Water holding capacity and enzymatic modification of pressed potato fibres

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ABSTRACT

Cell wall polysaccharides (CWPs) contribute to the water holding capacity (WHC) of fibre rich feeds, such as pressed potato fibres (PPF). However, the role of CWPs on the WHC of PPF was unidentified so far.

PPF was characterized to be abundant in arabinogalactan (AG) linked rhamnogalacturonan-I (RG-I), homogalacturonan (HG) and cellulose, next to which xyloglucan (XG) contributed the most of the hemicellulosic CWPs. The CWP network in potatoes was loosened upon starch extraction of potatoes and solubilized HG-RG-I-AG.

Analyses of the WHCs upon enzyme treatments indicated that the WHC of PPF was mainly caused by a network of insoluble, non-cellulosic CWPs such as pectic CWPs (HG-RG-I-AG) and XG. Findings in this thesis showed that AGs were better degraded than xyloglucans (XGs). Since XGs were found to be equally important in contributing to the WHC as AGs, the substantial removal of AGs, as well as XGs, should be advantageous to lower the WHC.

Other than lowering the WHC, the use of a pectinase-rich preparation improved the recovery of starch from potatoes by the degradation of mainly pectic CWPs, in particular pectic AG side chains and HG. The degradation of arabinan was observed to be inhibited by components in potato juice (PJ).

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Chapter 1

General Introduction

1.1 Preface

Annually, the industrial production of potato starch (1,600,000 tonnes) in the EU, yields about 135,000 tonnes (dry basis) of a fibre-rich by-product. This by-product is rich in fibres and pressed to remove potato liquids during the starch separation process. Therefore, the remaining solids are named “pressed potato fibres” (PPF) (Grommers and Krogt, 2009). Nowadays, PPF is mainly used as cattle feed. The application of PPF is hindered because of its high water holding capacity (WHC; Mayer and Hillebrandt, 1997). The high WHC makes PPF bulky, colloid-like and difficult to process. Therefore, a challenge lies in lowering the WHC of PPF. It is known that the WHC of fibres is influenced by the composition and interactions of the constituent cell wall polysaccharides (CWPs; Mayer and Hillebrandt, 1997). Enzymatic hydrolysis of these CWPs will lower the WHC.

The research described in this thesis was part of a large project carried out within the Dutch Carbohydrate Competence Centre. The project entitled “Pre-treatment of agricultural by-products” also aimed at investigating other non-enzymatic processing techniques, to help degrade plant cell walls in order to extract components enclosed within cells. The focus of the current research is on determining the composition of CWPs in PPF and how they affect WHC. Also, by the enzymatic hydrolysis of CWPs, the research aimed at increasing starch recovery from potatoes and lowering the WHCs of the corresponding fibre fraction.

1.2 Composition of potatoes (*Solanum tuberosum*), production of industrial starch and PPF

1.2.1 Composition of potatoes

The dry matter content of potatoes varies from 17 % to 30%, depending on the variety, period of storage, seasonal conditions and soil environment (Grommers and Krogt, 2009, van Dijk et al 2002, Burton, 1989). Potatoes have a high starch content, which is 76 w/w % based on dry matter (dm), and 4 w/w % (dm) is constituted by CWPs. The remaining dry matter comprises 2.7 w/w % mono-disaccharides, about 11 w/w % as

proteins, peptides and amino acids, 2.8 w/w % organic acids, 4 w/w % ash, 0.4 w/w % lipids, 0.13 w/w % phenolics and 0.1 w/w % Vitamin C (Grommers and Krogt, 2009).

1.2.2 Production of industrial starch and PPF

The industrial separation of starch from potatoes is illustrated in figure 1.

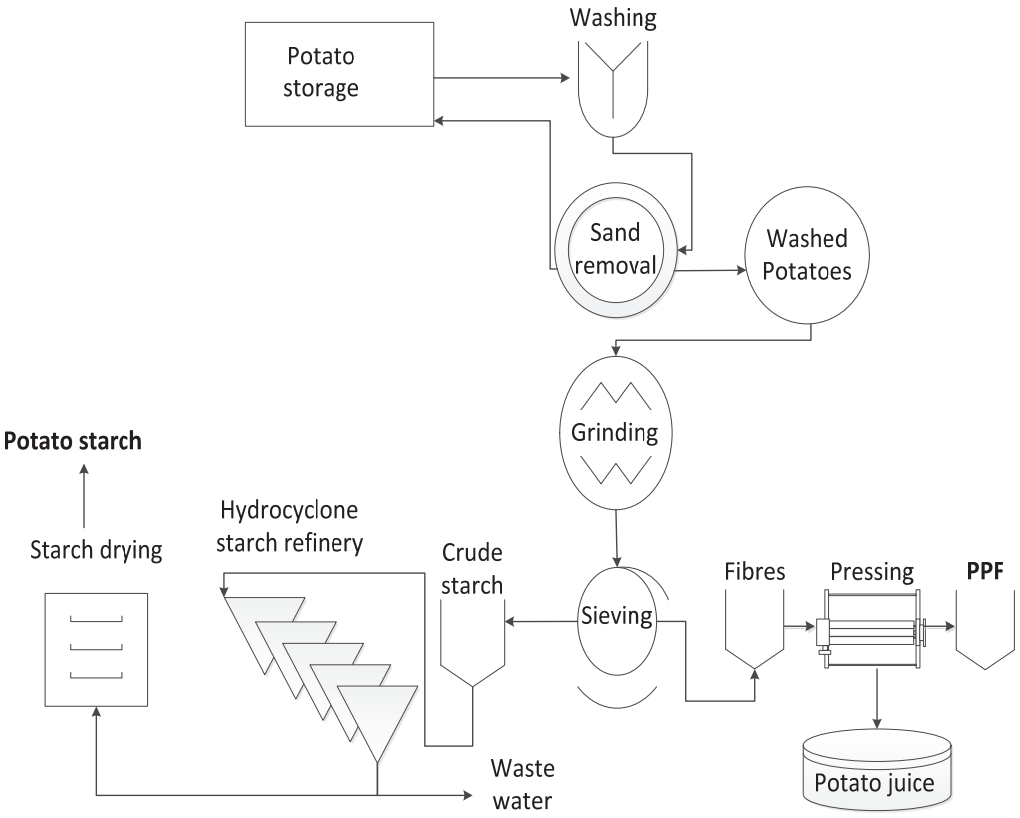

Figure 1: Simplified scheme of the process to isolate starch from potatoes (adapted from Grommers and Krogt, 2009)

During industrial production, about 97% of the total starch is recovered as a pure potato starch fraction (Figure 1), while the remaining 3% is lost as residual starch in the fibre fraction (Grommers and Krogt, 2009). It is a challenge to improve the recovery of potato starch even closer to 100%. The most difficult task is to extract the residual starch,

which is present within intact (or partially intact) potato cells remaining after the grinding step (Figure 1). Next to mechanical disruption, the use of CWP degrading enzymes can be a means to enhance starch recovery. An overview of enzymes used for releasing starch from potatoes described in literature is discussed in section 1.6.2.

The remaining fibre fraction after starch extraction, PPF, is rich in CWPs (45-65 w/w %), next to residual starch (20-40 w/w %)((Lesczczynski, 1989; Mayer and Hillebrandt, 1997; Meyer et al., 2009); Table 1).

Table 1: Composition of PPF based on dry matter

Component	% (w/w)
Total organic matter	96
Starch	20-40
Ash	4
Proteins/amino acids	2-6
CWPs total	40-65
Pectin	19-31
Hemicellulose	7-11
Cellulose	14-23

The CWPs in PPF are abundant in galactan (included among pectin in Table 1) and homogalacturonan (also pectic), which in one study (Meyer et al., 2009) constituted about 17 and 13 w/w % on dry matter, respectively. In that study, it was proposed that the galactan was abundantly present as side chains on rhamnogalacturonan-I (RG-I). Still, a detailed composition of CWPs in PPF has not yet been described as has been done for potato CWPs.

1.3 Composition and structure of cell wall polysaccharides in potato (*Solanum tuberosum*)

1.3.1 Composition of potato polysaccharides within the plant cell wall

In a typical dicotyledonous plant, such as potato, the plant cell wall is mainly composed of a primary cell wall layer. This layer can be envisaged as a cellulose-xyloglucan framework that entraps a number of pectic polysaccharides (Figure 2; McCann and Roberts, 1991).

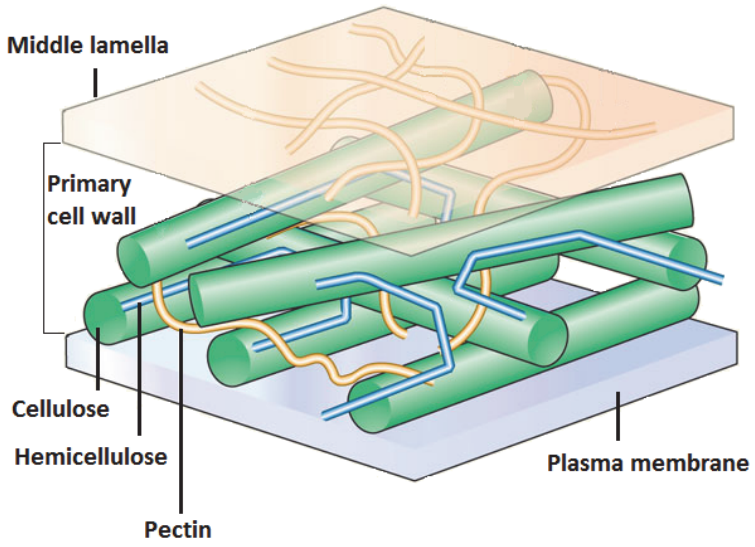

Figure 2: The three-dimensional network in the plant cell wall model (McCann and Roberts, 1991)

During the grinding step of potatoes (Figure 1), it is inevitable that the cell walls are disrupted (completely or partly) to release starch from the interior of cells. The remaining fibre fraction contains fragments of both potato flesh and potato peels (Leszczynski, 1989). The potato flesh is constituted of thin, parenchymatous tissue largely containing primary cell walls surrounding the starch granules (Figure 3). The potato skin is constituted of epidermal and suberized cells that next to primary cell walls also contain some secondary cell walls (McDougall et al., 1996).

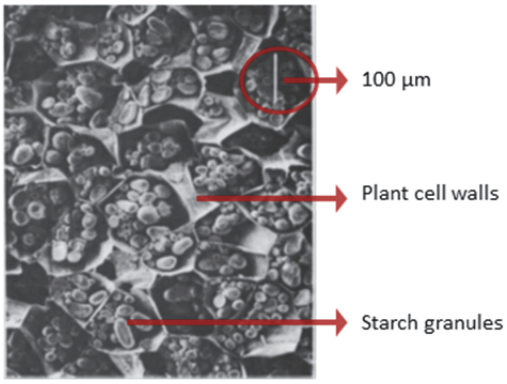

Figure 3: Scanning electron micrograph of potato tuber cells (McDougall et al., 1996). The bar represents 100 µm.

For potato CWPs (Table 2), the content of pectins has been shown to be the highest, followed by cellulose and hemicellulose (Harris et al., 2009, Vincken et al., 2000). Each of these potato CWPs is discussed in detail.

Table 2: Composition of CWPs in potato (Vincken et al., 2000).

CWP composition (on dry matter)			w/w %
Pectin			56
	Rhamnogalacturonan-I		
		Backbone	14
		Side chains ((Arabino)Galactan)	36
	Homogalacturonan		6
Hemicellulose			14
	Xyloglucan		11
	Mannan		3
Cellulose			30

1.3.2 Pectins are defined by the presence of galacturonic acid (GalA), comprise homogalacturonan (HG) and substituted galacturonans, namely, xylogalacturonan (XGA), rhamnogalacturonan type I (RG-I) and type II (RG-II). The most abundant pectic

polysaccharides in potato cell walls are galactan linked RG-I (table 2, figure 4; Caffall et al., 2009). The exact amounts in which XGA and RG-II occur in potato is not known.

RG-I constitutes a backbone structure of alternating α-1, 2-linked L-rhamnosyl (Rha) and α-1, 4-linked D-GalA_n residues, where n ≥ 100 and varies with the plant source (Figure 4, McNeil et al., 1980; Ralet and Thibault, 2009). In potato, RG-I is found to be highly acetylated at the O2 and/or O3 positions in the RG-I backbone with a degree of acetylation (DA) of around 90 (Schols and Voragen, 1994; Ishi 1997). Also, the RG-I backbone is substituted at the O-4 positions of Rha with side chains of arabinogalactans and to a lesser extent with arabinan. In table 2, all the side chains are grouped under the term "(arabino)galactan" and it is assumed that all "(arabino)galactan" is linked to RG-I. While it is known that the arabinogalactan side chains are the most predominant among other side chains, the exact ratio of arabinogalactan to arabinan is unknown (Oomen et al., 2003).

Arabinogalactans include arabinogalactan type I (AGI) and type II (AGII), distinguished mainly by the type of linkage of the polymer (Figure 4). AGI includes β-1, 4-linked D-Galpyranosyl (Gal*p*) residues forming the backbone structure, on which the O3 position is substituted with Gal*p* or Ara*f* residues (Huisman et al., 2001). AG-I, being the most abundant pectic CWP, is described rather well. The galactan chains have been suggested to be about 40 Gal residues long (Jarvis et al. 1981a). AGII is composed of a β-1,3-linked backbone of Gal*p* residues, connected with short side chains of 1,6-linked β-D-Gal*p* residues (1, 2 or 3 residues in length) and/or 1,6 linked α-L-Ara*f* residues, or more rarely, with a single β-D-Ara*p* residue (Huisman et al. 2001; Caffall et al., 2009). The β-1, 6-linked galactan could terminate with a β-D-Gal*p* or L-Ara*f* and to a lesser extent with L-Ara*p* (Huisman et al. 2001; Voragen et al., 2001; Luonteri et al. 2003).

