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THE HAGUE
UNIVERSITY OF
APPLIED SCIENCES

Graduation Project

Extraction and anti-oxidation activity of arabinoxylan from corn bran

Process and Food Technology graduation internship
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Chapter 1 Introduction

1.1 General situation of corn production

Corn is one of the world's three major food crops, and it is also the most extensive grain variety in China, accounting for 42% of the grain planting area. According to the data in 2019, corn production in China reached 255 million tons (USDA GAIN 2020). The total production just after the rice and wheat and its cultivated area and total production rank second in the world. Due to the high yield and strong adaptability, corn plays an essential position in agricultural production. However, the proportion of simple processing of corn as food is getting smaller and smaller, and the processing and utilization of corn have shifted to more intensive processing to make it more valuable. In addition, because corn kernels are rich in starch, which accounts for 72 to 73 percent of the kernel weight (Paraginski, R. et al. 2014), so they are often processed into corn starch, which has contributed significantly to the development of corn production.

1.2 Utilization of corn bran

Corn deep processing technology can be broadly divided into two categories: one is wet processing, and the second is dry processing. However, when milling the corn, many low-value by products are produced. The two main by-products produced by this operation are corn bran and corn fibre, which currently have low commercial value (Rose, D et al. 2010). Corn bran resources are abundant in China. Approximately 14% to 20% of the total corn bran is produced each year (Wenxia,W.et al 2016). However, the basic theory and application research of corn bran is still at a relatively low level. For a long time, most the corn bran has been consumed as feed materials or simply going to landfill. The high volume of waste occupies large spaces and thus does harm to the environment. Nowadays, the utilization of corn bran has aroused people's attention and has found that corn bran contains valuable compounds. In order to improve the use-value of corn bran, it is necessary to analyze its chemical composition and research deep processing.

Table 1. Composition (g kg^{-1}) of corn bran and corn fiber

Constituent	Corn bran	Corn fiber
Protein[a]	50–115[13–15]	100–130[16,17]
Starch	40–112[13,14]	150–200[16,17]
Oil	13.2–19[14,18]	17.2–36.8[18]
Ferulate phytosterol esters[b]	0.2[18]	0.61–1.28[18]
Ash	6–10[14,15]	6–20[16,17]
Total dietary fiber	732–860[13,19,20]	526–735[21]
Soluble fiber	2–26[19,20]	ND–3[21]
Insoluble fiber	706–863[19,20]	526–732[21]
Arabinose[c]	128–178[13,14]	113–117[12,16]
Xylose	217–243[13,14]	176–213[12,16]
Mannose	3[15]	ND–8.4[21]
Galactose	44–51[13–15]	35.9[16]
Glucose[d]	182–248[13,15]	300–372[12,16]
Uronic acids	39–42[13–15]	30–40[17]
Lignin	7–10[13,14]	78[16]
Total phenolics	55[13]	NR
Ferulic acid	28–31[13–15]	1.02–18.5[22,23]
Diferulic acid	6.8–32[15,24]	NR
p-Coumaric acid	3–4[13,14]	2[22]

ND, not detected; NR, not reported.
[a] Nitrogen × 6.25.
[b] Indentation indicates that this component is a component of the above constituent but is still reported as a proportion of the entire corn bran or corn fiber product.
[c] Neutral sugars and uronic acids reported in polysaccharide form.
[d] non-starch glucose.

Figure 1. Composition (g kg^{-1}) of corn bran and corn fibre (Rose, D et al. 2010)

As shown in figure 1, the corn bran contains a high amount of hemicellulose, cellulose, starch, protein and phenolic compounds, which is a good source of edible, medicinal plant active polysaccharides. Furthermore, studies have shown that corn bran water-soluble polysaccharides have some health benefits, such as significant hypoglycemia, weight loss and lipid reduction, anti-constipation and antioxidant (Ayala-Soto, F. et al 2014). From the table, the high amount of xylose and arabinose signifies the main components are arabinoxylans. However, it is difficult to show the physiological activity of the arabinoxylan in the corn bran without biological, chemical and physical processing, so it is necessary to remove the protein, fat and cellulose from the corn bran by separation to obtain the pure arabinoxylans.

1.3 An overview of arabinoxylans (AXs)

1.3.1 Structure of arabinoxylans

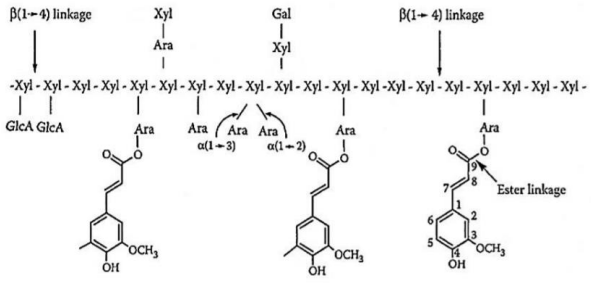

Figure 2. The general structure of the arabinoxylan (Zhurlova,O 2017)

Figure 2 shows that the basic structure of arabinoxylans is mainly composed of β-D-

xylopyranose residues (Xylp) forming a linear backbone and α-L-furanyl arabinose (Ara) is the side chain. The Ara group can be monosubstituted on the hydroxyl group at the C(O)-2 or C(O)-3 position of the xylose residue or double substituted on the hydroxyl groups at the C(O)-2 and C(O)-3 positions. In addition, a small amount of ferulic acid (FA) attached to the C(O)-5 position of the arabinose group is present in the ester bond, which has important effects on arabinoxylan functional properties (Cai, Z.et al 2021). However, the structure of arabinoxylans varies greatly among grains. For example, the structure of arabinoxylans in rice, sorghum and corn bran are more complex than those in wheat, rye and barley. This is because their lateral branches contain small amounts of xylose, galactose, α-D-glucuronide or 4-methyl-α-D-glucuronide residues in addition to arabinose residues (Zhurlova,O 2017). Besides, the amount of arabinoxylan varies from grains. Table 1 represents the content of the total arabinoxylans reaches the highest amount in the corn bran.

