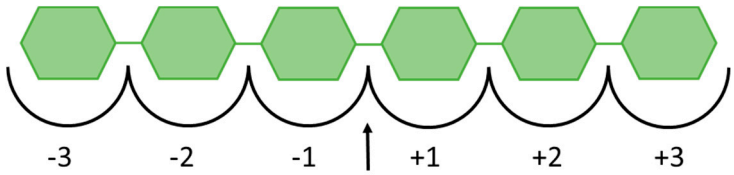


Figure 4.1. A schematic illustration of the sugar-binding subsites within the active site of GH enzymes. The arrow represent the site for cleavage, from which the subsite numbering is starting. The subsites are numbered from this point, towards the reducing end of the oligosaccharide (+1 to +*n*) and the non-reducing end in the opposite direction (-1 to -*n*).

The GH enzymes in focus of this thesis work are endo-1,4-β-xylanases from families GH10, GH11 and GH5. These xylanases can be utilized to break down AX into oligosaccharides, since they specifically hydrolyze the β-1,4-linkage between xylose sugars in the AX backbone [15, 69]. Xylanases within each enzyme family display unique specificities, which make them interesting to study for implementation in oat AX processing. Xylanases from GH10, GH11 and GH5 were investigated and employed for degradation of oat bran and fibre material in **Paper II** and **Paper V**, and a novel GH5 subfamily 34 arabinoxylanase, *Hh*Xyn5A, was characterized in **Paper III** and **Paper IV**.

4.1.1 Xylanases from family GH10 and GH11

Endo-1,4-β-xylanases (EC 3.2.1.8) from family GH10 differ from the endo-1,4-β-xylanases classified into family GH11, in terms of structure, substrate preference and hydrolysis products. Their common feature is the two catalytic glutamic acid residues acting as the nucleophile and general acid/base [59]. However, these enzymes are structurally different, where GH10 xylanases display a TIM barrel $(\alpha/\beta)_8$ fold (Figure 4.2 A) and GH11 xylanases display a β-jelly roll fold (Figure 4.2 B), thus leading to differences in the active site architecture and allowed ligand configurations.

The GH10 active site is small, showing high affinity for short XOS substrates. The conserved glycone subsites have strong binding affinity, in -3 to -1, but the aglycone subsites are less conserved and display weaker binding. Because of the binding orientation of the xylan ligand in the active site, arabinose substitution in the -1 subsite is not possible. Substitution can be accommodated in subsites -2 and +1 to +3 (Figure 4.2 C), although it is often only seen in the -2 subsite [59]. Since the aglycone subsites are less specific, it has a tolerance for glucose units and also β-1,3-linked polysaccharides. A GH10 xylanase that was used throughout this thesis is the commercial endo-1,4-β-xylanase *Geobacillus stearothermophilus* T6 (E-XYNBS, Megazyme), where it showed to be an efficient enzyme to hydrolyze and solubilize AX from extracted oat bran fibres (**Paper II**), as well as in an liquid oat

base process (**Paper V**). However, this GH10 xylanase displayed hydrolysis activity on β-glucan as well, which can be undesired in oat processing.