The ratio of AG-I to AG-II is unknown in potato. Nevertheless, the occurrence of small proportions of β-1,3-linked galactans in AG-I was observed (Hinz et al. 2005). Several possible branching patterns of the side chains on potato RG-I have been suggested. These include arabino-3,4-galactans (β-1,4-linked galactan to which α-1,5-linked L Ara*f* residues are attached at the O3 position; Ring and Selvendran, 1978) and arabino-3,6-galactans (β-1,6-linked galactan to which L-Ara residues are attached at the O3 position; (Jarvis et al.,

1981a; Harris et al., 2009)). On these Gal*p* residues, a single substitution at the O6 position with Gal*p* has also been observed (Voragen et al., 2001).

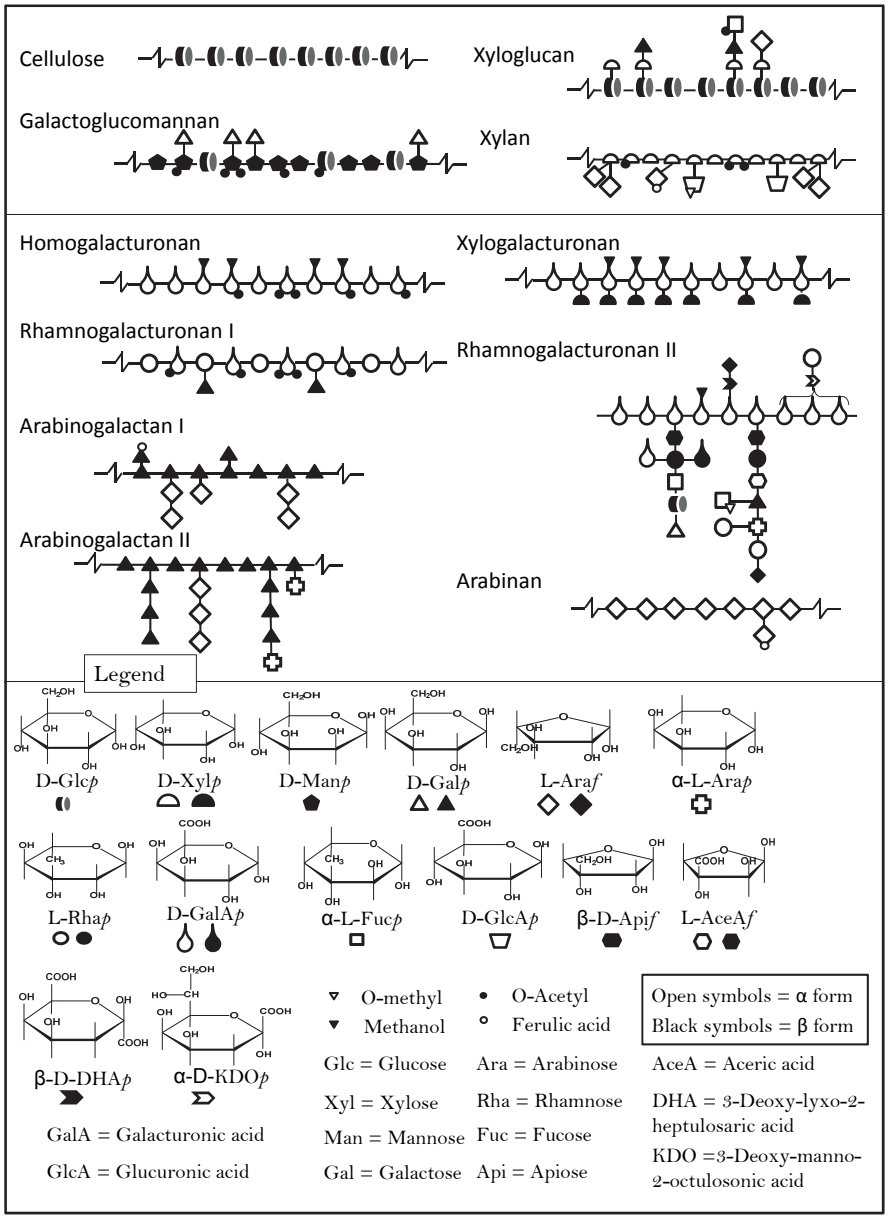

Figure 4: Composition and structure of cell wall polysaccharides (CWPs)

In addition, β-1,4-linked galactan oligosaccharides (four or more residues) are linked to the arabinan side chains of RGI in potato cell walls. Figure 5 summarizes the proposed structure of RG-I and its side chains in potato.

Arabinans have a backbone of α-1, 5-linked arafuranosyl (Ara*f*) residues, on which substitutions with Ara*f* via 1, 2 and/or 1, 3 linkages occur (figure 4; Voragen et al., 2001). In potato, assuming that all arabinose is a part of arabinan linked to RG-I, 8% w/w arabinan was found compared to 28% galactans (Vincken et al., 2000; Table 2).

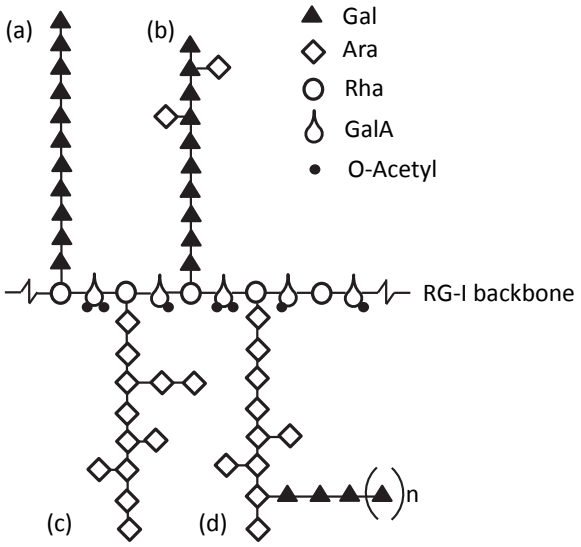

Figure 5: Proposed structure of potato RG-I, adapted from Obro et al., 2004. (a) Linear galactan, (b) AGI, (c) Branched arabinan, and (d) Hypothesized branched arabinan with galactan decorations, n≥1.

HG has a backbone of α-1, 4-linked galacturonic acid (GalA) residues (Figure 4). In potato cell walls, HG is suggested to constitute 30% of the pectic backbone versus 70% by RG-I (Table 2; Oomen et al., 2003; Mohnen et al., 2008). GalA residues in the backbone of potato HG may be acetylated or methyl esterified. Acetylation of GalA is found at the O3 position in HG in potato cell walls (Ishi 1997).

XGA, which contains one or more 1,2-linked or 1,4 linked Xyl chains attached to O-2 and O-3 of a galacturonan backbone (Figure 4), has been indicated to be present in potato cell walls (Zandleven, 2006), but has not been quantified yet.

RG-II has a backbone of nine galacturonan residues on which four side chains are attached that consist of rare carbohydrate residues, and is diagnostic for a species (Figure 4). RGII and its dimeric form have been found to be present in potato, but like XGA, not yet quantified (Ishi et al., 1999; Harris et al., 2009).

1.3.3 Cellulose is a polymer chain of β-(1, 4)-linked D-glucosyl (Glc) residues (Figure 4). Multiple cellulose chains are hydrogen bonded to each other and also held by Van der Waals forces to form microfibrils (Caffall et al., 2009). In potato cell walls, the cellulose microfibrils are mostly crystalline and are connected to give an interwoven network of microfibrils (Harris et al., 2009). The microfibrils are associated via hemicelluloses (Figure 2).

1.3.4 Potato hemicelluloses mainly include xyloglucans (XG) and mannans, of which XGs are the most abundant (Figure 4; Harris et al., 2009). XG is a polymer with a β-(1,4)-linked glucan backbone to which xylosyl (Xyl) residues are substituted on the O-6 position of the Glc residues (Figure 4). In potato cell walls, the pattern of Xyl substitutions is such that only two adjacent Glc residues are substituted. This is denoted as XXGG in which G is a glucosyl (Glc) residue and X is a xylosyl (Xyl) attached to a Glc residue. Further substitutions on Xyl have been observed with either Gal (XLGG) or Ara (XSGG) or Fuc (XTGG)(Vincken et al., 1996).

Mannans, in general, consist of a backbone of β-(1,4)-linked mannosyl (Man) residues that could be interrupted in between with Glc residues. Additionally, the Man residues may be substituted with galactosyl (Gal) residues (Figure 4; Bremner et al., 1971). In potato, mannans have been shown to be present, but have not yet been isolated or characterized (Harris et al., 2009).

In addition, xylans have been identified to be present in trace amounts in potato cell walls, but have neither been quantified nor characterized (Jarvis et al., 1981a). Xylans consist of β-(1, 4)-linked Xyl backbone, to which arabinose (Ara) and glucuronic acid (4-O-methyl) can be attached (Harris et al., 2009).

1.4 Polysaccharide interactions

The CWPs in the middle lamella, primary and secondary cell walls are interconnected via covalent and non-covalent interactions. The middle lamella comprises mainly **calcium pectin** interactions between HGs (Liners et al., 1992). These can also be expected to be present in the primary cell wall. The main non-covalent interactions in the primary and secondary cell walls include strong, hydrogen bonding stabilized interactions between microfibrils of **cellulose and xyloglucan** (Mort 2002). In addition, potato galactan side chains have been observed to adsorb to the surface of cellulose microfibrils, but to a lesser extent than xyloglucan, presumably mediated by hydrogen bonds (Zykwinska et al. 2005).

Mainly applying to the primary cell wall and middle lamella, covalent interactions have been observed between pectins, such as **di-ferulic acid (FA)** esters. These esters act as a bridge between FA attached to the O2 position of α-1,5-linked-L-Ara*f* residues in arabinan and another FA attached to the O6 position of β-1,4-linked Gal*p* residues in galactan for sugar beet cell walls (Ralet et al., 1994). FA esters have not yet been reported for potato CWPs. Also, **borate di-ester** crosslinks between apiose residues in RGII (Ishi et al., 1999) have been indicated to be present in potato cell walls (Ishii et al., 1999). Furthermore, it has been suggested that **uronyl ester** linkages between the carboxyl group in HG or RG-I and the hydroxyl group of another polysaccharide chain may occur (Voragen et al., 2009) but not proven for potato cell walls. Likewise, evidence for the covalent cross linking of **HG to XG** has been put forth involving RG-I and XGA, but not proven yet in plant cell walls (Mort, 2002; Fry, 2003). Covalent linkages have been described between HG in the primary cell wall/middle lamella and proteins via wall associated kinases (Wagner et al, 2001), or via amide linkages between the pectic carboxylic acid groups and protein amino groups (Perrone et al., 1998).

The secondary cell walls contain deposited lignin and suberin next to polysaccharides which are unknown (Harris et al., 2009). Some evidence of covalent linkages between xylans, HG and lignin complexes was shown and hint at involvement of ferulic acid ester crosslinks (Femenia et al. 1988, Waldron and Selvendran 1992; Fry, 2003).

1.5 Water holding capacity

1.5.1 Definition of water holding capacity and other hydration capacities

Hydration of fibres is usually represented as 1) water holding capacity (WHC) or water absorption capacity (WAC), 2) water retention value (WRV) or water binding capacity (WBC), and 3) swelling.

The WHC is defined as the ability of a material to absorb water and is measured using a Baumann method or with a dialysis method (Thibault et al., 2000; Stephen and Cummings, 1979). In the Baumann's method, the WHC is measured by the quantitative uptake of water (or any other solvent) by a known amount of dried material, usually in the form of a powder. The apparatus consists of a thermostat water jacket in the form of a funnel that is connected to a calibrated capillary. The sample is placed on top of the funnel via a wetted glass filter, with an additional filter paper above the glass filter, and the water absorbed by the sample at equilibrium is read from the capillary (Baumann, 1967). The Baumann's method is used for measuring the WHC of dried samples as such (without a re-distribution of water), mostly for small amounts of samples (in the mg range depending on the sample)(Chen et al., 1984; Kneiffel et al., 1991).

The dialysis method for measuring WHC is used mainly in fermentation studies, for which, the WHC of fibre rich materials *in vitro* is correlated to the WHC observed in the colon. The method determines the amount of solvent (simulating the content and composition of colonic fluids) held by a known amount of fibre sample placed in a dialysis bag (Stephen and Cummings, 1979). The numerical values obtained from this method cannot be compared to that of the Baumann's method due to procedural differences that influence the way fibre particles uptake water. Therefore, the choice of a method depends on the purpose of measuring the WHC.

The WBC and WRV are defined as the amount of water retained by subjecting the material to external stress such as centrifugation or ultra-filtration (Thibault et al. 2000). Therefore, the actual values of WBC are influenced by the g force applied.

Swelling is defined as the ratio of the volume (mL) of water occupied by a material in excess of water (or any other solvent) to the dry weight of the material (g) (Robertson et al., 2000). The method for analyzing the swelling capacity briefly involves soaking a known amount of fibre sample in a solution contained in a graduated cylinder for a fixed time interval, after which the volume occupied by the fibre is read.