Table1. Content of total arabinoxylans in various grains and grain tissues (Biliaderis, C 2007)

Source	Total arabinoxylans (%)
Barely	
Whole grain	6.11
Whole grain	3.4-4.1
Whole grain	-
Pearled grain	4.45
Pearlings	14.14
Pearled flour	-
Wheat	
Whole grain	5.77
Whole grain	-
Bran	19.38
Flour	1.37-2.06
Durum wheat	4.07-6.02
Rye	
Whole grain	7.6
Whole grain	8-12.1
Bran	-
Flour	3.2-3.64
Oats	
Whole grain	2.73
Hulls	8.79
Bran	3.50
Pearled grain	3.00
Rice	
Whole grain	2.64
Hulls	8.36-9.24

Bran	4.84-5.11
Sorghum Whole grain Pearlings	 1.8 5.4
Corn Bran	 29.86
Soybean Hulls	 13.10

1.3.2 Functional properties of arabinoxylans

- Antioxidant activity:

Ferulic acid is a vital component of arabinoxylan and is also an internationally recognized natural antioxidant. Therefore, arabinoxylan contains ferulic acid moiety has a good antioxidant effect. Ferulic acid can quickly form a conjugated structure with a hydrogen atom, thus stabilizing or scavenging free radicals, inhibiting lipid oxidation in food and other functions (Libo,W et al.2016). Through moderate enzymatic hydrolysis, feruloyl oligosaccharides (FOs) can be released from arabinoxylans. Feruloyl oligosaccharide is a compound formed by combining ferulic acid and oligosaccharide through ester bonds, combining the physiological functions of both ferulic acid and oligosaccharide. It has been shown in research from Kikuzaki, H et al (2002) that feruloyl oligosaccharides have a more potent antioxidant capacity than free ferulic acid.

- Regulating intestinal function：

Prebiotics as a dietary supplement can positively affect human health by regulating intestinal flora and immunity, alleviating autoimmune diseases, and enhancing immune defence. As a type of prebiotic, dietary fibre can be fermented by intestinal flora to promote the selective growth of beneficial flora and regulate short-chain fatty acids (SCFA), while dietary fibre can also interact directly with human mucosa to influence cytokine production and play an immune role in improving chronic diseases such as constipation, colitis and ulcers (Makki, K et al. 2018) Arabinoxylan is an crucial dietary fibre in corn bran, and as a new prebiotic, arabinoxylan has been certified by the USFDA, which can specifically promote the proliferation of beneficial bacteria in the intestinal tract of humans and animals. Also, it enhances the intestinal epithelial barrier, relieves constipation, and improve lipid and glucose metabolism and other biological activities (Wu M, Mcnulty N P, Rodionov D A, et al.2015).

- Other functions:

In addition to the above two main functions, it also has lowering blood sugar, immune

regulation, anti-cancer and weight loss functions (Libo,W et al.2016). In a report by Lupton, J. et al (1999), it was found that of all the high-fibre supplements tested, arabinoxylan-rich bran cellulose was the most effective in protecting against tumours. Diabetes mellitus is a chronic hyperglycemic metabolic syndrome caused by defective insulin secretion, defective action or reduced sensitivity of target cells to insulin. Its incidence is increasing year by year. Arabinoxylan, as a significant component of cereal dietary fibre, can play a role in regulating blood glucose.

Because of its many physiological functions, arabinoxylan can be used as an active ingredient in dietary supplements, functional foods and drugs, and is used in food chemicals and bioengineering. Although some scholars have studied arabinoxylan, their understanding is not deep enough, and some physiological functions have not been thoroughly studied, and the mechanism of action is not yet precise. Therefore, it is necessary to do further research on the physiological functions of arabinoxylan.

1.4 Method of extraction

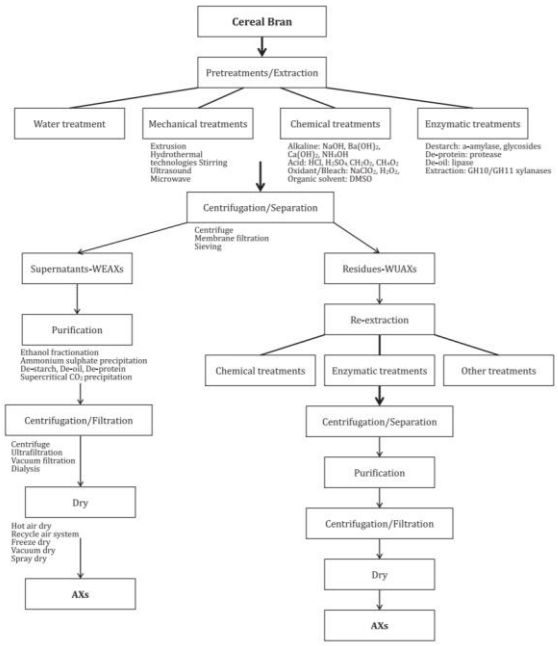

Figure 3. The flow chart of the general extraction process for AXs from cereal bran (Zhang, Z.et al.2014)

Figure 3 points out the general extraction process for arabinoxylans from cereal bran. Because arabinoxylan is also widely present in the cereal bran, these basic extraction methods (water treatment, mechanical treatments, chemical treatments and enzymatic treatments) can be

applied to corn bran. In this paper, the effects of various extraction methods on the extraction yield of arabinoxylan will be introduced. Also summarize the advantages and disadvantages of each method and choose the optimal extraction method for this project.

1.4.1 Water extraction

Water extraction is one of the most common methods to separate arabinoxylans from corn bran. The study of Ganguli and Turner (2008) used wheat flour as raw material, extracted arabinoxylan under the conditions of 1:10 ratio of material to water and stirring at room temperature for 90 minutes, and removed starch with amyloglucosidase. Then, it was purified with 65% ethanol. Finally, the extraction rate of arabinoxylan obtained by precipitation was 0.43%. Moreover, through orthogonal experiments, Xiaoqing and Haizhou (2003) found that the extraction yields of arabinoxylan achieved at 60℃ for 2 hours (1:5 w/v) was 1.78% of rye. Although the extractant in water extraction is safe and non-toxic, cheap and easy to obtain, the yield of arabinoxylan obtained by using the water extraction method according to previous research is low.

1.4.2 Chemical solvents extraction

Due to the arabinoxylan in corn bran and the cellulose and protein in the system, many covalent ester bonds are connected with ferulic acid, which reduces its soluble components. So this requires the extraction solvent to have good solubility, and it also needs to have the function of hydrogen bond breaking. Therefore, chemical treatment method usually uses alkaline solvents or acidic solvents to extract arabinoxylan (Weixu.W et al.2016).

In the presence of an alkaline solvent, the covalent ester bond will be broken, and the previously water-insoluble arabinoxylan will become water-soluble. Commonly used alkali solvents are NaOH, $Ca(OH)_2$ and $Ba(OH)_2$ (Zhang, Z.et al.2014). However, the effect of extracting arabinoxylan varies greatly due to their different degree of action on the cell wall. Xue.L et al (2012) compared five different solvents on the extraction effect of arabinoxylan from wheat bran, which includes $Ba(OH)_2$ and NaOH solvents. The results indicated that the yield of arabinoxylan was different with different solvents. The highest yield reached 14.3% when used $Ba(OH)_2$, while the yield of using NaOH was 10.22%. Bergmans et al (1996) used two alkaline extractant to extract the water-insoluble arabinoxylan from wheat bran. The reagents were $Ba(OH)_2$ and $Ca(OH)_2$. The experimental results showed that the extraction yield obtained using $Ca(OH)_2$ was not as high as that obtained using $Ba(OH)_2$. Because the $Ca(OH)_2$ is less

destructive to the cell wall and incomplete for arabinoxylan extraction. While the reason why $Ba(OH)_2$ can be used as a solvent is that Ba^{2+} can interact with β-glucan in the cell wall to form a substance that is insoluble in saturated solution $Ba(OH)_2$, which can be removed by centrifugation to obtain pure arabinoxylan (Cyran, M., Courtin, C. and Delcour, J., 2004). However, $Ba(OH)_2$ is only suitable for laboratory extraction because of its high cost and complicated subsequent processing.