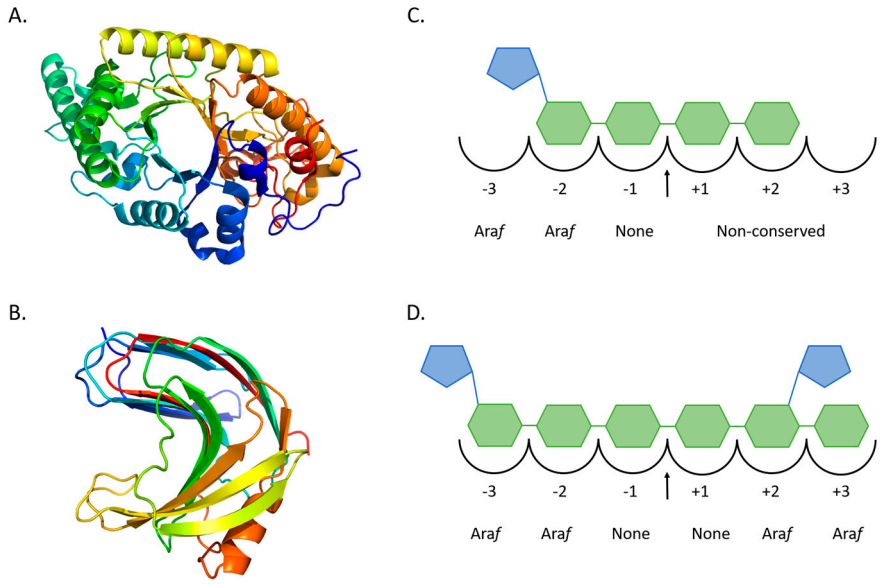


Figure 4.2. A. Tertiary structure of xylanases from family GH10 (E-XYNBS, PDB 1HIZ). B. Tertiary structure of xylanases from family GH11 (*Tl*Xyn11A, PDB 1YNA). C. Suggested substrate and potential products from AX hydrolysis using GH10 xylanase, resulting in products A^3X and X^2 from substrate A^3XXX. D. Suggested substrate and potential products from AX hydrolysis using GH11 xylanase, resulting in products A^3XX and XA^2X from substrate A^3XXXA^2X. Allowed substitution of the Xyl*p* unit for each subsite is stated below the subsite number.

GH11 xylanases are smaller compared to those in GH10, which are often multidomain enzymes. The binding cleft is narrow, long and deep, typically containing five subsites, although up to seven subsites have been suggested [59]. As a result, these enzymes exhibit a preference for longer polysaccharides, showing increased affinity for ligands with a DP ranging from DP3 to DP5 [59]. Due to the narrow cleft, arabinose substitutions on the xylan ligand are not allowed in subsites, -1 or +1, but can be accommodated in -3, -2, +2 and +3 (Figure 4.2 D). In **Paper II**, the GH11 xylanases *Tl*Xyn11A (Pentopan Mono BG, Novozymes) from *Thermomyces lanuginosus* and Feed xylanase (exact source unknown, Novozymes) were utilized for degrading bran fractions and demonstrated their effectiveness in synergy with other enzymes to solubilize AX.

It has been suggested that GH11 xylanases have higher efficiency in degrading insoluble xylan compared to GH10 xylanases, and this may be attributed to their smaller size [59, 73]. In contrast, the more open active site cleft of GH10 xylanases

enables them to accommodate more substituted substrates, whereas GH11 xylanases exhibit a preference for longer substrates, resulting in the generation of alternative reaction products. Furthermore, it is important to note that even within the same xylanase family, different enzymes can exhibit varying substrate specificities. As demonstrated in **Paper II**, enzyme activity and synergy are highly substrate dependent. Therefore, when applying enzymatic processing of biomass using GH enzymes, considerations should be given to the choice of xylanase, reaction conditions, and the substrate matrix, as these factors significantly influence efficiency and the resulting hydrolysis products.

4.1.2 Arabinoxylanases from family GH5 subfamily 34

Some variants of xylanases can accept certain substituents on the xylan chain, however, some even require these moieties for hydrolytic activity. The arabinoxylanases (EC 3.2.1.-) in family GH5 subfamily 34 (GH5_34) are endo-β-1,4-xylanases that are arabinose specific, requiring this substitution in the active site for hydrolytic activity [74, 75]. Similar to GH10 xylanases, the GH5_34 arabinoxylanases display a $(\alpha/\beta)_8$ TIM barrel fold (Figure 4.3 A), with catalytic glutamic acid residues and a highly conserved active site. Many of the GH5_34 enzymes are multidomain enzymes, having two to three carbohydrate binding modules (CBM) in addition to the catalytic domain, which can influence enzyme stability, efficiency and specificity.