WHC, WAC, WBC, WRV and swelling, are all expressed as the mL of water held per g of material. For PPF, the hydration values reported are a water binding capacity (WBC) of 7 mL/g and a swelling of 10 mL/g freeze dried material (Serena and Knudsen, 2007).

1.5.2 Factors influencing the WHC

Fibres hold water as “bound” water or “free” water (Eastwood et al. 1983). Water is bound via molecular interactions. Free water, also called embedded water, is held in the fibre lumen, which is an elongated cavity within the cell wall or within fibre particles, and in pores in the fibre matrix. The size and distribution of pores in the matrix determines the amount of free water held. In PPF, the cell wall material present is modified by the grinding and pressing (Figure 1), which increases the possibility for formation of lumens and pores.

The WHC of fibres is influenced by several factors. One of them is the *drying condition.* For example, freeze drying was found to lower the WBC and swelling of fibres (Serena and Knudsen, 2007)). Another factor that is mostly seen to influence upon mechanical treatments is the *particle size*. A decrease in *particle size* lowers the WHC by decreasing the insoluble dry matter (Thibault et al., 2000; Teimouri Yansari and Primohammadi, 2009). It is also observed that *thermo-mechanical* and *enzymatic treatments* increase the amount of soluble fibre and modify the hydration properties of the insoluble residue by modifying the CWP network. For example, in the case of wheat cell wall material, treatment with a xylanase increased the WHC of the residue (Gruppen et al., 1993), while the WHC of the total dry matter (soluble and insoluble) decreased. Besides, the *composition of CWPs* influences the hydration. The presence of non-cellulosic polysaccharides showed a positive correlation with hydration capacities (Serena and

Knudsen, 2007). In particular, HG and xyloglucans, have been indicated to contribute to a high WHC (Boulos et al., 2000; Nishinari et al., 2000). In hydrated cell walls, the higher mobility of arabinans and galactans compared to cellulose, as determined by ^{13}C NMR, indicated a higher possibility of the side chains to interact with water (Ha et al. 2005). Besides the composition, the *ionic state* of CWPs is important. Ionized HG hold more water than their non-ionized forms due to repulsion between the carboxyl groups, and their salt forms (Na^+, Ca^{2+}, K^+, Mg^{2+}) hold even more water than their H^+ forms (Michel et al. 1988). The presence of salts, such as NaCl decreased the WBC and swelling of de-esterified beet pulp (Renard et al. 1994).

1.6 Enzymes degrading potato cell wall polysaccharides

1.6.1 Potato CWP degrading enzymes

The ability of enzymes to degrade CWPs could facilitate starch release from potato cells and lower the WHC of PPF. For this, degradation of the polysaccharide by endo-acting enzymes are expected to have more effect than exo-acting enzymes. Since pectins are the most abundant among potato CWPs (Table 2), enzymes degrading them are described in more detail in this section. These enzymes, pectinases, can be divided based on the pectin-structure they are active on. In each category, these enzymes can be sub-divided into esterases (methyl and acetyl; CE), hydrolases (GH) and lyases (PL), based on the type of reaction they catalyze. Nowadays, an online database called carbohydrate-active enzyme database (CAZy; www.cazy.org) is commonly used for CWP degrading enzymes. In CAZy, families of structurally related catalytic modules of enzymes that degrade, modify or create glycosidic bonds are described (Cantarel et al., 2009).

Hydrolysis of homogalacturonan (HG) as well as its esterified forms in potato requires the use of **endo-polygalacturonanases (PGase)** or **pectin lyase** and/or **pectate lyases (PLase).** **PGases** (GH family 28) hydrolyze glycosidic linkages adjacent to free carboxyl groups in low methyl esterfied-polygalacturonic acid and in pectic acid. **Pectate lyases** are present in the PL families 1-3,9,10 and hydrolyze glycosidic linkages adjacent to free carboxyl groups (Roy et al. 1999). **Pectin lyases,** PL family 1, split between methyl

esterified GalA residues, all by trans-elimination reactions (Pilnik and Voragen, 1991; Voragen et al., 2001). **Pectin methyl esterases** (PMEs; CE family 8) split off methyl esters and **pectin acetyl esterases** (CE families 12,13 and 16) split off acetyl groups from the HG backbone (Voragen et al., 2001). De-acetylation enhances the activity of PME on potato HG (Voragen et al., 1995).

The RG-I backbone degrading enzymes include **RG hydrolases** (GH family 28) that split the α-galactopyranosyluronic acid-(1→2)- α-rhamnopyranosyl linkages in RG (Voragen et al. 2001). Also, **RG lyases** (PL families 4 and 11) act on RG-I by splitting the α-rhamnopyranosyl-(1→4)-α-galactopyranosyluronic acid linkage by a transelimination reaction (Voragen et al. 2001). In addition, the exo-acting **RG rhamnohydrolases** (GH family 28) release rhamnosyl groups and **RG galacturonohydrolases** (GH family 28) release galacturonyl groups from the non-reducing end (Beldman 1996; Mutter 1997; Voragen et al., 2001). **RG acetyl esterases** (CE family 12) act as accessory enzymes for **RG hydrolases** and **RG lyases** as it specifically removes acetyl groups at O-2 and O-3 from GalA residues in the RG backbone (Searle-van Leeuwen et al. 1996). All the RG modifying enzymes cause solubilization and hydrolysis of the hairy regions of pectins. Therefore, they could be of technological importance for solubilizing hairy pectins from potato.

Hydrolysis of the side chains of potato RG-I, being dominated by (-arabino)galactan (Table 2), is expected to produce significant changes in the architecture of potato CWPs. Galactan degrading enzymes comprise the **endo-(1→4)-β-D-galactanases** (GH family 53) that cleave 1,4-linked galactan side chains, **exo-(1→4)-β-D-galactanases** and **β-galactosidase** (GH families 1, 2, 35 and 42). The latter can release β-(1→6)-linked-D-galactosyl side chains and β-(1→4)-linked-D-galactosyl residues from the non-reducing end of a galactan backbone. Also, **endo-** (GH family 16) and **exo-(1→3)-β-D-galactanases** (GH family 43) and **endo-(1→6)-β-D-galactanases** (GH family 5) have been identified (Voragen et al., 2001; Luonteri et al. 2003). In addition, arabinan degrading enzymes could assist the degradation of RG-I. These enzymes include **endo-(1→5)-α-L-arabinanase, α-L-arabinofuranosidases (Ara*f*ase)** and **exo- α-L-arabinanase** enzymes (all belonging to GH family 43)(Beldman et al. 1997).

Cellulases hydrolyze β-1, 4 glucosidic bonds in cellulose. In general, three types of cellulases are needed to completely degrade cellulose. These include **endo-glucanases**

(GH family 5, 9, 51) that cleave the internal sites of amorphous cellulose (Lynd et al., 2002) as well as crystalline cellulose (Gilad et al., 2003), **cellobiohydrolases** (CBH; GH family 6,7, 48) that release cellobiose from crystalline cellulose and **β-glucosidases** (GH family 1, 3 or 9) that degrade cellobiose to glucose (Lynd et al. 2002).

XGs can also be degraded by such cellulases. Also, enzymes specific for hydrolyzing the glucan backbone of XGs have been identified. These include the **XG specific β-(1→4)-glucanases** (GH families 5,12, 74; Grishutin et al. 2004; Powlowski et al. 2009). It has been shown that these enzymes are hindered by fucosylated elements on XG, by the presence of Ara substitutions on Glc, or by the presence of acetyl groups. Accessory enzymes, such as **α-xylosidases** (GH family 31), **β-galactosidases** (GH families 1,2,35 and 42), **α-arabinofuranosidases** (GH families 3,43,51,54 and 62), **α-fucosidase** and **acetyl esterases** (CE family 16), are required to debranch or linearize the glucan backbone (Vincken, 2002) and facilitate breakdown of XGs.

Since mannan and xylan are present to a much lesser extent in potato than XG, their degradation alone is not expected to affect the functional properties of potato CWPs. Therefore, these enzymes are not described in this chapter.

1.6.2 Commercial enzyme preparations

Most commercial enzymes are produced from microbial sources, as these can be produced on a large scale. To degrade potato CWPs, commercial enzyme preparations need to contain enzyme activities that degrade the most abundant CWPs, which are galactan, RG-I, HG and cellulose. While several enzyme preparations are available, only a handful of them are abundant in endo-galactanases or contain these enzymes and HG or RG-I degrading enzymes. These enzymes produced by *Aspergillus* species include SP249 (*Aspergillus niger var aculeatus*; Lahaye et al., 1991), SP584 (*Aspergillus sp*., Bonnin et al., 2002), SP585 (*Aspergillus sp.*, Micard et al., 1996) and Pectinex Ultra-SP L (*Aspergillus aculeatus*; Luonteri et al., 2003), which are abundant in endo-galactanase and polygalacturonase. Novozym 342 is produced from *Humicola insolens* and contains endo-galactanases along with cellulases (Bonnin et al., 2002). Driselase, containing RG-I degrading enzymes, is produced from *Basidiomycete sp.(Irpex lacteus)* and could

depolymerize both acetylated (33 nkat/mL) and non-acetylated RG-I (853 nkat/mL) (Picataggio et al., 1983, Normand et al., 2010), making it suitable for the solubilisation of hairy regions from potato.

Only few publications describe the effect of commercial enzymes on PPF, and most of them are aimed at the release of carbohydrates, and not at reducing the WHC or releasing starch. The enzymes described and conditions for treating potato fibres or PPF are summarized in table 3. The treatment of potato fibres with cellulase resulted in 25 g/L of reducing sugars from 40 g/L of potato fibres (Singh et al., 1991) implying not only the solubilization of cellulose but also of other CWPs. The application of only Viscozyme L to a de-starched, de-proteinated fibre solubilized 24% of the dry matter (Meyer et al., 2009). Viscozyme L is a β-glucanase rich enzyme preparation with a wide range of side activities (see table 3). This implied that the potato fibre CWPs expected to be degraded include mainly XGs and cellulose. Also, the application of Ultra-SP, a pectinase rich preparation including galactanase activity, hemicellulolytic and cellulolytic activities, to the de-starched, de-proteinated fibre residue, released 34% of the dry matter (Norsker et al., 1999). In another example, galactanases from *Phytophthora infestans* were able to disrupt the network of CWPs. This was observed from the release of 23% of original cell wall material in potato cell walls, but only after extraction of calcium bound HG (Jarvis et al., 1981b). It seems likely that in order to solubilize at least 20% cell wall material from potatoes, more than one type of enzyme activity is essential.

The combination of enzymes has been observed to be useful in lowering the WHC for other materials as well. For example, the viscosity and WHC of dehulled lupins were reduced by 18% and 14%, respectively, upon incubation with a combination of polygalacturonase supplemented with pectin methyl esterase (Ali et al., 2005). For PPF, the effect of degrading CWPs on the WHC is not yet known. Nevertheless, the de-watering of PPF has been observed upon incubating PPF (6.5 w/w % of dry matter) with 2 w/w % of polysaccharide degrading enzymes (SP249; *Aspergillus niger*, Novozymes, Bagsvaerd, Denmark; Slominska and Starogardzka, 2006) for 1 hour at 45°C. The de-watering was represented as a decrease in the volume of the solids phase (in mL) after centrifugation, which was found to be twice lower than the non-enzyme treated solids phase.

A synergistic increase in cellulose hydrolysis was observed for a combination of Celluclast 1.5 L (containing cellulases; Novozymes, Bagsvaerd, Denmark) and Rohapect (pectinolytic and cellulolytic activities; Roehm, Darmstadt, Germany), applied on sweet potato (Kallabinski and Balagopalan, 1994). The synergistic effect was explained by the fact that cellulose was more accessible for cellulases after hydrolysis of pectins. In the same study, an 18% improved recovery of starch was observed. In another study, 90% of the total starch from potatoes was released upon combining pectinases with cellulases from *Pencillium funiculosum* (Gayal and Hadge, 2003). However, the actual degradation of CWPs was not studied.

The performance of enzymes not only depends on the type and accessibility of targeted substrates, but can be influenced by other components present. In this respect, potato juice components, such as proteins and phenolic acids have been pointed out in several studies to inhibit enzyme activity. Proteins, such as PGIP-1, and phenolic acids, such as chlorogenic acid, have been indicated to inhibit polygalacturonase (Bulantseva et al., 2005; Lyon and McGill, 1989). Phenolic acids contribute to plant resistance against phytopathogens by inhibiting CWP degrading enzymes secreted by these pathogens. However, the inhibitory effect of phenolic acids on CWP degrading enzymes is not fully understood (Lyon, 1989 and Friedman, 1997).

Research is needed to investigate the effect of enzymes that can degrade CWPs to lower the WHC of PPF and similar materials, and to improve starch recovery. An experimental enzyme (Erbsloh; Geisenheim, Germany) and a commercial enzyme, (Pectinex Ultra-SP L, Novozymes) showed to be successful in liquefying PPF during a proof of principle research (Van Gool and Schols, 2008) that preceded the research in this thesis. Therefore, these enzymes were used further on in this thesis, in order to study their effect on the WHC of PPF and release of starch.

Table 3: Effect of commercial enzymes applied on PPF or similar fibres from potato.