In contrast, NaOH is more suitable for industrial production owing to its low price and simple process. However, the color extracted by using alkali solvent is dark, which is not favorable for application. Therefore, some people choose to use acidic reagents to extract arabinoxylan. Xu et al (2006) analyzed the impact of different acidic solvents including acetic acid, formic acid, methanol, and ethanol joined with 0.1% HCl at 85 °C for 4 h on arabinoxylan yields from wheat sources. The results showed that when formic acid/acetic acid/water (30/60/10) was used as the extractant, the highest yield of hemicellulose was 29.6% (of the dry basis weight of wheat straw), with 78.1% of arabinoxylan content. The two organic reagents (without acid solvent) yielded lower arabinoxylan yields of 5.5% and 8.6%, respectively, so that high yields of arabinoxylan were obtained using acid solvent. Nonetheless, suppose large amounts of acid solvents are used to treat the grains. In that case, some of the arabinoxylan may be degraded to low molecular weight compounds and dissolved in organic reagents, reducing the yield of arabinoxylan (Zhang, Z.et al.2014). Moreover, one thing common to the use of acids and the use of alkaline is that they both eradicate starch and protein completely, a process that results in almost 100% synthetic cellulose, more than 50% hemicellulose, and 10% to 30% loss of cellulose. However, soluble hemicellulose has complementary physiological functions, so it is unsuitable for chemical extraction (Qinqin.L.,Xin.G, 2012).

1.4.3 Mechanical extraction

In order to improve the efficiency of arabinoxylan extraction, various mechanical techniques have been widely used as a pretreatment process, including steam blasting, microwave-assisted, and ultrasound-assisted methods.

For instance, in steam blasting, the raw material is treated by high-pressure steam, the pressure is rapidly released, leading to explosive depolymerization. This separates the lignocellulosic structure, and the hemicelluloses are then basically removed. Sun et al (2005) compared wheat straw steam blasting at 200 ℃ for 10 min and 33 min, and at 220 ℃ for 3 min, 5 min and 8 min, and then treated with 2% alkaline hydrogen peroxide at pH 11.5 at 50 ℃ for 5 h. The

results showed that wheat straw pretreated by steam blasting obtained hemicelluloses rich in oligosaccharides and monosaccharides. The hemicellulose rich in oligosaccharides and monosaccharides was obtained after pretreatment with steam blasting. The alkali hydrogen peroxide treatment degraded 77.0%-87.6% of the total hemicellulose in the raw material, of which the average hemicellulose content was 38.7%. Although steam blasting is an environmentally friendly and effective method for destroying the structure of lignocellulose, the color of the material after steam blasting is black and sunken. It has also been shown in study by Glasser et al (2000) that steam blasting causes severe degradation of the polymer and result in low arabinoxylan yields.

The ultrasound-assisted method is a mild physical method whose greatest advantages are lower temperature, short extraction time and high yield. However, it should be noted that the ultrasonic time should not be too long, probably due to the strong shearing effect of ultrasonic waves, which may lead to the breakage of chemical bonds of large polysaccharides for a long time and reduce the extraction rate of polysaccharides (Rengen,S.et al.2011).

The microwave-assisted method is mentioned in the experiment of Rose et al (2010). They used corn husk as raw material, treated with microwave at different time and temperature. The results showed that the arabinoxylan content reached a maximum of 50% at 180 ℃ microwaves for 10 min or 200 ℃ microwaves for 2 min. Thus, compared with the traditional alkali-solvent method, the use of microwave assistance significantly reduces the extraction time and does not lead to excessive decomposition of arabinoxylan. However, it has been shown in the literature that some undesirable substances can be produced at high temperatures in this auto-hydrolysis so that steps such as refining and purification are required (Vegas, R et al.2004).

1.4.4 Enzymatic extraction

Enzymatic extraction of arabinoxylans is attractive and harmless to the ecosystem extraction choice that has now been broadly examined. Endoxylanases and cellulases are generally used to separate and debase arabinoxylans from corn bran (Zhang, Z.et al.2014). Under the action of biological enzymes, the initially insoluble arabinoxylan also becomes soluble due to the degradation by enzymes.

Xylanases are used in AX extraction to attack the main polysaccharide backbone and release (solubilise) portions of insoluble-water arabinoxylan. Endocleavage -β - (1,4) -xylanase is the most commonly used, which can break the main chain of xylan, randomly evident the internal

β - (1,4) -junction bond and penetrate the network structure of the cell wall, degrade water-soluble arabinoxylan to produce mixed substituted or unsubstituted xylo-oligosaccharides and increase the solubility to obtain part of water-insoluble arabinoxylan (Alyassin, M., 2016). E. Escarnot et al. (2012) studied the treatment of wheat bran with different commercial xylan endonucleases in combination with commercial cellulases. The results showed that cellulase could improve the arabinoxylan yield. The arabinoxylan yield without cellulase was 38.9%-68.6%, and with cellulase was 62.6%-74.8%. This may be due to the ability of cellulase to digest cell wall β-glucan and break the non-covalent bond between arabinoxylan and β-glucan, releasing more arabinoxylan.

The enzymatic extraction technique stands out among these extraction methods because of its mild conditions, green and non-polluting nature, and maximum recovery of active ingredients. However, the use of different enzymes and process conditions for corn bran can significantly impact the arabinoxylan yield. Therefore, this project decides to use cellulase to extract arabinoxylan from corn bran, and the enzymatic process will be optimized by changing the enzymatic temperature, time and enzyme dosage.

1.5 Research question

To promote the in-depth research, development and utilization of corn bran arabinoxylan, this project aims to extract arabinoxylans from corn bran, optimize the method, analyze its structure and molecular weight characteristics to evaluate the antioxidant capacity of degraded AX. So the research question and sub-questions are:

How to effectively extract arabinoxylan from corn bran?