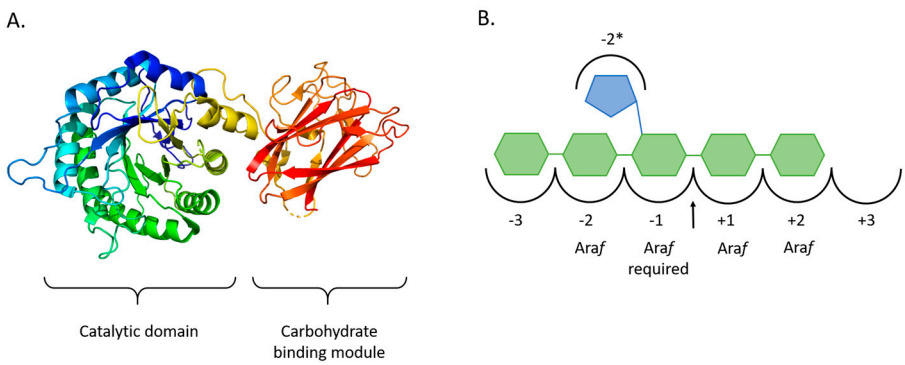


Figure 4.3. A. Tertiary structure of a xylanase from family GH5_34 (*Ct*Xyn5A, PDB 5LA2). B. Active site subsites with suggested substrate and potential products from AX hydrolysis using GH5_34 xylanase, resulting in products XXA^3 and X^2 from substrate XXA^3XX.

There are currently only 9 entries of GH5_34 classified enzymes in the CAZy database, where only one of them has been extensively characterized, *Ct*Xyn5A from *Clostridium thermocellum* [74, 75]. *Ct*Xyn5A displays an open cleft active

site, allowing arabinose substitution in all subsites, from -2 to +2. Arabinose substitution is required in subsite -1, where the ligand has the strongest affinity, with the arabinose sugar binding in a distinct subsite termed -2* (Figure 4.3 B) [16, 75]. Common hydrolysis products from *Ct*Xyl5A include short AXOS with a xylose DP of 1 to 2, and Ara*f* substitution at the O-3 position of the reducing end Xyl*p* residue [16, 74]. In **Paper II**, a commercially available, truncated version of *Ct*Xyn5A was utilized. Its synergistic effects with xylanases from GH10 and GH11 were observed, leading to the solubilization of AX and indicating the possibility to degrade highly substituted substrates.

In **Paper III,** a novel GH5_34 from *Clostridiales* bacterium (initially *Herbinix hemicellulosilytica*), *Hh*Xyn5A (Figure 4.4 A), for the intended use in oat processing, was characterized and compared to *Ct*Xyn5A. The truncated, two-domain variant of *Hh*Xyn5A, showed to be highly storage stable and reaction stable, displaying an optimum temperature around 50-55 °C. Thermostability is an advantageous quality for enzymes to be used in processing, since high temperatures are used to increase solubility and decrease viscosity of AX substrates [59]. *Hh*Xyn5A additionally showed to be arabinose specific and therefore correctly classified to GH5_34, not being able to hydrolyze beechwood xylan or β–glucan, but displaying activity on rye AX, wheat AX and extracted oat bran fibre. In the particular case of oat processing, glucanase side-activity is often an undesired characteristic of the enzymes employed, since it is desired to limit the hydrolysis of valuable high-molecular weight oat β-glucan. *Hh*Xyn5A and *Ct*Xyn5A are both truncated two-domain versions of their native multidomain architecture, comprising of the catalytic domain but also a CBM from family 6 (CBM6). Interestingly, *Hh*Xyn5A and *Ct*Xyn5A demonstrated a difference in oligosaccharide product profile (Fig. 4.4 B), although their catalytic domains and active sites are very similar. Since additional modules of the enzyme can influence specificity, we hypothesized that the CBM6 was the main factor contributing to the observed differences.