Enzyme	Enzyme composition	Source	Enzyme dose (w/w %)	Conditions, Process	Effect on fibres	Reference
Viscozyme L, Novozymes	β-glucanase and side activties of arabinanase, cellulase, xylanase	*Aspergillus aculeatus*	0.27	62.5°C, pH 3.5, 1 hour Enzyme treatment of de-starched, de-proteinated fibre residue	24% of dry matter solubilized. Mainly RGI with galactan side chains. Abundance of Ara relative to Gal increases above 100 kDa	Meyer et al., 2009
Pectinex Ultra-SP, Novozymes	Pectolytic, some hemicellulytic and cellulolytic activties present	*Aspergillus aculeatus*	0.005	45°C, pH 4, 1 hour Enzyme treatment of de-starched, de-proteinated fibre residue	34% dry matter solubilized	Norsker et al., 1999 Mutter et al., 1998
Cellulase TC, Serva	Cellulolytic	*Trichoderma reesei*	0.75 (enzyme protein to substrate)	50°C, 24 hours	62% dry matter solubilized as reducing sugars	Singh et al., 1991
Termamyl 120 L+ Viscozyme L+ Celluclast 1.5L (Novozymes)	Termamyl: α-amylase	*Bacillus licheniformis*	10 ((v/w) %, Termamyl)	2% (w/w) of potato peel waste treated in three steps:	Increasing the dosing of Viscozyme by 4 times and supplementing cellulase increased the release of reducing sugars from 10.3 g/L to 18.4 g/L	Arapoglou et al., 2009
	Viscozyme L: β-glucanase	*Aspergillus aculeatus*	1.2 (enzyme protein to substrate , Viscozyme)	1) Liquefaction with Termamyl at 85°C, pH 6, 1 hour		
	Celluclast 1.5 L: Cellulase	*Trichoderma reesei*	2 ((v/w) % Celluclast)	2) Saccharification with Viscozyme at 44°C, pH 4.6, 2.5 hours 3) Cellulose degradation with Celluclast 1.5 L at 50°C, pH 5 for 2 hours		

1.7 Objective and outline of this thesis

As discussed above, information on the structural composition of CWPs and their effect on the WHC of pressed potato fibres PPF are lacking. In addition, although it is known that enzymes can be used to improve starch recovery from potatoes, the effects of such treatments at a much larger scale (*pilot* scale), and on the WHC of corresponding "fibre" fraction have not been studied. Therefore, the objective of this thesis was to characterize CWPs in PPF, and to understand the effect of these CWPs (and their enzymatic degradation) both on the WHC of PPF and on starch recovery at the *pilot* scale, and the consequences for the industry. The main **hypothesis** of this thesis is that the **solubilization** of one or more types of **CWP(s)** by **disintegrating** CWP **networks** in PPF or "fibre" (in the case of potato pulp), facilitates **starch recovery** from "fibre" and **reduces** the **WHC** of PPF or "fibre". Furthermore, the performance of CWP degrading enzymes in the presence of potato juice is studied to investigate if the enzymatic solubilization of CWPs is affected by components in potato juice.

The structural characteristics of CWPs from PPF and their influence on the WHC are described in **chapter 2**. In **chapter 3**, the detailed effects of soluble and insoluble CWPs, and the effect of enzyme liquefaction on the WHC of PPF are discussed. In **chapter 4,** the effects of two commercial enzyme cocktails on starch recovery from potatoes and on lowering the WHC of corresponding "fibre" in a *pilot* scale set-up are shown. Suppression of these commercial enzymes by components present in potato juice was observed and is studied in **chapter 5**. In **chapter 6**, the overall findings of this research are discussed and consequences are described.

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Chapter 2

Structural features and water holding capacities of pressed potato fibre polysaccharides

Abstract

Pressed potato fibre (PPF) has a high water holding capacity (WHC) affecting its processing as an animal feed. The aim of this study was to characterize cell wall polysaccharides (CWPs) in PPF and investigate their WHC. This was done via sequential extractions. Half of all CWPs were recovered in the hot buffer soluble solids extract as pectins (uronic acid and rhamnose) and galactans wherein most pectins (76%) from PPF were water soluble. Most likely, the network of CWPs is loosened during processing of potatoes. PPF showed a WHC of 7.4 expressed as the amount of water held per g of dry matter (mL/g). Reconstituting hot buffer soluble solids with buffer insoluble solids in water gave a WHC comparable to that of PPF. Removal of alkali soluble solids, which mainly comprised xyloglucans, lowered the WHC of the final residue. The results indicated that interactions between CWPs could affect the WHC of PPF.

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2.1 Introduction

Pressed potato fibre (PPF) is a major by-product of industrial potato starch production. PPF exhibits a high water holding capacity (WHC) that makes it colloid-like, bulky and difficult to process (e.g. pumping). This hinders its use as cattle feed. An approach to enable the handling of PPF would be to reduce its WHC. WHC is affected by factors related to the potato cell wall, such as polysaccharide composition and architecture (Serena and Knudsen, 2007). Other factors include particle size, pore volume, thermo-mechanical treatments and drying conditions (Pejic et al., 2008; Serena and Knudsen, 2007; Thibault et al., 2001).

PPF is composed of potato flesh and potato skin fragments (Lisinska and Leszczynski, 1989). Potato flesh is largely composed of parenchyma that contains thin walled, non-lignified, primary cell walls. Skin fragments typically consist of periderm, which includes epidermal and suberized cells containing some secondary cell walls (McDougall, 1996). PPF contains 20-40 % (w/w) starch (Leszczynski, 1989) and 48 % (w/w) cell wall polysaccharides (CWPs; Mayer and Hillebrandt 1997). Detailed structural characteristics of PPF polysaccharides are not yet known.

In general, the plant cell wall architecture in dicotyledonous plants, such as potato, consists of a firm network of cellulose and xyloglucans that entrap pectic polysaccharides (McCann and Roberts, 1991). Pectins include linear 1,4- linked galacturonans, also named homogalacturonans (HG) or smooth regions, and branched galacturonans, such as xylogalacturonans, rhamnogalacturonan type I (RG-I) and rhamnogalacturonan type II (RG-II). RG-I and RG-II are often referred to as hairy regions.
In potato cell walls, RG-I constitutes about 75 % of total pectins (Oomen et al., 2003). The backbone of RG-I has repeats of [→α-D-GalA-1,2-α-L-Rha-1,4→] (Voragen et al., 2009). Most of the structural analyses on potato cell walls has been related to RG-I and its side chains predominantly composed of (1,4)-β-galactans next to arabinogalactans (Harris et al., 2009). The degree of acetylation (DA) and the degree of methyl esterification (DM) of potato pectins were reported to be 14 and 31, respectively (Voragen et al., 1986). Analysis of other cell wall polysaccharides reveal the presence of crystalline microfibrills of cellulose in an interwoven network (Kirby et al., 2006), and the occurrence of XXGG type

of xyloglucans where the repeating unit consists of two glucosyl residues substituted with xylose (X) adjacent to two un-substituted glucosyl (Glc) residues (G). Substitutions on X gives several forms of XXGG as XSGG (S is arabinosyl substitution of X), XLGG (L is galactosyl substitution of X) and LSGG (Vincken et al., 1996). Also, heteromannans and heteroxylans have been indicated to be present in potato cell walls, but have not yet been characterized (Jarvis et al., 1981).

Hydration of CWPs has been expressed in terms of swelling, water retention power and WHC. The arabinan side chains of potato RG-I have been suggested to be hydrated more readily than galactan side chains, although long galactan side chains have been implicated to be extremely mobile. Also longer side chains were shown to impart a hydrophilic nature to RG-I (Larsen et al., 2011). In hemp fibres, a removal of 59% of the hemicelluloses decreased the water retention power by 7 % (Pejic et al., 2008). For PPF, hydration capacities previously reported were water binding capacity of 7g/g and swelling of 10 mL/g freeze dried material (Serena and Knudsen, 2007).

However, no information is available yet about the contribution of individual CWPs to the WHC of PPF. Such information is important if the WHC of PPF is to be lowered. In addition, the composition of CWPs, their organization and structural aspects in PPF is unknown. In this study, CWPs from PPF have been extracted and characterized for sugar composition, levels of esterification and types of CWPs present. In addition, the WHCs of CWPs in different residues from PPF has been investigated.

2.2 Materials and methods

Pressed potato fibre (PPF) was provided by AVEBE, Foxhol, The Netherlands on January, 2010. The potatoes were harvested in autumn 2009.

2.2.1 Extraction of non-starch polysaccharides from pressed potato fibre

2.2.1.1 De-starching pressed potato fibre

Freeze dried PPF (particle size < 1 mm; 90 g; provided by AVEBE, Foxhol, The Netherlands) was suspended in 0.2 M sodium acetate buffer, pH 5.2 (1520 mL) heated to 85°C and 0.006 g of α-amylase (Sigma A4551-1G, USA; 0.548 U/g, *Bacillus licheniformis*) was added. The suspension was stirred at 85°C for 30 min. After adjusting the pH to 5.5 (using 1 M NaOH), residual starch was hydrolyzed with 0.006 g of α-amylase and 0.3 g of amyloglucosidase (Sigma; 11.6 U/g, *Rhizopus mold*) and stirred at 30°C for 20 h. The suspension was centrifuged (20 min, 29,400 × g, 20°C) and the procedure was repeated for the residue. The residue was washed 3 times with demineralized water; freeze dried and denoted as buffer insoluble solids (BIS). All washings and supernatants were combined into one extract and dialyzed. Dialysis was performed against distilled water (cutoff size 12-14 kDa; Visking, Medicell International Ltd., UK) and freeze dried. After dialysis, the extract was denoted hot buffer soluble solids (HBSS).

2.2.1.2 Extraction of buffer insoluble solids (BIS)

BIS was extracted with 0.05 M EDTA and 0.05 M sodium acetate in 0.05 M sodium oxalate at pH 5.2 at 70°C for 1h. The suspension was centrifuged (20 min, 38,000 × g, 20°C). The residue was re-extracted once and all supernatants were combined. Supernatants and residue were dialyzed twice against 0.1 M ammonium acetate buffer, pH 5.2 and subsequently against demineralized water and after freeze drying denoted as chelating agent soluble solids (CHSS) and chelating agent un-extractable solids (CHUS). CHUS was treated with 0.05 M NaOH containing 20 mM $NaBH_4$ at 0°C for 1 h. The suspension was centrifuged (20 min, 38,000 × g, 20°C), the residue was re-extracted twice and all supernatants were combined. Supernatants and residue were neutralized with acetic acid, dialyzed twice against 0.05 M sodium acetate buffer, pH 5.2 and against demineralized water. After freeze drying, they were denoted dilute alkali soluble solids

(DASS) and dilute alkali un-extractable solids (DAUS). Finally, DAUS was treated with 4 M NaOH + 20 mM $NaBH_4$ (concentrated alkali) at 0°C for 1 h. The suspension was centrifuged (20 min, 48,000 × g, 20°C) and the residue was re-extracted twice. Supernatants and residue were treated as described above for DASS and DAUS and after freeze drying denoted concentrated alkali soluble solids (CASS) and Res.

2.2.2 Analytical methods

All analyses were performed in duplicates.

2.2.2.1 Carbohydrate composition: Samples were pre-hydrolysed with 72% (w/w) sulphuric acid for 1 h at 30°C followed by hydrolysis with 1 M sulphuric acid for 3 h at 100°C. The monosaccharides released were derivatized into their corresponding alditol acetates and determined by gas chromatography (Englyst and Cummings, 1984) using inositol as an internal standard. The total uronic acid content was determined using an automated m-hydroxydiphenyl assay (Thibault, 1979). Starch content (including resistant starch) was determined enzymatically using the Megazyme total starch kit (Megazyme, Ireland). The starch glucose (Glc) was subtracted from the total Glc to obtain cell wall Glc.

2.2.2.2 Protein content (N x 6.25 (Van Gelder, 1981)) was determined on a Thermo Quest NA 2100 Nitrogen analyzer (Interscience, Breda, The Netherlands). D-Methionine (Acros Organics, USA) was used as an external standard.

2.2.2.3 Acetic acid and esterified methanol content were determined using HPLC after saponification of 0.01 g sample by 0.4 N NaOH (1 mL) in isopropanol / water (ratio 1:1) for 4 h. For HPLC, an Ultimate 3000 system (Thermo Scientific, Sunnyvale, CA, USA) equipped with an Aminex HPX-87H ion exclusion column (300 mm x 7.8 mm; Bio-rad Laboratories, Hercules, CA, USA) was used in combination with a self-packaged guard column (50 mm x 7.8 mm, AG 50W-X4 Resin; Bio-Rad). Elution was performed with 0.005 M sulphuric acid at a flow rate of 0.6 mL/min. 20 µl of each sample was injected and eluted at 40°C and a refractive index detector, Shodex type RI 101 was used for detection. The degree of acetylation (DA) and methyl esterification (DM) were calculated as moles of acetic acid or

methanol per 100 mol of uronic acid, respectively, and were corrected for free acetic acid and methanol.

2.2.2.4 Ferulic acid content: PPF, BIS and HBSS (0.01 g each) were suspended in 200 µL methanol and 1 mL 0.5 M KOH (flushed with N_2). Samples were submerged in N_2 and stored in the dark at 20°C for 16 h. After 16 h, 0.75 mL of 6 M HCl was added to adjust the pH to <2. The ferulic acid released was extracted twice using 4 mL of ethyl acetate. The ethyl acetate fractions were combined and dried under N_2. The dried residue was dissolved in 1 mL of methanol prior to analysis. Analysis was performed on an Acella UHPLC system (Thermo Scientific, Sunnyvale, CA, USA) as described by Appeldoorn et al. (2010). Ferulic acid (FA) was identified and quantified using standards and corrected for free FA. The recoveries (n = 3) were 89.5 % (±0.003 standard deviation) for FA.