- *How to establish an effective extraction method with high yield and minimal structural damage of arabinoxylan from the corn bran?*

- *Regarding anti-oxidation, what is the relationship between activity and structure features of arabinoxylan?*

Chapter 2 Materials and methods

2.1 Arabinoxylans extraction process

2.1.1 Corn bran pretreatment

Before the pretreatment, the mass, starch content and protein content of the raw material were measured. In the beginning, the corn bran was washed with water, drained and mixed with distilled water in the ratio of 1:4, then adjusted to pH 3.5 with 4 mol/L HCl solution and let to stand at room temperature for 24 h. Afterwards, the solution was washed with distilled water to neutral, drained and dried in an oven at 105 °C for 4 h. Then the dried bran was crushed repeatedly with a grinder until all the brans passed through 40 mesh sieve. After that, a suitable amount of bran was prepared in suspension with water at 60 °C, sterilized for 15 min and then cooled to room temperature. Next, the prepared bran suspension was heated to 75 ℃ and stirred well. At the same time, added a certain amount of high-temperature resistant α-amylase and neutral protease to remove the starch and protein hydrolysis, and the constant temperature was maintained for 4 h. Then, the water temperature was raised to 100 ℃ for 15 min. Finally, the enzyme was inactivated, and the filtrate and residual solids were filtered after cooling to room temperature. The mass, starch content and protein content of the raw materials were also tested after pretreatment. The detailed detection method for protein, starch and β-glucan is described in the following.

The Megazyme total starch assay kit was used to detect starch, and the testing experiment was processed according to the detailed data booklet provided. The figure 4 describes the process of measuring the total starch content. Then the starch content can be calculated based on the formula and absorbance value. The formula is put in the appendix.

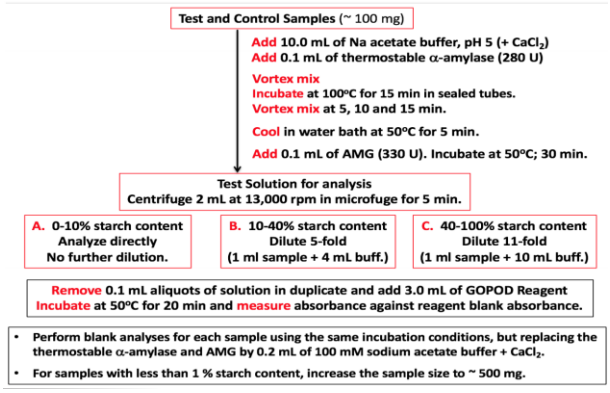

Figure 4. Measurement of the total starch content (Megazyme. 2021)

The protein was tested by using the BCA protein assay (Smith,P et al.1985). Under alkaline conditions, the protein can complex with copper ions to form complexes, and reducing Cu^{2+} to

Cu^+ at the same time. The concentration of the protein in the sample can be calculated by measuring its absorption value at 562 nm and compare it with the standard curve. The BCA assay can be roughly divided into four steps.

S1: Preparing diluted albumin standards (BSA standards)

S2: Making working reagent according to the assay kit instruction and calculating the total volume by using formula:

Total volume = (the total number standard samples + the total number of tested samples) * number of duplicate tests * the volume of working solution required for each test)

S3: Operating the microplate

S4: Using the instrument to measure the absorbance at 562 nm, draw a standard curve and then use the standard curve to determine the protein concentration of the sample.

As for the β-glucan, the content amount can be tested by using Congo red method. It can be briefly summarized as the following steps ((Jia,W.et al 2015): S1: Preparing the standard β-glucan solution and the tested β-glucan solution sample, also the Congo red solution

S2: Making the standard curve, measuring the absorbance of the tested solution, and calculating the β-glucan content in the sample according to the standard curve.

S3: Using the standard β-glucan concentration as the horizontal coordinate and the absorbance as the vertical coordinate, the β-glucan standard curve is plotted to obtain the regression equation so that the β-glucan content in the sample can be calculated based on the regression equation.

2.1.2 Enzymatic extraction process

After pretreatment, the corn bran was mixed with NaAc buffer in a specific ratio, and a certain amount of cellulase was added for extraction. The reaction was carried out at 40 ℃ for 60 min, and the enzyme was inactivated by heating in a water bath at 100 ℃ for 10 min. After cooling to room temperature, centrifuging the solution at 3000 rpm for 5 min to obtain the supernatant. Finally, the arabinoxylan from corn bran can be achieved after filtration, concentration and freeze-drying step.

At the same time, alkaline extraction was carried out as a comparison. First, a certain amount of destarched and deproteinised corn bran was weighed and added to saturated $Ba(OH)_2$ solution. In this step, the extraction was carried out at room temperature for 16 h using a 1:10 ratio of material to liquid. Then obtained the supernatant from centrifuging and concentrated by spinning and dialyzed in distilled water. Afterwards, the precipitate was lyophilized overnight and the arabinoxylan was obtained. After the experiment, the arabinoxylan extraction

rate was calculated according to the following formula:

$$\text{The yield of arabinoxylan} = \frac{the\ mass\ of\ extract\ arabinoxylans\ (g)}{the\ mass\ of\ destarched\ and\ deproteinised\ corn\ bran(g)}$$

2.2 Optimization of the enzyme extraction process

In order to improve the yield of arabinoxylan, the enzymatic extraction process needs to be optimized. Therefore, several single-factor experiments were carried out to separately investigate the effects of feed-to-liquid ratio, cellulase amount and extraction time.

- **The experiment of feed-to-liquid ratio:**

The arabinoxylan was extracted at a temperature of 40 ℃ and an extraction time of 1h at a ratio of 1:7, 1:10, 1:15 and 1:20 respectively.

- **The experiment of cellulase amount:**

The arabinoxylan was extracted at 40 °C and the feed-to-liquid ratio of 1:10 with cellulase additions of 0.01, 0.02, 0.04 and 0.06 g/g (per gram of corn bran), respectively.

- **The experiment of extraction time:**

The arabinoxylan was extracted at 40 ℃ and the feed-to-liquid ratio of 1:10 with the extraction time is 30 min, 60 min, 90 min and 120 min, respectively.

2.3 Structure determination

2.3.1 Determination of monosaccharide composition

A pre-column derivatization with 1-phenyl-3-methyl-5-pyrazolone (PMP) reagent combined with high-performance liquid chromatography (HPLC) was utilized to determine monosaccharide components in arabinoxylans. This experiment referred to Ai, Y et al (2016) and Xuehao.W et al (2016) with slight modifications which mainly contains these major steps

- **Hydrolysis of arabinoxylans:**

The arabinoxylan extracted by enzymatic method (2 mg) was dissolved in 2 ml of 2M trifluoroacetic acid in ampoule. After sealing, the solution was hydrolyzed at 110 ℃ for 4 h, spin-dried and washed several times with methanol to remove the residual trifluoroacetic acid. Finally, added 10 ml of distilled water to obtain 1.0mg/ml hydrolyzed sample for the following experiments.