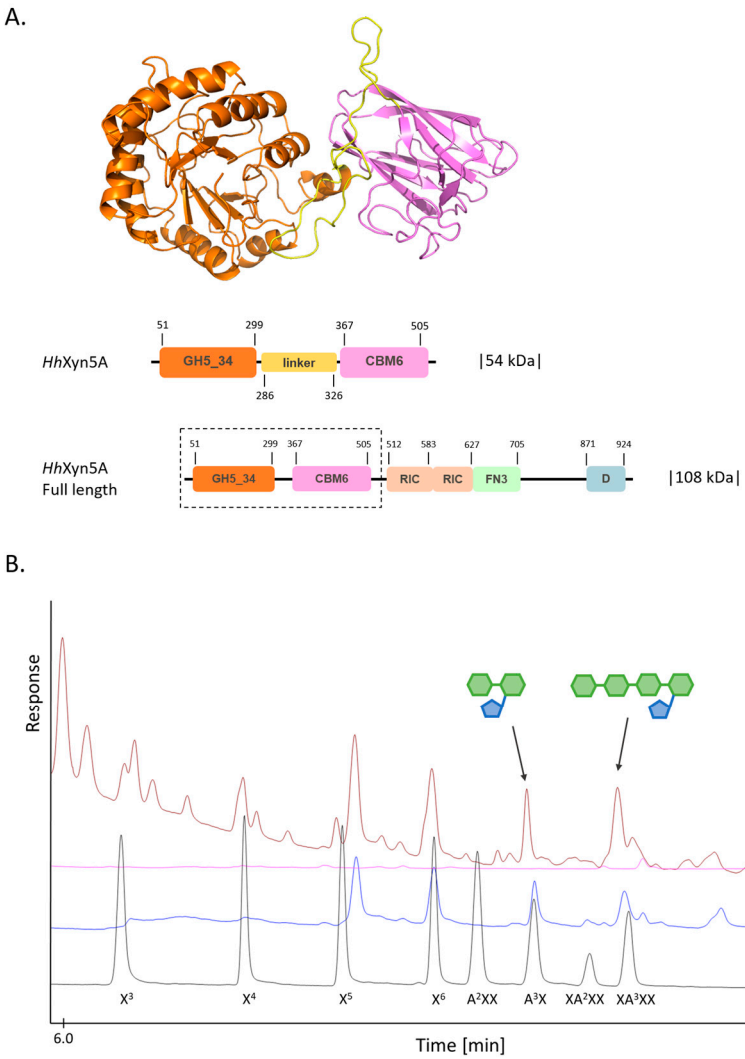


Figure 4.4. A. Modelled tertiary structure of GH5_34 arabinoxylanase *Hh*Xyn5A and the schematic representation of the domain organization of the truncated two-domain version, with catalytic domain and carbohydrate binding module family 6 (CBM6), as well as the full length enzyme. RIC = ricin-type beta-trefoil lectin domain; FN3 = fibronectin type 3 like domain; D = dockerin domain; B. HPAEC-PAD chromatogram of hydrolysed oat bran AX using *Ct*Xyn5A (brown) and *Hh*Xyn5A (blue). Two peaks identified as (A)XOS products, XA^3 and $XXXA^3$, are indicated by arrows and structure.

4.2 Carbohydrate binding modules

Carbohydrate acting enzymes are often multiple domain enzymes, comprising of additional modules to the catalytic domain [76]. These domains are often non-catalytic, instead providing important function to recognize and attach to various biomass (i.e cellulose), and others have yet unknown function. For example, dockerin domains help to link the GH enzymes together in a so called cellulosome complex, for efficient bacterial degradation of cellulose rich biomass [77].