2.2.3 Molecular weight characterization of cell wall polysaccharides

2.2.3.1 High performance size exclusion chromatography: Analyses of molecular weight distribution of oligo- and polysaccharides were performed using high performance size exclusion chromatography (HPSEC). For this, an Ultimate 3000 HPLC (Thermo Scientific) was used with three TosoHaas TSK-gel columns in series (4000, 3000 and 2500 SuperAW; each 150 mm x 6 mm; TosoHaas, Japan) preceded by a TSK Super AW-L guard column (3.5 cm x 4.6 mm; TosoHaas). 20 µL of sample (0.002 g/mL) was injected and eluted with 0.2 M sodium nitrate at a flow rate of 0.6 mL/min at 55°C. Detection was performed with a refractive index detector Shodex R101 (Showa Denko, Kawasaki, Japan). The system was controlled using Chromeleon (version 7) software (Dionex, now ThermoScientific). For calibration, pullulan standards (Sigma; mass range of 180 Da to 790 kDa) and pectin standards (mass range of 2.8 - 100 kDa) were used.

2.2.3.2 Matrix assisted laser desorption/ionization time of flight mass spectrometry: Oligosaccharides were profiled using Matrix Assisted Laser Detection Time of Flight Mass Spectrometry (MALDI-TOF MS). An Ultraflextreme workstation (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser of 337 nm was used in positive mode. After a delayed extraction time of 200 ns, the ions were accelerated to a kinetic energy of 12 kV

and were detected using reflector mode. Data were collected by averaging at least 100 laser shots. The lowest laser power needed to obtain sufficient spectra intensity was used. The apparatus was calibrated using a mixture of maltodextrins (AVEBE). Samples (0.001 g/mL) were desalted using ion exchange material (AG 50W-X8 resin; Bio-Rad Laboratories, CA, USA). 1 µL of desalted sample was mixed with 1 µL of matrix solution on an MS target plate. The matrix solution used was 2,5-dihydroxybenzoic acid (Bruker Daltonics) in a concentration of 0.01 g/mL of acetonitrile/water ratio of 3:7. The sample mix was dried under a stream of warm air before analysis.

2.2.4 Enzymatic profiling of cell wall polysaccharides

Pure and well characterized enzymes (Table 1) were used to specify the type of polysaccharides present in the HBSS, CHSS and CASS extracts. 0.002 g/mL (total sugars) of HBSS, saponified HBSS (sHBSS), CHSS and CASS were prepared in 10 mM sodium acetate buffer, pH 5.0. Saponification of HBSS was carried out by incubating HBSS in 0.05 N NaOH for 24 h at 4°C. Afterwards, the solution was acidified with 0.1 M acetic acid to pH 5.0 and the volume was adjusted with 10 mM sodium acetate buffer, pH 5.0 to obtain a final concentration of 0.002 g/mL (total sugars).

Table 1: Enzymes employed in screening of polysaccharide structures in extracts.

Enzyme	Organism	Specific activity	Literature
Polygalacturonase II (PG II)	*Aspergillus niger*	8700U/mg	Kester and Visser (1990)
Endo-galactanase (Endo-Gal)	*Aspergillus aculeatus*	988U/mg	Van de Vis et al. (1991)
Betagalactosidase (Exo-Gal)	*Aspergillus niger*	0.8U/mg	Laboratory of Food Chemistry, unpublished data
Endo-Xylanase I	*Aspergillus awamori*	7.8U/mg	Kormelink et al. (1993)
Endo-mannanase	*Aspergillus niger*	86.4U/mg	Dusterhoft et al. (1993)
Xyloglucan specific endo-glucanase (EGII)	*Aspergillus aculeatus*	2259U/ml	Pauly et al. (1999)

All enzyme digestions were carried until endpoint incubation. Polygalacturonase (PGII) and a combination of endo-galactanase (endo-Gal) and β-galactosidase (exo-Gal) were applied to HBSS and sHBSS in a dose of 0.01% (w/w) of enzyme protein to total sugar. PGII was also applied to CHSS in the same dosage and all incubations were carried for 24 h. CASS was incubated with 0.0128 mL/g (total sugars) of a xyloglucan specific endo-glucanase (EGII, Table 1) for 48 h. After this, CASS was incubated separately with 0.01% (w/w of enzyme protein to total sugar) endo-xylanase (xylanase, Table 1) and separately with 0.01% (w/w of enzyme protein to total sugar) endo-mannanase (Mannanase, Table 1). The incubations were carried at 40°C in a head over tail mixer and enzyme digests were inactivated at 100°C for 10 min. Samples were centrifuged (20 min, 19,500 × g, 20°C) before analysis on HPSEC and MALDI-TOF MS.

2.2.5 Water holding capacity

WHC measurements were performed using the Baumann's apparatus (Baumann, Germany; Baumann,1966). A small quantity (minimum 0.01 g) of dried sample was placed on the filter (G2; Duran, Germany) and the volume of water absorbed to hydrate the sample until saturation was recorded.

Reference substrates tested were potato starch (Sigma Aldrich, USA), polygalacturonic acid (ICN Biochemicals, Aurora, OH, USA), cellulose (filter paper; Whatman, Grade 3, UK), tamarind seed xyloglucan (Dainippon Sumitomo Pharma, Osaka, Japan), linear arabinan and branched arabinan (British Sugar, Peterborough, UK) and potato galactan (Megazyme, Wicklow, Ireland).

For HBSS, a filter paper (grade 595 Whatman, Dassel, Germany) was used above the filter and water absorbed by the filter was corrected accordingly. In addition, the WHC was also measured for a combination of HBSS and BIS (in a ratio of 1:1) and a combination of HBSS and BIS mixed with water (in a ratio of 1:1; 0.07 g/ml) at 200 rpm in a shaking incubator (Innova 40 R, New Brunswick, NJ, USA) for 2.5 h at 40°C before freeze drying. The WHC was expressed as the volume of water held in mL per g dry matter of sample analyzed. Corrections were made for evaporation losses by measuring water uptake without sample. All measurements were performed in triplicates.

2.3 Results and discussion

2.3.1 Cell wall polysaccharides in PPF extracts

PPF contains 74% (w/w) carbohydrates of which 30% is starch (Table 2). The most abundant CWPs in PPF are built from glucosyl (Glc), galactosyl (Gal) and uronyl (UA) residues.

2.3.1.1 Distribution and composition of CWPs in extracts

After de-starching, 46 % of all CWPs from PPF are recovered in HBSS (dialyzed), while 41% remains in BIS. This is consistent with earlier data on PPF where Thomassen et al. (2011) reported a high solubilization of dry matter in phosphate buffer. From undialyzed HBSS (51 % of all CWPs from PPF), 5 % of CWPs are removed upon dialysis as Rha, Ara, Gal and UA. Oligomers of these CWPs were present prior to de-starching PPF (data not shown). Since HBSS is composed of 85 % (w/w) of CWPs, of which 41 mol% is Gal, 8 mol% is arabinose (Ara) and 43 mol% is UA, it is expected to be rich in pectins (Table 2). About 76 % of pectins (UA and Rha) from PPF were extracted into HBSS.

Earlier studies on cooking or de-starching of intact potato cell walls solubilized much less pectins. Ball milling of uncooked cells released much more cell wall material as small and large fragments than decompression of cooked potato cells (Van Marle et al., 1997b). De-starching of non-processed potato cell walls show much less solubilization of CWPs (less than 6%; Ring and Selvendran, 1978) than that of processed cell walls (our data). The composition of pectins in HBSS is similar to the Na_2CO_3 extract of intact potato cell walls as shown by Jarvis et al. (1981) after extraction of calcium bound pectins. Pectin solubilization into HBSS without prior chelation is possible if the middle lamella (rich in calcium bound pectins (Caffall and Mohnen, 2009)) is ruptured. This indicates that disruption of the cell wall interactions in potatoes may have occurred during processing in industrial starch extraction. In PPF, a lower molar ratio of Rha:Gal (about 1:7) is observed compared to whole potato cell walls (about 1:35), indicating a relatively lower occurrence

of Gal in PPF cell walls (Van Marle et al., 1997a). This could imply differences in the packaging of CWPs between PPF and potato cell walls.

Less than 2 % (w/w) CWPs are solubilized into CHSS and DASS, 9.7 % in CASS and 30.6 % remains in Res. In prior studies on potato cell walls, 50 % CWPs were left in Res (Ryden and Selvendran, 1990 and Van Marle et al., 1997a). This difference supports the observation that a less rigid CWP architecture exists in PPF compared to non-processed potato cell walls.

Only 0.7% of starch remains in BIS, which contains 59 % (w/w) as CWPs, mainly comprising cell wall Glc and Xyl. UA is the major sugar in CHSS (64 mol%, Table 2), which contains 2.7 % (w/w) of HG (based on UA) from PPF. A Rha:UA ratio of 1:17 points that CHSS is rich in HG.

Dilute alkali (0.05 M NaOH) treatment of CHUS extracts mainly Gal, UA, Ara, and Rha (Table 2) in DASS and most of the starch from BIS (4.6 % on dry matter; Table 2). Compared to HBSS, the ratio of (Ara+Gal)/Rha (8.5) is lower, indicating lesser branching of RG and an Ara:Gal ratio of 1:2 indicates lesser dominance of Gal residues. The ratio of Rha:UA (2:7) is higher than that of HBSS suggesting that DASS is richer in RG. DASS is thus distinct from HBSS with respect to abundance and composition of RG.

CASS mainly comprises Xyl (19 mol%) and Glc (54 mol%)(Table 2) pointing to the presence of xyloglucans (XGs) reported earlier as XXGG type, in which the Xyl:Glc ratio is 1:2 for potato cell walls (Vincken et al., 1996). Since CASS shows a ratio of 1:3, it indicates possible presence of β-glucans or glucomannans. In previous studies, a Xyl:Glc ratio of 1:9 for the 6M NaOH extract by Jarvis et al. (1981) suggests differences in alkali soluble glucans between PPF in our study and potato cell walls.

Res is abundant in cell wall Glc (90 mol%)(Table 2) and contains 87% of all cell wall Glc from PPF which is possibly cellulose.

In general, our results suggest that most pectins are easily extractable from PPF due to a less rigid cell wall network. Few pectins are extracted in CHSS and DASS extracts, while hemicelluloses are mainly extracted in the CASS extract, leaving glucans, most likely cellulose, in Res.

Table 2: Composition of CWPs in PPF

Fraction	Yield% CWPs (dry PPF)	NSPs : Sugar composition (mol%); yield % sugar residues[1,2]								mol%		w/w % dry matter		
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc[d]	UA	DA[3]	DM[3]	Starch	CWP	Protein
PPF	100	4	0	8	3	2	27	32	24	25	17	30	44	4
		(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)					
HBSS	46	5	0	8	1	1	41	1	43	20	1	0.4	85	4.4
		(53)	(19)	(48)	(9)	(26)	(68)	(2)	(79)					
BIS	41	2	0	5	7	3	4	73	6	156	2	0.7	59	5.7
		(23)	(23)	(25)	(92)	(66)	(6)	(96)	(10)					
CHSS	1.2	4	0	6	2	3	13	9	64	6	3	n.d	32	n.d
		(1)	(0.9)	(0.9)	(0.7)	(2)	(0.6)	(0.3)	(3)					
DASS	0.5	5	1	15	9	2	31	19	18	n.d	n.d	4.6	21	n.d
		(0.6)	(2)	(1)	(1)	(0.8)	(0.7)	(0.3)	(0.4)					
CASS	9.7	2	0	7	19	8	7	54	2	n.d	n.d	0.1	42	n.d
		(5)	(9)	(9)	(60)	(51)	(3)	(18)	(0.9)					
Res	30.6	2	0	3	1	0	1	90	3	n.d	n.d	1	68	n.d
		(12)	(17)	(10)	(13)	(6)	(1)	(87)	(4)					

[1] Values in brackets indicate yields of sugar residues; "n.d" is not determined.
[2] Rha: Rhamnose, Fuc: Fucose, Xyl: Xylose, Man: Mannose, Gal:Galactose, Glc:Glucose, UA: Uronic Acid
[3] DA is Degree of Acetylation and DM is Degree of Methyl esterification calculated as moles of acetyl / methyl esters per 100 moles of uronic acid.
[d] is cell wall Glc

2.3.2 Esterification of cell wall polysaccharides

The degree of acetyl esterification (DA) and methylation (DM) of PPF is 25 and 17, respectively. This is consistent with earlier data (Turquois et al., 1999). Since PPF has a lower DM than reported for potato cell walls (49-53, Van Marle et al., 1997a), most likely methyl esters are removed during the industrial starch extraction process. Also, although the experimental set up should have inactivated the native pectin methyl esterase, extracts from PPF even show a lower DM due to losses in methyl esters. The DA of BIS (156, Table 2) suggests a high level of substitution of acetic acid ester-groups over pectin as reported previously for potato pectic hairy regions (DA of 90; Schols and Voragen, 1994). However, it cannot be excluded that non-pectic CWPs could also be acetylated, such as xyloglucans in tomato (Jia et al., 2005). Next to acetylation and methyl esterification of CWPs, ferulic acid esterification was determined for potato CWPs. Not much information on feruloyl substitution is known for potato CWPs. The ferulic acid (FA) content is 0.01 % ((w/w) dry matter) in PPF and 0.03 % in BIS, which is slightly higher than those for potato peels (0.007 %; Nara et al., 2006). 8-*O*-4 linked di-FA (m/z 385 (M-H)), which was one-fifth the response of FA, was observed in PPF and BIS, and has not been reported previously for potato cell walls. In BIS, 1 mol of FA corresponds to 233 mol of Ara+Gal. For sugar beet pulp, pectin populations are reported containing 1 mol of FA per 67 mol of Ara+Gal (Oosterveld et al., 1996). For wheat arabinoxylans, in which 1 mol of FA corresponds to 308 mol of arabinose+xylose (Gruppen et al., 1989), feruloyl substituents were indicated to maintain the quarternary structure of CWPs (Smith and Hartley, 1983). In our PPF and BIS, LC-MS detection of (-di)FA esters could indicate presence of quarternary structures in PPF. Further research on these (-di)FA's may prove their contribution to quarternary structures and effect on WHC.