- **Preparation of standard monosaccharide solutions:**

Weighed 10 mg of mannose, glucose, galactose, galactonic acid, gluconic acid, xylose and arabinose separately, dissolved with water and fixed the volume to 10 ml. The comparison

solution with the concentration of 1mg/ml of monosaccharide mixed standard was obtained. Diluted step by step as needed when using.

- **Derivatization of hydrolyzed arabinoxylans with PMP:**

Took 0.2 ml of monosaccharide mixture standard solutions and arabinoxylan hydrolysis solutions respectively in a 10 mL centrifuge tube. After that, added 0.2 ml of 0.3 mol/L NaOH solution, mixed well and then added 0.2 ml of 0.5 mol/L PMP methanol solution. It was then derived under 70 ℃ in a water bath for 1 h. Next, cooled to room temperature to neutralize NaOH in the solution, added 200 μL of 0.5 mol/L HCl and mixed well. Afterwards, added 1.0 ml of trichloromethane and vortex extract 3 times to remove the excess PMP solution. At last, took the supernatant and fixed the volume to 2 ml.

- **HPLC analysis conditions:**

The solution obtained from the above treatment was filtered through a 0.22 μm microporous filter membrane. According to the following chromatographic conditions, the analysis was performed: Philomon Gemini C_{18} column (4.6 mm×250 mm, 5 μm). The detection wavelength: 245 nm; Column temperature: 30 ℃； Injection volume: 20 μL; Flow rate: 0.8 mL/min; Mobile phase was acetonitrile: phosphate buffer solution (12 g/L, pH 6.8) = 18:82 with isocratic elution.

2.3.2 Determination of molecular weight

The relative molecular mass distribution of arabinoxylan samples obtained under enzymatic extraction conditions was determined by gel permeation chromatography (GPC).

- **Chromatographic conditions:**

The Ultrahydrogel liner column (7.8 mm*300 mm) was used in this experiment. The temperature of the column and detector temperature was set at 45 ℃. The mobile phase selected 0.10 mol/L $NaNO_3$ solution, and the flow rate was 0.9 mL/min.

- **Detection method:**

The dextran standard (1.0 mg/ml) with known relative molecular mass and the arabinoxylan samples (10 mg/ml) obtained under enzymatic extraction conditions were dissolved using mobile phase. Then the supernatant was filtered through 0.45 μm membrane after high-speed centrifugation for analysis. After that the retention time of arabinoxylan was measured, and the relative molecular mass of arabinoxylan was obtained using the standard curve.

2.4 The degradation of arabinoxylan

The complete degradation of arabinoxylans requires the synergistic action of multiple enzymes to be accomplished. They mainly include endo-xylanases, xylosidases, and xylan branched

chain decomposing enzymes. The differences in amino acid sequences and spatial structures determine the differences in their enzymatic cleavage sites when hydrolyzing xylans, so xylanases can be classified into different glycosidic hydrolases (GH) depending on the hydrolysis of glycosidic bonds (Zhao,L 2017). In this experiment GH43 and GH51 were chosen to degrade arabinoxylans. As shown in Figure 5, GH43 and GH51 are both xylan branched chain-degrading enzymes that mainly act to break down various branched chains on the xylan side chain.

In this degradation experiment, four separate experiments will be tested and compared, namely the original assay, addition of GH51 and GH43 separately and addition of both GH43 and GH51. The general flow has been briefly drawn as shown in Figure 6 and the product fragments will be analyzed after degradation is completed.

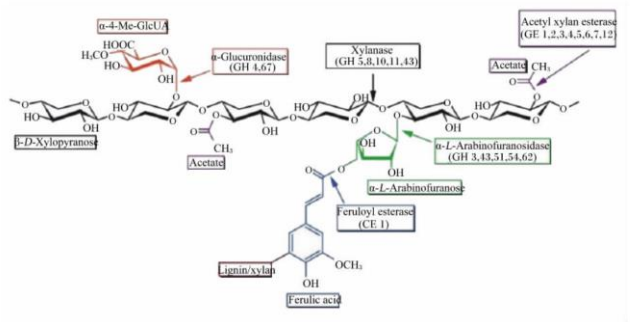

Figure 5. The cutting site of the xylanase degradation system (Zhao,L 2017)

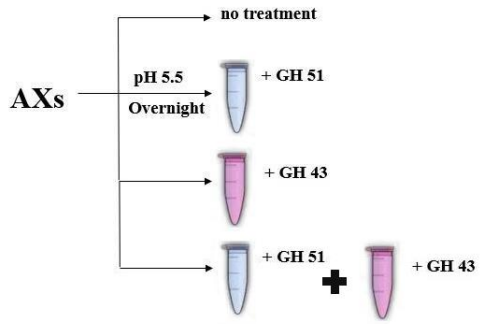

Figure 6. The process of degradation the arabinoxylan

2.5 The antioxidant activity experiment

In this experiment, the inhibition rate and inhibition capacity of the samples were determined using the hydroxyl radical assay kit, which is based on the principle that the amount of hydroxide ions and H_2O_2 produced by the Fenton reaction ($H_2O_2+Fe^{2+}=\cdot OH+ H_2O+Fe^{3+}$) is proportional to the amount of hydroxide ions, and when the electron acceptor is given, the color

is developed with the Griess reagent to form a red substance whose color presentation is proportional to the amount of hydroxide ions.

The degraded arabinoxylan, the samples with GH43 and the samples with GH51 were selected for experiments, respectively. The detail of the experiment is introduced below: added ultrapure water to prepare the sample into 1 mg/mL of degraded arabinoxylan solution, and then a part of it was diluted into 0.5 mg/mL and 0.2 mg/mL of arabinoxylan solution by adding ultrapure water. After distillation, took 0.2 ml of each sample solution from the concentration of 1 mg/mL, 0.5 mg/mL and 0.2 mg/mL in a test tube and placed them in a water bath at 37 ℃. Then added the configured application solution to it and mixed it well. Removed it after timing for 1 minute and then added 4mL of color developer, mixed well and left it for 20 minutes. The absorbance of each tube was measured at a wavelength of 565 nm and an optical diameter of 1 cm, and zeroed by RO pure water. Finally, the corresponding inhibition rate and inhibition capacity were calculated according to the formula.

Chapter 3 Results and discussion

3.1 Pretreatment

The purpose of the pretreatment process is to reduce the content of impurities in the corn bran and to increase the yield and purity of arabinoxylan. In this step, 50 g of raw wheat bran was first taken to determine of the corresponding components. The starch content before pretreatment was 10.89%, the β-glucan content was 5.56% and the protein content was 10.60%. After the corresponding enzyme treatment, the mass of bran decreased to 40 g, where the content of starch was reduced to 2.06%, the content of β-glucan to 1.24%, and the content of protein to 1.68%. The changes in the content of each component are shown in Table 1. From the data, it indicates that the content of starch, β-glucan and protein in the non-pretreated corn bran is high. In contrast, after the addition of protease and amylase treatment, the content of impurities in the bran is greatly reduced, which is more favorable to improve the extraction rate of arabinoxylan.