Some of these domains are carbohydrate binding modules (CBMs), which are non-catalytic modules facilitating substrate binding or association to the catalytic domain for increased catalytic efficiency [78]. CBM classification is based on amino acid similarity, but CBMs within the same family do not necessarily share the same specificity. Many families of CBMs display specificity towards several different ligands. Common substrates include cellulose, starch, mixed-linkage glycans, xylans and their oligosaccharide derivatives. Often, CBMs are found attached close to the catalytic domain, to specifically aid in degradation of insoluble substrates [78].

The CBM function varies depending on the family and substrate to be recognized, by either providing proximity to the substrate and concentrate enzyme on the substrate surface, by targeting the ligand at a specific region (for example the reducing end of a polysaccharide), or even by disrupting the substrate [78]. Apart from the classifications into families by sequence similarity, CBMs can additionally be divided into types depending on their structure-function relationship; surface-binding (type A), glycan chain binding (type B) or small sugar binding (type C) [78]. The truncated GH5_34 enzymes *Hh*Xyn5A and *Ct*Xyn5A are both composed of a catalytic domain with an appended family 6 CBM (CBM6), whose significance for function and specificity was studied in detail in **Paper III** and **Paper IV**.

4.2.1 Carbohydrate binding module family 6 (CBM6)

The CBMs in family 6 adopt a common β-sandwich/jelly-roll fold, with two proposed type B binding sites; one located on the edge loops of the sandwich (cleft A) and another on the concave sheet side (cleft B) [78-81]. The CBM6 specificities are highly varied, displaying affinity to cellulose, the ends of β-1,4-glucans, β-1,3-glucans and xylan in cleft A, and cellulose, internal parts of β-1,4-glucans and mixed β-1,4-1,3-glucans in cleft B [78-81]. Cleft A is made up of a few aromatic residues, which can form stacking interaction with sugar rings in the substrate, and additional hydrogen bonds assist in binding the ligand (Figure 4.5). The mode of binding in cleft A vary between CBM6 modules, which display different specificities, however, the conserved aromatic residues have an essential role in binding for example xylo-oligosaccharide ligands [79-81]. A CBM6 from a *Clostridium*

thermocellum GH11 xylanase has shown to bind with high affinity to decorated xylan and XOS with DP 5 [81]. CBM6 from *Ct*Xyn5A has also shown important for affinity to xylan substrates, although, the isolated CBM6 alone did not show affinity to any of the xylan and XOS substrates [74].

In **Paper III** we proposed that the CBM6 domain in *Ct*Xyn5A and *Hh*Xyn5A could potentially serve as the key determinant for the variations observed in their oligosaccharide product profiles. Specifically, we noted that *Hh*CBM6 lacked the conserved aromatic residues in cleft A, which are believed to play a significant role in XOS substrate affinity (Figure 4.5). In **Paper IV** we further explored this hypothesis by switching the two CBM6 domains, *Ct*CBM6 and *Hh*CBM6, between the enzymes, as well as the inter domain linker region connecting the CBMs to the catalytic domain. The results demonstrated that the CBM6 modules were crucial for catalytic function and the hydrolysis product patterns observed. Moreover, it was discovered that the linker region plays a vital role in facilitating proper enzyme folding, while also contributing to the determination of the resulting oligosaccharide product profiles.