2.3.3 Size distribution of polysaccharides in soluble extracts

To compare the molecular weight (Mw) distributions of extracts, HPSEC was performed (HBSS in figure 1). For HBSS, two major populations of CWPs are observed where the larger population is distributed over a broad molecular weight (Mw) range of 50

-570 kDa next to a smaller population of 12-170 kDa. Based on sugar composition, these populations could represent pectin of different Mw. For CHSS and DASS (not shown), a large population from 50 to 570 kDa is observed. CASS (in figure 2A) shows a broad population distributed from a low to high Mw range (5-572 kDa) representing hemicelluloses. Thus, HPSEC profiling of extracts show main differences in Mw distributions of HBSS, CHSS and DASS on one hand and CASS on the other hand. The HPSEC data indicates that next to sugar composition data, pectins and hemicelluloses have distinct Mw populations.

2.3.4 Enzymatic profiling of cell wall polysaccharides in extracts

To obtain more information on the structure of CWPs present in extracts, pure and well characterized enzymes (Table 1) were used. It was confirmed that the digestion was an endpoint incubation based on elution patterns of defined substrates treated with these enzymes. Therefore, it was assumed that CWPs were targeted by the enzymes as far as possible, although the extent of degradation of the extract is far from complete. Nevertheless, the enzymatic profiling described is well usable to study CWP structural characteristics in various PPF extracts.

2.3.4.1 Profiling CWPs in HBSS

Sugar composition data of HBSS shows dominance of UA and Gal. Therefore, HBSS was profiled using polygalacturonase (PGII) and a combination of endo-galactanase (endo-Gal) and beta-galactosidase (exo-Gal). CWPs in HBSS are acetylated (DA of 20). Therefore, after saponification, PGII shows more degradation (Figure 1) in the high Mw region eluting between 7.5 and 8.5 min. Irrespective of saponification, comparable profiles are observed in the low Mw region eluting between 11.5 and 12.5 min. This low Mw region in undigested HBSS may be abundant in low Mw HG. Surprisingly, only saponified HBSS (s.HBSS) shows a complete shift in HMw material when digested with a combination of endo-Gal and exo-Gal. This indicates high esterification of RG-I (Table 2) in HBSS and is in line with earlier findings where for potato RG-I (Schols and Voragen, 1994). Removal of acetyl groups increases accessibility of these enzymes to galactan side chains.

Due to this, it is possible that a significant distortion in the secondary structure of pectic hairy regions occurs resulting in a complete shift in the high Mw region of HBSS.

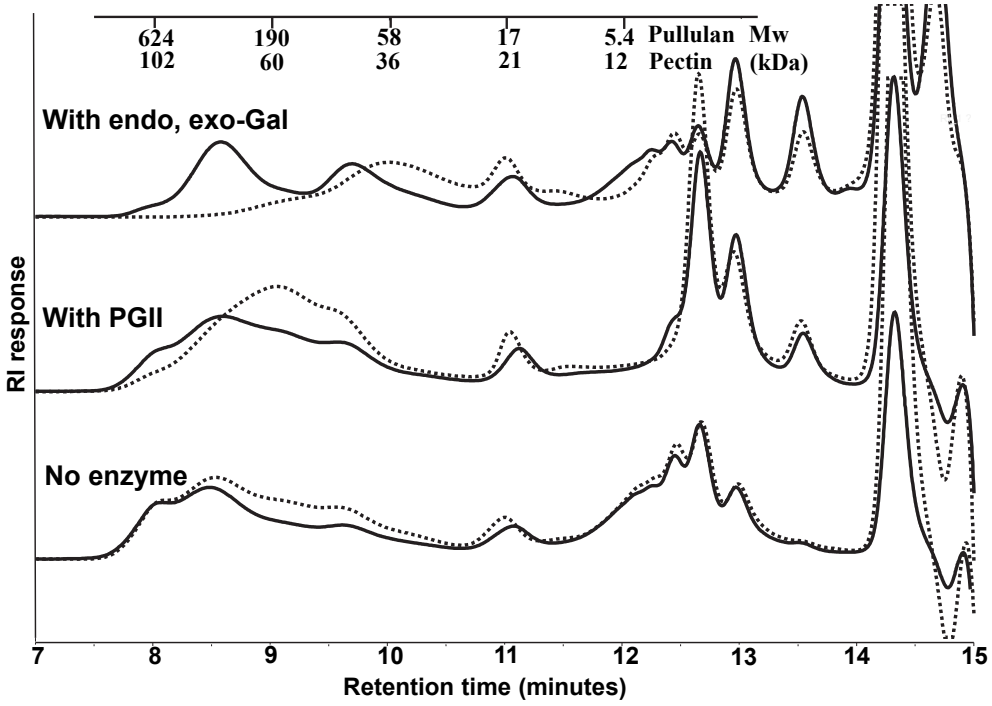


Figure 1: HPSEC elution patterns of enzymatic profiling after 24 hours of HBSS (—) and saponified HBSS (s.HBSS;••••) with polygalacturonase (PGII) and a combination of endo-galactanase and β-galactosidase (endo, exo-Gal). Molecular weight (Mw) annotations on top x-axis are based on pullulan and pectin standards.

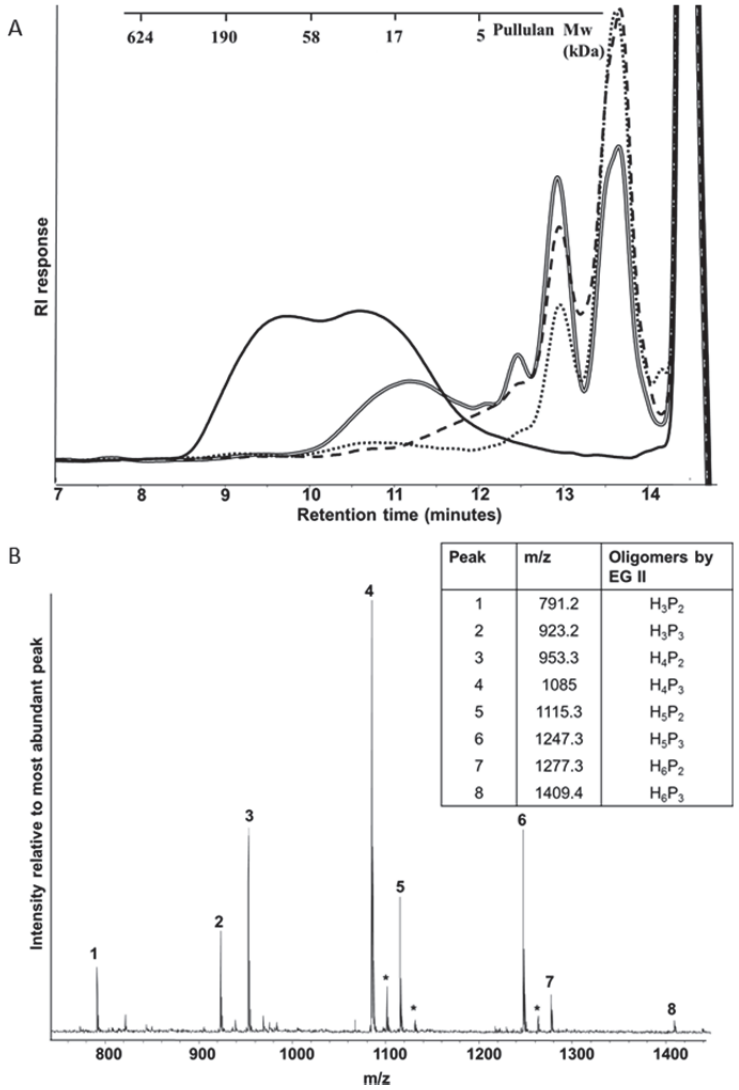
These results suggest that HBSS contains acetylated CWPs mainly rich in pectic galactans and HG.

2.3.4.2 Profiling CWPs in CHSS using polygalacturonase

As the degree of esterification of HG in CHSS is very low (Table 2), PG II seems to degrade CHSS effectively to produce low Mw material less than 10 kDa (added as supplementary figure). This data supplements sugar composition of CHSS indicating that it is mainly dominant in HG.

2.3.4.3 Profiling CWPs in CASS using hemicellulases

Digestion of CASS with xyloglucan specific endoglucanase (EGII) degrades 59 % of soluble material eluting between 8 and 12 min (Figure 2A).



Peak	m/z	Oligomers by EG II
1	791.2	H_3P_2
2	923.2	H_3P_3
3	953.3	H_4P_2
4	1085	H_4P_3
5	1115.3	H_5P_2
6	1247.3	H_5P_3
7	1277.3	H_6P_2
8	1409.4	H_6P_3

Figure 2: A) HPSEC elution patterns of CASS before (–) and after digestion with EGII (—), EGII and xylanase (––) and EGII and mannanase (••••). B) MALDI-TOF-mass spectrum of CASS after digestion with EG II. P is pentose and H is hexose (sodium adducts),* represent potassium adducts.

This indicates that CWPs in soluble CASS largely consist of xyloglucans (XGs). The abundance of xyloglucans in CASS is supported by previous studies on potato xyloglucans by Vincken et al. (1996), wherein linkage analyses of a purified alkali extract showed to consist exclusively of terminal and 1,2-linked Xyl, together with 1,4 and 1,4,6-linked Glc. Oligomer profiling of EGII treated CASS using MALDI-TOF MS shows that the main fragments in the low Mw region are H_3P_2, H_3P_3, H_4P_2, H_4P_3, H_5P_2, H_5P_3, H_6P_2 and H_6P_3 (H is hexose and P is pentose (Figure 2B)). These are expected to correspond to XXG, XSG, XXGG or LXG, XSGG, XLGG, LSGG and LLGG (where G is glucose, X is xylose linked to glucose, L is galactose and S is arabinose linked to xylose-glucose) building blocks reported previously by Vincken et al. (1996). After treatment with EGII, treatment of CASS with mannanase and xylanase further degrades CASS, indicating the presence of mannans and xylans. Earlier studies on potato CWPs also have reported the presence of 4-linked mannans and 4-linked xylans (Jarvis et al., 1981), which are yet to be confirmed. Further investigations are necessary to characterize their structures.

In general, enzymatic profiling revealed that HBSS mainly contains acetylated pectins comprising HG and pectic galactans which were easily extractable while CHSS exclusively contains HG. On the other hand, CASS is abundant in hemicellulose, mainly xyloglucans with lesser abundance of mannans and xylans.

2.3.5 Water holding capacity

The water holding capacity (WHC) is defined as the amount of water held (in mL) per g dry matter of sample. The WHC is determined using the Baumann's method based on the principle that a certain amount of water hydrates a known amount of dried sample material (usually in the form of a powder) until equilibrium or saturation. The amount of water held by defined substrates and PPF substrates (extracts and residues sequentially derived from PPF and representing different populations of CWPs) was determined (Figure 3 and 4). Also, the relative contribution of each of the PPF substrates to the total water held by PPF was determined (Figure 4) and expressed as "the WHC contributed by a substrate to the WHC of PPF". This relative contribution of PPF substrates to the total WHC of PPF (Figure 4) is calculated based on the CWP yield per substrate (extract or

residue) and the mL of water held per g of CWPs in the substrate (denoted in figure 4 as "mL/g CWPs in sample for 1g total CWPs in PPF").

2.3.5.1 Water holding capacity of reference substrates

Of all reference substrates tested, linear arabinan shows the highest WHC (4.6 mL/g; α-(1, 5)-linked backbone of L-arabinosyl residues (Beldman et. al (1997)). The WHC of branched arabinan (0.4 mL/g); arabinan substituted with α-(1,2) and / or α-(1,3) linked L-arabinosyl residues (Beldman et. al (1997)) is much lower than that of linear arabinan. This could be related to the observation that longer chains of linear arabinan are more flexible than highly branched arabinan, and, therefore, more mobile to interact with water giving them a higher radius of gyration and hydrodynamic volume (Chaplin, 2003). Potato galactan has a lower WHC (3.5 mL/g) than linear arabinan, but higher than branched arabinan. This may be due to increased mobility of galactan side chains in solution. The results are in line with prior studies in which arabinan side chains hydrate more readily than galactan and shorter side chains impart RG-I with a lesser affinity to water (Larsen et al., 2011).