Table 2. Changes of each content before and after pretreatment

	Mass (g)	Starch content (%)	β-glucan content (%)	Protein content (%)
Origin	50 ± 0.13	10.89 ± 0.25	5.56 ± 0.03	10.60 ± 0.47
After pretreatment	40 ± 0.26	2.06 ± 0.18	1.24 ± 0.09	1.68 ± 0.31

3.2 Optimal enzymatic extraction method

- **The study of the effect of feed-to-liquid ratio on arabinoxylan yield**

The linear trend in figure 7 shows that the arabinoxylan yield increased and then stabilized with the increase of extractant volume. The concentration difference at the contact interface between the crude extract and the solvent increases as well as the mass transfer rate which leading to an improvement in the yield. When the feed-to-liquid ratio reached 1:10, the arabinoxylan yield reached 11.8%. The arabinoxylan yield stabilized when the extractant volume continued to increase. Therefore, the optimum ratio of 1:10 is chosen in the extraction process.

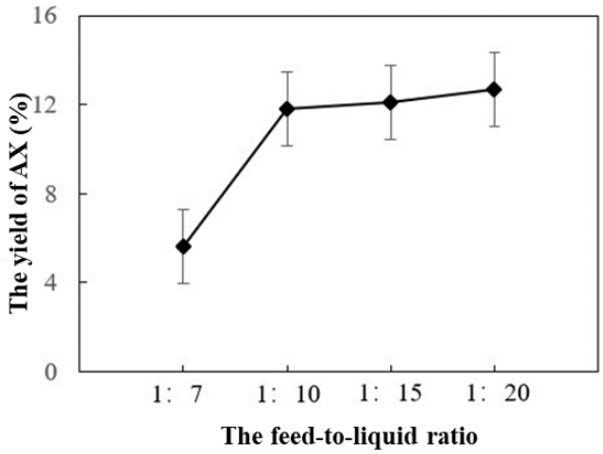

Figure 7. The relationship between the feed-to-liquid ratio and the arabinoxylan yield

- **The study of the effect of cellulase addition on arabinoxylan yield**

It can be seen from figure 8 that the extraction rate increased continuously with the increase of enzyme addition, but the growth of extraction rate levelled off after 0.04 mg/g. It is inferred that there is enough cellulase to digest the cell wall cellulose and break the non-covalent bond between arabinoxylan and cellulase to release arabinoxylan. According to the perspective of energy saving, the optimal amount of enzyme addition is selected as 0.04 mg/g.

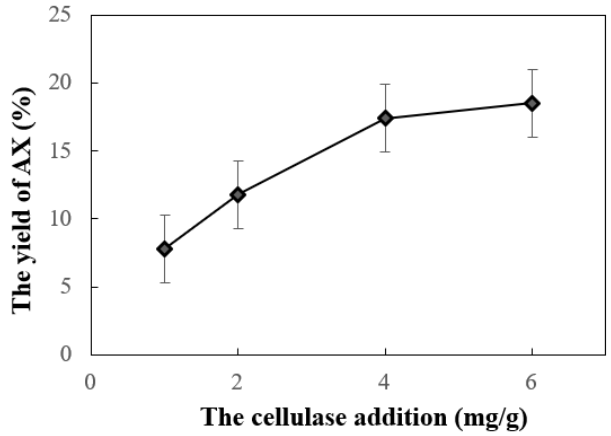

Figure 8. The relationship between the cellulase addition and the arabinoxylan yield

- **The study of the effect of extraction time on arabinoxylan yield**

Figure 9 reveals that when the extraction time was less than 1h, there was an upward trend from 30min to 60min. This is because the extraction time is too short and the enzyme is not entirely in contact with the crude extract, so increasing the extraction time is beneficial to increasing the extraction rate. However, when the extraction time reached 1h, the increasing trend was slowed down, signifying that the extraction was completed after 1h. Therefore, from the analyses above, we can conclude that optimal extraction time is chosen as 1h.

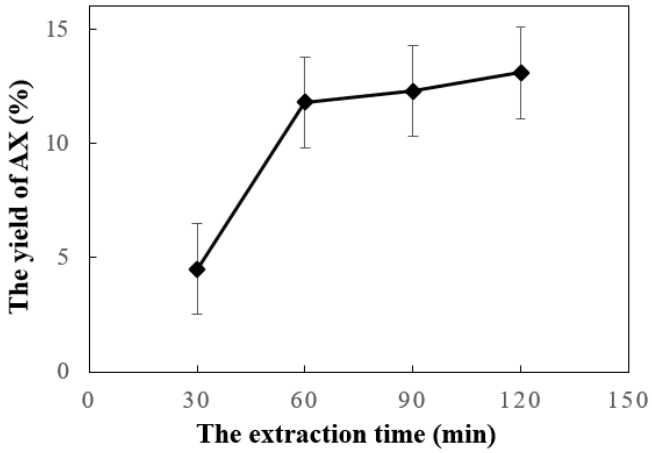

Figure 9. The relationship between the extraction time and the arabinoxylan yield

3.3 The yield of arabinoxylans

Arabinoxylan is divided into water-soluble arabinoxylan (WEAX) and water-insoluble arabinoxylan (WUAX). Although the yield of both types of arabinoxylan are calculated, only the water soluble arabinoxylan was used in the further experiments. Therefore, in this part, only the WEAX is discussed.

As can be seen from the table 3 and table 4, the yield of arabinoxylan obtained by enzymatic extraction was higher than that obtained by alkaline extraction. Although small amounts of protein, as well as starch and β-glucan were still present, the content was significantly reduced compared to the pretreated. Moreover, the arabinoxylan extracted by enzymatic extraction had less starch content, so it can be concluded that the samples extracted by enzymatic extraction were purer than the alkaline method.

Table 3. Extraction rate of arabinoxylan by using the enzymatic method

	The yield of arabinoxylan (%)	Starch content (%)	β-glucan content (%)	Protein content (%)
WEAX	**46.7±0.23**	**0.92±0.03**	**0.58±0.02**	**0.72±0.09**
WUAX	32.5±0.56	0.2±0.01	0.18±0.01	0.15±0.03

Table 4. Extraction rate of arabinoxylan by using the alkaline method

	The yield of arabinoxylan (%)	Starch content (%)	β-glucan content (%)	Protein content (%)
WEAX	**35.7±0.12**	**1.10±0.05**	**0.58±0.21**	**0.72±0.17**
WUAX	29.9±0.3	0.68±0.04	0.18±0.04	0.15±0.01

3.4 The monosaccharide composition

The extracted WEAX was subjected to HPLC analysis, in which the results of WEAX and residues were analyzed as shown in Figure 10 and table 5. Since the peak area obtained on the chromatogram could not directly reflect the content of the monosaccharide components, the histogram was utilized to present it in more clearly way.