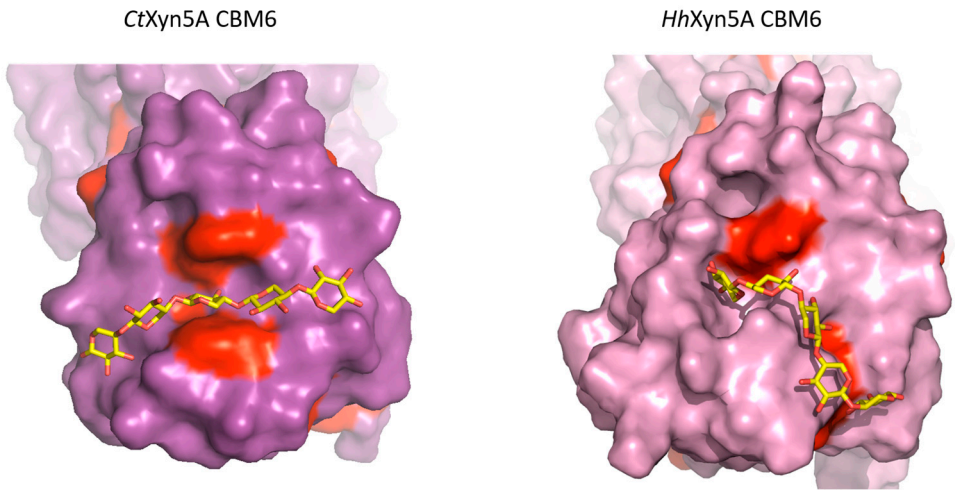


Figure 4.5. Tertiary structure of CBM6 of *Ct*Xyn5A and modelled tertiary structure of *Hh*Xyn5A, with cleft A aromatic residues coloured in red and potential binding mode of ligand X^5.

5 Concluding remarks and future perspectives

By applying specific carbohydrate active enzymes during oat processing, insoluble dietary fibre can be valorized into health-promoting foods, offering consumers more dietary options to enhance their health while promoting a sustainable bioeconomy. This research not only advances the understanding of oat processing but also introduces novel insights into utilizing arabinoxylan (AX) and enzymatic tools to develop sustainable processes and innovative products, in an effort to exploit the full potential of Swedish oats.

The under-utilized AX fibres from oats are resilient to kernel kilning (**Paper I)**, but can be chemically extracted and further efficiently solubilized into oligosaccharides using combinations of lignocellulose degrading enzymes, specifically xylanases (**Paper II**). Synergistic effects between enzymes are highly substrate dependent, and ferulic acid esterase and laccase may be needed to solubilize AX in recalcitrant materials, such as insoluble fibres from oats. By introducing xylanases in a liquid oat base (LOB) process, the soluble AX amount can be significantly increased, creating a high fibre plant-based milk alternative (**Paper V**), while simultaneously reducing insoluble fibre waste.

Identifying and characterizing new xylanases for AX valorisation is important in order to develop efficient bioprocesses, for tailored fibre products. The novel GH5_34 arabinoxylanase *Hh*Xyn5A was highly stable, tolerating high temperatures and showed promising activity on oat AX fibres without having β-glucan side activity (**Paper III**), all of which are desired enzyme qualities for successful industrial application. Interestingly, *Hh*Xyn5A displayed contrasting (A)XOS oligosaccharide product formation compared to the commercial homologue, *Ct*Xyn5A. Swapping of the CBM6 domain and inter-domain linker region, between the two enzymes revealed the importance of CBM6 for substrate specificity as well as the linker region influence on enzyme stability, catalytic activity and oligosaccharide product profile produced by respective enzyme (**Paper IV**). Since small differences in domain structure of xylanases greatly influence specificity and stability, this aspect should be of immense interest, when considering enzyme engineering to optimize activity and tailor enzyme application for specific AX processing.

Continued research and deeper analysis is needed in order to utilize oat AX to its full capacity, and create fibre rich and prebiotic oat products in a cost-efficient manner. In addition to exploring new xylanase candidates, elucidating structure-function relationships for enzyme engineering and optimizing conditions for enzyme synergy, the resulting (A)XOS mixtures should be evaluated on their prebiotic and health-promoting properties, by for example *in vitro* fermentation studies and human trials. Other characteristics of oat (A)XOS such as molecular weight and viscosity of the fibres may affect prebiotic activity as well as consumer acceptance, and should therefore be studied in the product development process. Ultimately, the imperative lies in the ongoing development of enzymatic tools and processes, their adaptation across diverse oat varieties and fractions, all while considering the desired characteristics of the final products.

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