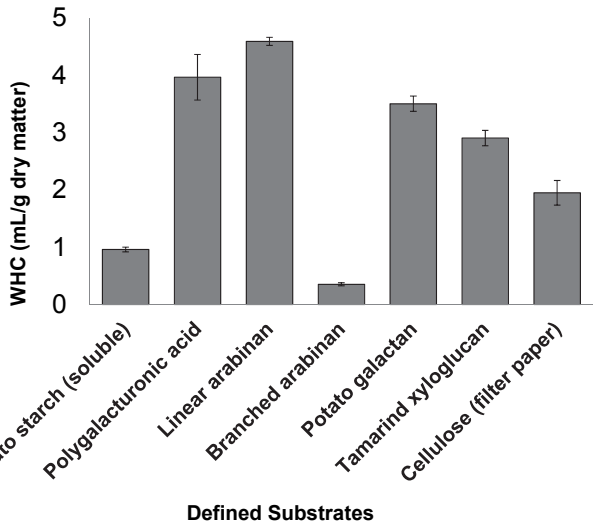


Figure 3: Water Holding Capacity (WHC) values (mL/g) of reference substrates with the Baumann's apparatus. See section 2.2.5 for more details on substrates.

Compared to galactan, filter paper cellulose shows a lower WHC. Our observation matches with the finding that for onion cell wall material solid state NMR revealed a lower hydration for cellulose than for pectic galactan. This was not only due to increased mobility of the latter but also due to easier penetration of water into galactan (Hediger et al., 1999).

2.3.5.2 Water holding capacity of substrates derived from PPF

In general, substrates derived from PPF have higher WHCs than reference substrates (figure 4 versus figure 3). This was expected since PPF substrates represent different populations of CWPs, rather than pure reference substrates. Either entrapment of water within the matrix of CWM or water binding due to interactions between CWPs, most likely contributes to the high WHC (Figure 4).

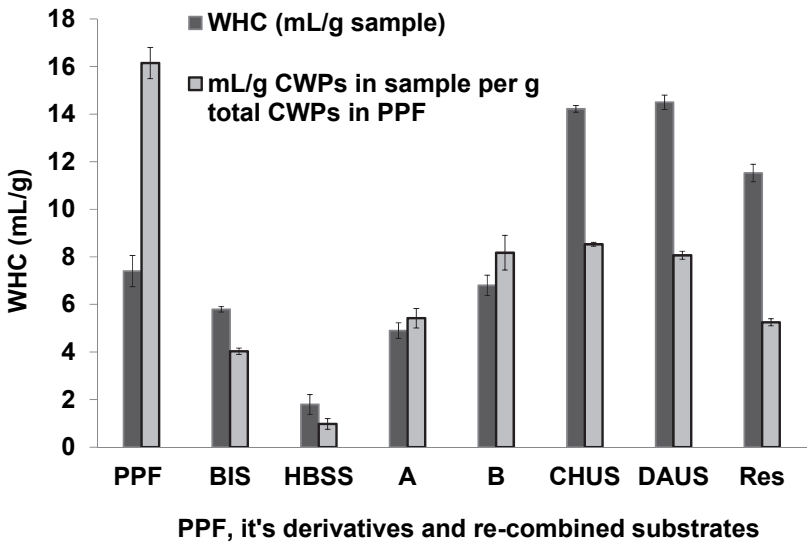


Figure 4: Water Holding Capacity (WHC) values (mL/g) of PPF and substrates derived from PPF its sequentially derived residues. "A" is reconstitution of HBSS and BIS by mixing them without water and "B" is re-constitution of HBSS and BIS in water. See section 2.2.5 for more details on A and B.

PPF has a WHC of 7.4 mL/g. After extracting 50% CWPs from PPF in HBSS, the cell wall network is loosened. The resulting BIS has a WHC, which is 22% lower than the WHC of PPF. HBSS, in which both UA and Gal (Table 2) are abundant and present in equal proportions, shows a lower WHC than that of reference substrates potato galactan and polygalacturonic acid. When a mix of HBSS and insoluble BIS is reconstituted by hydrating for two and a half hours followed by freeze-drying, the WHC of the recombined sample is higher than the mix reconstituted in the absence of water. In presence of water, it is likely that galactan side chains in HBSS become mobile due to which RG-I in HBSS is hydrated as observed previously (Larsen et al., 2011). After hydration, these polysaccharides may interact with CWPs in BIS forming a network similar to that in PPF. This indicates that the interactions are at least partly restored in this mix and play a role in the WHC. The exact role of HG remains unclear, although HG and galactan are present in equal amounts in HBSS. Since soluble potato starch had a very low WHC (1 mL/g; Figure 3), the presence of residual amounts of starch is not expected to have much effect on the WHC of PPF. Removal of calcium bound pectins from BIS modifies the cell wall network resulting in CHUS with a very high WHC (14.2 mL/g). Modification of the network could be because of spatial rearrangement of the packaging of CWPs instead of only removing HG. Therefore, the role of HG is not discussed further. Removal of hairy pectins from CHUS did not have any major effect on the WHC seen in DAUS. Removal of CASS from DAUS, lowers the WHC further by 21% seen for Res. CASS is found to consist of hemicelluloses mainly comprising xyloglucans (XGs), which could contribute positively to a higher WHC. Alkali treatment is expected to increase the swelling and therefore, most likely, the apparent WHC of the Res which is abundant in cellulose (Pott, 2003). In view with these findings and as discussed earlier for reference substrates, cellulose alone is not expected to contribute much to the WHC of PPF.

In general, the results suggest that among the most influential interactions of CWPs in regulating the WHC of PPF, are those between HBSS and BIS CWPs. In more detail, the results implicate that galactans, and xyloglucans in the CHUS and DAUS residues could be important in regulating the WHC of PPF.

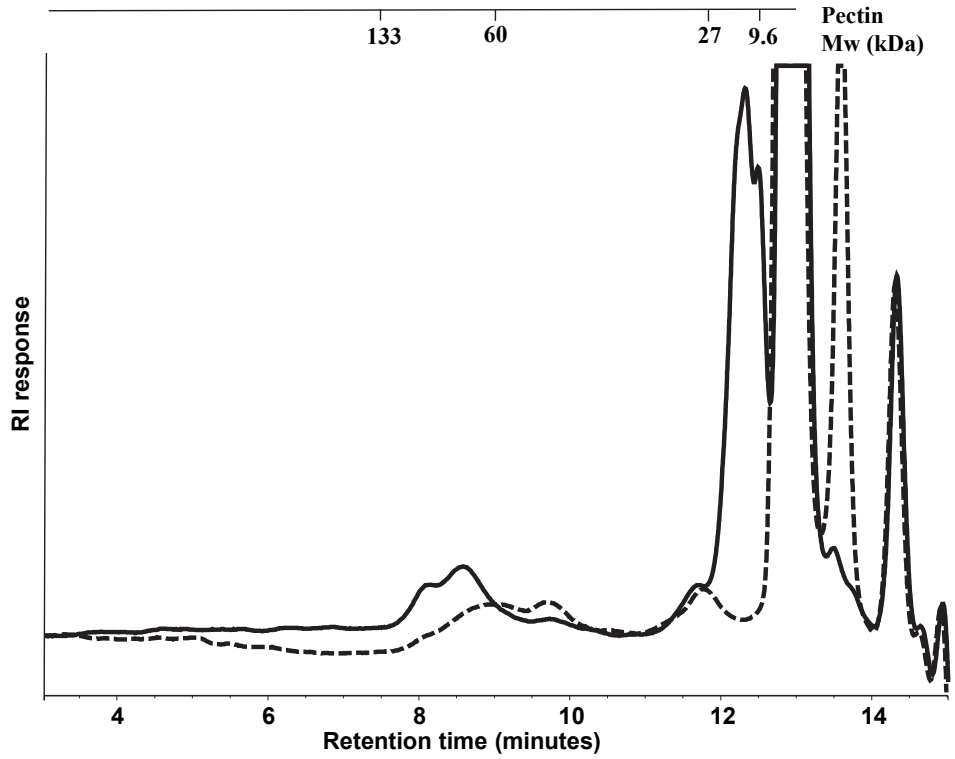
2.4 Conclusions

The cell wall architecture in PPF is less rigid than in non-processed potato cell walls as observed from the high solubilization of pectins and pectic galactans in HBSS. In addition, the composition of CWPs in PPF is different than in non-processed potato cell walls with respect to alkali soluble glucans and a lower proportion of galactans. Profiling of the main CWP extracts, HBSS and CASS, using enzymes showed that the most easily extractable CWPs from PPF are acetylated homogalacturonan and rhamnogalacturonan rich in galactans while hemicelluloses, mainly xyloglucans, are extracted under harsher conditions. The WHC of PPF is restored at least partly after hydrated mixing of HBSS with BIS. The WHC of PPF may be governed by interactions between CWPs in which the water soluble galactans and alkali soluble xyloglucans are implicated to be important.

2.5 Acknowledgement

This project is jointly financed by the European Union, European Regional Development Fund and The Ministry of Economic Affairs, Agriculture and Innovation, Peaks in the Delta, the municipality of Groningen, the province of Groningen as well as the Dutch Carbohydrate Competence Centre (CCC WP8), Wageningen University, AVEBE, Royal Cosun and Duynie B.V.

Supplementary data:



Supplementary figure 1: HPSEC elution patterns of CHSS before (——) and after enzymatic profiling (– –) with polygalacturonase.

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195.

Chapter 3

Water holding capacity of soluble and insoluble polysaccharides in pressed potato fibres

Abstract

Pressed potato fibres (PPF), a by-product of starch production, has a high water holding capacity (WHC). In this study, it is shown that the WHC is caused by a network of mainly insoluble, non-cellulosic cell wall polysaccharides (CWPs). Despite the solubilization of one-fourth of the CWPs from PPF, representing 40-60 w/w % of pectic CWPs (rhamnosyl, uronyl, galactosyl and arabinosyl residues) present in PPF, the insoluble residues still had similar WHCs as PPF. Only after enzymatic hydrolysis of mainly non-cellulosic CWPs, the WHC decreased substantially (by 61%). Combining the cellulose-rich residue obtained after enzyme hydrolysis with a polymeric homogalacturonan(HG)-rhamnogalacturonan-I (RG-I)-arabinogalactan(AG) extract increased the WHC. This increased hydration is suggested to result from the adsorption of the soluble HG-RG-I-AG to the insoluble cellulose-rich residue. Adsorption of the HG-RG-I-AG was not detected for the insoluble residue enriched in non-cellulosic CWPs.

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3.1 Introduction

Pressed potato fibres (PPF), a by-product of industrial starch extraction from potatoes, is difficult to process because of its high water holding capacity (WHC). PPF contains 20-40% (w/w) starch (Leszczynski, 1989), about 40-60% (w/w) cell wall polysaccharides (CWPs), 3-6% (w/w) proteins, about 4% (w/w) ashes, about 4% (w/w) lignin and the remaining (6% (w/w)) is unknown (Mayer and Hillebrandt, 1997, Meyer et al., 2009; Ramaswamy et al., 2013).

The CWPs in PPF comprise arabinogalactan rich rhamnogalacturonan-I (RG-I), homogalacturonan (HG), hemicellulosic CWPs, such as xyloglucan (XG), glucomannan, and xylan (the least among hemicelluloses), and cellulose. Previously, the WHC of PPF has been suggested to be influenced by interactions between CWPs, in which water soluble arabinogalactans associated with RG-I and insoluble XGs were indicated to be the most important (Ramaswamy et al., 2013). In another study, it was found that side chains of galactan and arabinan imparted a high hydrophillicity to the RG-I backbone unless they were truncated (Larsen et al., 2011). Apart from these pectic components, the effect of XGs and their interactions with other CWPs on the WHC remains unclear. The adsorption of XG to cellulose (Vincken et al., 1995) and arabinogalactan to cellulose (Zykwinska et al., 2005) have been proposed, but whether this type of interaction also contributes to the WHC of the insoluble residue is not known.

Enzymatic hydrolysis has been shown to reduce the WHC. For example, the WHC of dehulled lupins was reduced by 14% after treatment with a combination of polygalacturonase and pectin methyl esterase (Ali et al., 2005). Also, treatment of wet milled rapeseed meal with commercial pectinolytic enzymes reduced the amount of water bound by 38% (Pustjens et al., 2012). From these studies, it seems that depletion of CWPs in the cell wall network by enzymatic hydrolysis causes less water to be retained by the resulting insoluble residue. However, it has also been shown that despite a reduction in the WHC for xylanase-treated wheat cell wall materials, the WHC of the insoluble residue after enzyme incubation increased by a factor of 2.5 (Gruppen et al., 1993). Therefore, it is evident that the type of CWPs degraded determines the extent to which the WHC of the residual material is reduced.

In the present study, we hypothesize that the WHC of the CWP network in PPF results mainly from a network of insoluble non-cellulosic CWPs. For this purpose, the WHCs of PPF and its insoluble residues before and after treatment with an enzyme, and their combinations with soluble CWPs, were studied.

3.2 Materials

Pressed potato fibres (PPF) was provided by AVEBE, Foxhol, The Netherlands. PPF was frozen immediately after provision until use. The enzyme used for hydrolysis of PPF was an experimental enzyme preparation, described to contain acetyl esterase and β-glucanase activities (*Penicillium funicolusum*; Erbsloh; Geisenheim, Germany) and denoted as Ebs. Substrates were polygalacturonic acid (PGA, Fluka Biochemica, Buchs, Switzerland), high methyl esterified pectin (HMP, from citrus, degree of methyl esterification of 67 (Zitko and Bishop, 1965), Eastman Kodak, Rochester, NY, USA), arabinan (British Sugar, Peterborough, UK), arabinogalactan (from potato, Megazyme, Wicklow, Ireland), xyloglucan (XG; from tamarind seed, Dainippon Sumitomo Pharma, Osaka, Japan) and cellulose (carboxy methyl form, CMC; Sigma, St. Louis, MO, USA). Soluble arabinogalactan, arabinan and PGA were prepared by mixing a solution of these polysaccharides (2 mg/mL) head over tail for 30 minutes, followed by centrifuging (18,000 x g, 20 min, 20°C) and freeze drying the soluble fraction. PNP-β-galactopyranoside and PNP-α-arabinofuranoside were obtained from Sigma. Potato starch was obtained from AVEBE.