The monosaccharide fraction of arabinoxylan extracted by enzymatic extraction was relatively simple, consisting mainly of xylose, arabinose, glucose and galactose. The arabinoxylan content in WEAX was 75.63%, with a slightly higher content of arabinose than xylose, with A/X of 1.05. The arabinose and xylose accounted for about 60% of the total monosaccharaides composition, along with some glucose, galactose, etc., and a tiny amount of mannose and Glc A. While, the arabinoxylan content in the residue was low and the percentage is about 42.11, with A/X of 0.97. The glucose content in the residue was higher, occupying nearly 50%, while the remaining majority was still arabinose and xylose, and a small amount of Glc A and galactose. This reveals that some arabinoxylans are not extracted from corn bran, and the extraction process needs to be improved.

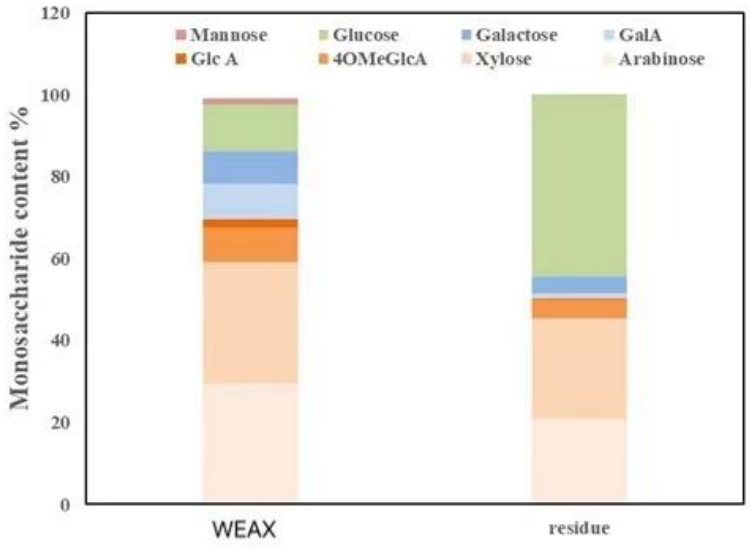

Figure 10. Results of monosaccharide composition analysis of arabinoxylan

Table 5. Extraction rate and structural features

	Arabinoxylan	A/X (Ara/Xyl)
Sample	75.63%	1.05
Residue	42.11%	0.97

3.5 The molecular weight

The extracted WEAX by gel permeation chromatography method was shown in figure 11. The graph indicates two peaks, indicating that the molecular mass of arabinoxylan has two main distribution ranges. The first peak appeared at around 20 min, and the peak time was calculated from the standard curve, which obtained the molecular weight was 156690 Da. The second peak appeared at about 27min, and the area of the peaks was calculated from the standard curve, and the molecular weight was obtained 7625.3 Da. The first peak increased significantly compared to the second peak, which implies that the arabinoxylan macromolecules in corn bran were mainly aggregated at this stage. The coworkers in our group purified the AXs of 156690 Da using SEC column, which was utilized as the material in enzymatic degradation and antioxidant assay.

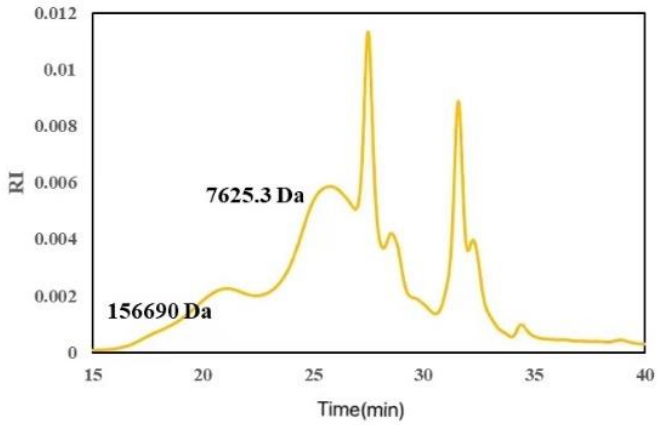

Figure 11. The relative molecular weight distribution of arabinoxylan by GPC

3.6 The analysis of degraded arabinoxylan

It was confirmed in the paper by Preece and Macrenzie (1952) that grain feeds contain two main types of anti-nutritional factors: xylan and β-glucan. Xylan, also known as arabinoxylan, acts as a physical barrier together with other cell wall constituents to prevent the release of intracellular nutrients. WEAX absorbs water in the digestive tract to form a gelatinous solution, gradually forming large molecules that interfere with the digestive process in animals through their viscous effect, reducing the absorption of fats and proteins, promoting increased intestinal microbial reproduction, and leading to increased animal morbidity (Benguang,Z 2008). Therefore, WEAX has an anti-nutritional effect, where xylanase are chosen to degrade xylan, reducing the harmful effects on the organism and enhancing the beneficial effects.

The histogram in figure 12 clearly shows that the structural analysis of the degraded arabinoxylans differs depending on the enzyme added. GH 43 refers to α-Arabinofuranosidase,

exhibits highly specific hydrolysis of α-1,3-linked L-arabinofuranose residues from doubly substituted D-xylosyl or L-arabinosyl residues of arabinoxylans and branched arabinans, respectively. The other enzyme named GH51, could remove α-1,2 and α-1,3-linked L-arabinofuranose residues from arabinoxylans and branched arabinans. There was no free araf unit in the original arabinoxylan (Fig. 13). However, after adding GH43, the araf, X6 and xylose content started to appear.

The amount of free araf, X2 and X3 unit increased significantly after the addition of GH51. When adding the GH43 and GH51 together, the proportion X5 content occupied the second place in the total contents. Therefore, we can know that most araf connected to the main chain through α-1,2 or α-1,3 linkage. Furthermore, a small number of them linked by double substitution.

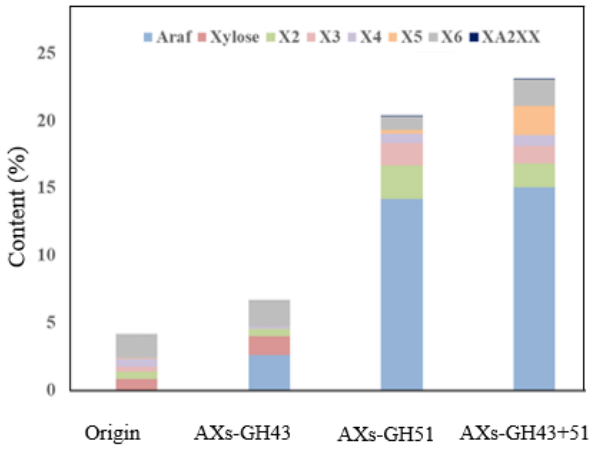

Figure 12. Analysis of products without and after enzyme treatment

3.7 The evaluation of antioxidant ability

The result in the figure 13 points out that the ability to scavenge hydroxyl radicals increases with increasing AXs concentration. Also, these results confirm the antioxidant activity of AXs which are able to donate electrons or hydrogen atoms to free radicals, leading to the conversion of free radicals into stable products. According to the comparison between AXs-GH43 and AXs-GH51 (the The free small molecular groups degraded by enzyme have been removed, only the backbone structure left), the AXs-GH43 exhibited higher hydroxyl radical scavenging activity. The difference in inhibitory ability may be related to their structure, especially ferulic acid content. Just like the fragment analysis done in the previous experiment, the type and content of groups revealed by adding GH43 and adding GH51 were not the same. An amount of ferulic acid group linked to the α-1,2 and α-1,3-linked L-araf residues was removed by GH51,

resulting the lower hydroxyl radical scavenging rate. It was confirmed by the ferulic acid content of hydrolysate of GH43 and GH51, which was done by coworkers.

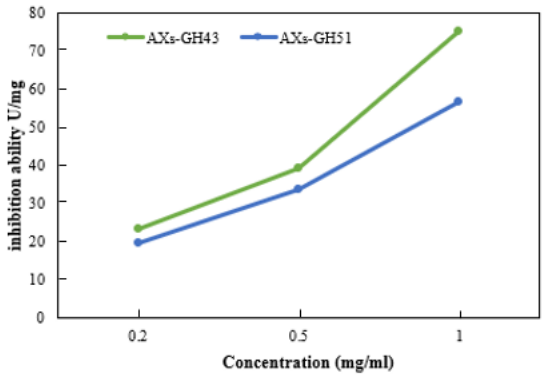

Figure 13. Hydroxyl radical-scavenging effects of arabinoxylans.

Chapter 4 Conclusion

In this project, corn bran was used as raw material, and the refined corn bran was first obtained by pretreatment to remove the protein and starch that were more abundant in corn bran. Then two extraction methods, alkaline extraction and enzymatic extraction were selected to extract arabinoxylan from corn bran. In order to maximize the yield of arabinoxylan, several single-factor experiments were designed to optimize the enzymatic extraction method. As a result, we found optimal conditions: the amount of cellulase added was kept at 0.04 mg/g, the extraction time was controlled at 1 h, and the feed-to-liquid ratio was fixed at 1:10. The arabinoxylans extracted from corn bran were divided into WEAX and WUAX. In this project, only WEAXs were investigated. The yield of WEAX was 46.7% by enzymatic extraction and 35.7% by alkaline extraction, which proved that the use of enzymatic extraction is an environmentally friendly process and effective in improving the yield of arabinoxylan.

After that, the structural analysis of WEAX was carried out to determine the monosaccharide composition by PMP-HPLC method. The results showed that the monosaccharide composition of arabinoxylan was mainly arabinose and xylan, with small amounts of glucose and galactose. Also, the molecular weights of arabinoxylan were measured by GPC method as 7625.3Da and 156690Da. The larger one was purified and utilized for the enzymatic degradation. Since WEAX has an anti-nutritional factor in it, so it is necessary to degrade. In the degradation experiments, two enzymes, GH43 and GH51, were selected for the degradation of arabinoxylan, and the structure of fragments was analyzed. Afterwards, their degraded fragments were subjected to antioxidant analysi The product degraded by GH51 exhibited lower antioxidant activity, because an amount of ferulic acid groups linked to the α-1,2 and α-1,3-linked L-araf residues was removed. Therefore, based on these experiments above, the main research question and the sub-question of this project can be easily figure out.

Chapter 5 Recommendation

The extraction method for obtaining arabinoxylans from corn bran is still inadequate, as evidenced by the determination of the monosaccharide composition, which still leaves some of the AX unextracted. Therefore, the enzymatic extraction method can be further improved to enhance the yield of arabinoxylan. The current extraction method is limited to a single method, and then a combination of multiple methods can be tried to improve the extraction rate. It is mentioned in the introduction of extraction methods that mechanically assisted methods have higher extraction efficiency and can be combined with enzymatic methods to improve the yield of arabinoxylan.

In the antioxidant analysis of degraded arabinoxylans, only the GH43-added and GH51-added were analyzed. However, the fragments with both GH43 and GH51 additions were not analyzed. This fragment was not analyzed in this project because of the complexity and time-consuming nature of the experiments to analyse the simultaneous addition of two enzymes. However, if this fragment could be analyzed, it would be possible to have a better understanding of the relationship between the structure of arabinoxylan and the antioxidant properties.

AX is considered prebiotic, as demonstrated by its ability to promote the proliferation of probiotic bacteria in the human intestine. However, here, only the antioxidant properties of AX have been investigated, and the aspects concerning prebiotics have not been studied in depth. Therefore the following study could explore the relationship between the structure of AX and the probiotic function.

Appendix

Calculations for total starch content

- **Solid samples:**

$$Starch, \% = \Delta A * F * \frac{EV}{0.1} * D * \frac{1}{1000} * \frac{100}{W} * \frac{162}{180}$$

$$= \Delta A * F * EV * \frac{D}{W} * 0.9$$

Where:

ΔA =Absorbance of sample solution read against reagent blank, less the absorbance of the sample blank read against (only where a sample blank is determined)

F = Factor to convert absorbance values to µg glucose

EV = Sample extraction volume

0.1 = Volume of sample analyzed

D = further dilution of sample solution

1/1000 = conversion from µg to mg

100/W = conversion to 100mg sample

162/180 = factor to convert from free glucose, as determined, to anhydroglucose, as occurs in starch

- **Liquid samples (mg/100mL)**

$$Starch = \Delta A * F * \frac{DSV}{SV} * \frac{100}{0.1} * \frac{1}{1000} * \frac{162}{180} * D$$

$$= \Delta A * F * DSV/SV * 0.9$$

Where:

ΔA = Absorbance (reaction) read against the reagent blank minus the absorbance of the sample blank read against the reagent blank.

F = Factor to convert absorbance values to mg of D-glucose

DSV = Diluted Sample Volume

SV = Volume of sample taken for analysis

100 = Conversion to 100 mL sample volume

0.1 = Volume of sample analyzed

1/1000 = Conversion from µg to mg

162/180 = Adjustment from free D-glucose to anhydro D-glucose

D = further dilution of the incubation mixture

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