3.3 Methods

3.3.1 Fractionation and de-starching of PPF

Soluble and insoluble fractions of PPF were prepared by incubating PPF in water at a dry matter concentration of 8% (w/w), enabling separation of all soluble dry matter from the residue. Incubations were performed at 40°C for 47 h with head over tail mixing after which the solution was centrifuged (20 min, 38,000 x g, 20°C). After three washing

steps with water, the residue was denoted as PPF Res, and after combining the corresponding wash water with the supernatants, this was denoted as PPF Sup. PPF was also de-starched as described previously (Ramaswamy et al., 2013) to obtain BIS (buffer insoluble solids; dialyzed) and HBSS (hot buffer soluble solids; dialyzed), after which their starch contents were determined. The residues and supernatants were freeze dried prior to analysis. PPF, PPF Res, PPF Sup, BIS and HBSS were analyzed for carbohydrate content and composition and used for WHC studies. Part of BIS was milled (BIS^{m}; 50% intensity for 1 min using a MM 2000 mill (Retsch, Haan, Germany)), washed further with water and centrifuged (38,000 x g, 20 min, 20°C). The residue was freeze dried and denoted as BIS^{mw}, before combining with soluble polysaccharides.

3.3.2 Enzymatic liquefaction of PPF

About 310 g of dried PPF was mixed thoroughly in a Mark 5 high intensity mixer 9820 of a capacity of 2L (Quantum Technologies, Marysville, OH, USA) at a dry matter content of 15% (w/w) in water (pH was 5.2). After this, PPF was incubated with Ebs at a dose of 1.2 % (w/w of enzyme liquid weight on dry matter of PPF). The protein content of Ebs was 12.9 % (w/w; N%*6.25). The incubation was carried out under continuous stirring at 40°C for 47 h. After incubation, PPF was homogenized and aliquots of Ebs treated PPF, denoted as Ebs PPF, and were further used. Ebs PPF was centrifuged (20 min, 38,000 x g, 20°C). After three washing steps with water, the residue after Ebs treatment was denoted as Ebs Res. After combining the corresponding wash water with the supernatants, this was denoted as Ebs Sup. Ebs Res and Ebs Sup were freeze dried prior to carbohydrate analysis and WHC studies.

3.3.3 Identification of enzyme activities in Ebs enzyme preparation

Enzyme activities present in Ebs were screened by studying the effect of Ebs on the hydrolysis of various reference substrates. These substrates, PGA, HMP, arabinan, arabinogalactan (AG), xyloglucan (XG) and cellulose, were used at concentrations of 5 mg/mL in 50 mM NaOAc buffer, pH 5.2. Ebs was applied at a dose of 1% (w/w) enzyme

liquid on dry matter of substrate. Incubations were carried out at 40°C for 16 h, head over tail. Enzymes were inactivated by heating at 100°C for 5 min. Samples were centrifuged (20 min, 19,500 x g, 20°C), before analysis on HPSEC.

HBSS (1.5 mg of total carbohydrates/mL) was incubated with 0.1 % (w/w) of Ebs at 40°C for 16 h in 10 mM NaOAc buffer, pH 5.0. Enzymes in Ebs were inactivated by heating at 100°C for 5 min. Ebs treated HBSS was centrifuged (20 min, 19,500 x g, 20°C), before analysis on MALDI-TOF MS.

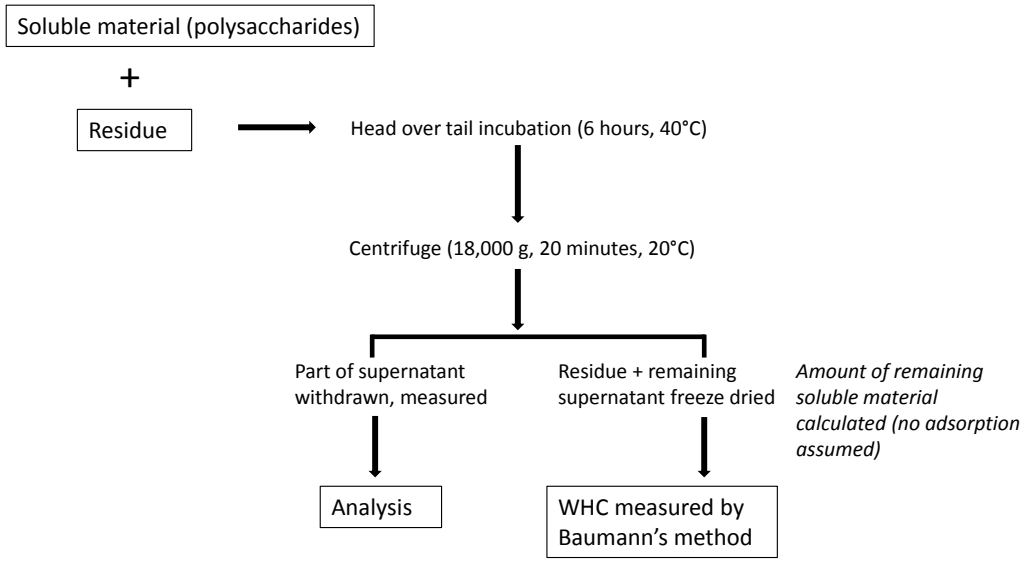
In addition, it was verified whether Ebs was active on starch. For this, the enzymes were incubated at a dose of 1% (w/w) of enzyme liquid on dry matter of potato starch (6 % (w/w) in milliQ water) at 40°C for 24 h with head over tail mixing and frozen until further analysis. Samples were centrifuged (30 min, 18,000 x g, 20°C) before analysis on HPSEC. No differences in the HPSEC profiles were seen upon incubating starch with Ebs (supplementary figure 1). Therefore, the starch contents in PPF Res and Ebs Res were calculated assuming that all starch originally present in PPF was recovered in these residues.

Ebs was also tested for exo-activities by using p-nitrophenyl (PNP) glycosides. About 25 µl of the enzyme or diluted enzyme solution was mixed with 0.025 ml of 0.1% (w/w) of the PNP-glycoside solution. The mixture was incubated with 0.075 ml of 0.05 M NaOAc buffer, pH 5.0 for 1 h at 40°C. After this, 0.125 ml of 0.5 M glycine NaOH buffer, pH 9.0, containing 0.002 M EDTA was added and the absorbance of the PNP released was measured at 405 nm. The exo-activity of the enzymes was expressed as Units (U) of PNP released per mL of enzyme solution, where 1U corresponds to 1µmol of PNP released per min.

3.3.4 Substrate combinations for WHC studies

For WHC studies, residues obtained from PPF (3.3.1 and 3.3.2) were combined with soluble polysaccharides. All combinations were performed in duplicate. The scheme followed for combining samples and preparing them for WHC measurements is shown in figure 1. In this scheme, a known amount of residue was combined with a known amount of soluble polysaccharide, to which water was added to obtain a final concentration of

about 30 mg/mL. After vortexing, the sample was incubated head over tail at 40°C for 6 h. After incubation, the samples were centrifuged (18,000 x g, 20 min, 20°C), and a part of the supernatant was withdrawn for analyses on HPSEC to check for adsorption.



$$\text{WHC} = \frac{mL\ of\ water\ held}{g\ of\ dry\ matter}$$

$$\text{WHC}^{i} = \frac{WHC\ of\ 1\ g\ of\ dry\ matter}{g\ of\ insoluble\ material\ in\ 1\ g\ of\ dry\ matter}$$

$$g\ of\ insoluble\ material\ in\ 1\ g\ of\ dry\ matter = \frac{g\ of\ residue\ added}{(g\ of\ residue\ added + g\ of\ remaining\ soluble\ material)}$$

$$\text{WHC}^{P} = \frac{WHC\ of\ 1\ g\ of\ dry\ matter - WHC\ of\ soluble\ material\ in\ 1\ g\ of\ dry\ matter}{g\ of\ insoluble\ material\ in\ 1\ g\ of\ dry\ matter}$$

$WHC\ of\ soluble\ material\ in\ dry\ matter$ = WHC x % of soluble material in dry matter

Figure 1: Scheme of preparation of combinations of samples for WHC measurements. Inset box shows the calculations used for expressing the WHC of the insoluble residue (WHC^{i} and WHC^{P}; mL/g dry matter of residue).

The absence of adsorption, in most cases, overruled the possibility of preferential binding of soluble polysaccharides to the residue. Therefore, the amount of soluble polysaccharide (on dry matter) present with the residue was calculated based on the amount of supernatant withdrawn and the amount of soluble polysaccharide (dry matter) added to the residue. The residue and remnant supernatant containing samples were freeze dried for WHC measurements. Combinations of PPF and Ebs PPF with water, PPF Res with PPF Sup and Ebs Res with Ebs Sup were freeze dried directly for WHC measurements, after incubation for 2.5 h.

The expressions used for understanding the water held by the insoluble residue were WHC^{i} and WHC^{P} and shown in figure 1 (inset box). The WHC^{i} is calculated as the g of water held by the total sample (inclusive of soluble polysaccharides) expressed per g of dry insoluble residue. With WHC^{P}, the water held only by the insoluble residue is expressed per g of dry insoluble residue (Figure 1). The distinction between WHC^{i} and WHC^{P} is that a higher WHC^{i} occurs when the WHC of soluble polysaccharides additionally contributes to the WHC of the total sample. For samples that showed binding of soluble polysaccharides to the residue, the WHC^{i} and WHC^{P} were not calculated.

3.3.4.1 Combination of residues with water

BIS^{mw}, PPF Res and Ebs Res were incubated in water (30 mg/mL) as blanks. After incubation (Figure 1), the supernatant was withdrawn and the residue was freeze dried for WHC measurements. Additionally, PPF and Ebs PPF were also incubated in water to verify if prolonged incubations in water altered their WHCs. These samples were directly freeze dried after incubation for WHC measurements.

3.3.4.2 Recombination of BIS^{mw} with soluble polysaccharides

For all combinations with BIS^{mw}, the concentration of total dry matter in water or buffer was constant (30 mg/mL).

About 50 mg of BIS^{mw} was combined with different amounts of HBSS (see 3.3.1), with a maximum of 37 mg. After combining, the samples were incubated head over tail at 40°C for 6 h and centrifuged (20 min, 19,500 x g, 20°C; Figure 1). A part of the supernatant

was withdrawn and the remaining sample (residue and residual HBSS) was freeze dried for WHC measurements. The amount of residual HBSS remaining with BIS^{mw} was 5, 9, 14 and 19 mg and was calculated via the supernatant withdrawn.

Likewise, about 40 mg of BIS^{mw} was incubated with different concentrations of a combination of arabinogalactan (AG) and PGA (0.1 M NaOAc buffer, pH 5.0). After incubation and centrifugation (20 min, 19,500 x g, 20°C; Figure 1), the amounts of residual AG and PGA were 4 mg (2 mg of AG and 2 mg of PGA), 5 mg (3 mg of AG and 2 mg of PGA) and 11 mg (6 mg of AG and 5 mg of PGA) and were calculated via the supernatant withdrawn.

Furthermore, 50 mg of BIS^{mw} was combined with 12 mg of galactan, 1 mg of arabinan, and 12 mg PGA (in 0.2 M NaOAc buffer, pH 5; all polysaccharides were soluble) in the amounts in which these polysaccharides were expected to be present in HBSS. After incubation and centrifugation (20 min, 19,500 x g, 20°C), a part of the supernatant was withdrawn. About 6 mg of AG, 0.7 mg of arabinan and 5 mg of PGA remained with BIS^{mw} and were calculated via the supernatant withdrawn. All residues of BIS^{mw} were freeze dried for WHC measurements.

3.3.4.3 Recombination of PPF Res with soluble polysaccharides

For all combinations with PPF Res, the concentration of total dry matter content in water or buffer was constant (30 mg/mL).

PPF Res was recombined in water with PPF Sup in the same ratio as they were obtained: 0.4 mg of PPF Sup per mg of PPF Res. These combinations were incubated at different time intervals from 30 min to 24 h and directly freeze dried for WHC measurements.

In addition, PPF Res (50 mg) was recombined in water with soluble defined polysaccharides in the amounts that they were expected to be present in PPF Sup (19 mg), based on the carbohydrate composition (w/w %) of PPF Sup. The polysaccharides used were soluble AG (6 mg), arabinan (1 mg) and PGA (4 mg). Incubation with PGA was performed in a 0.2 M NaOAc buffer of pH 4.2 at the same pH of PPF Sup. Additionally, PPF Res was also separately incubated with the buffer at pH values 4.2 and 3.6 (the pH of Ebs

Sup) to understand the effect of pH on WHC. After incubation (Figure 1), the samples were centrifuged (20 min, 19,500 x g, 20°C) and a part of the supernatant was withdrawn and quantified. All residues were freeze dried for WHC measurements.

3.3.4.4 Recombination of Ebs Res with soluble polysaccharides

For all combinations with Ebs Res, the total dry matter content in water or buffer was constant (30 mg/mL).

Ebs Res was recombined in water with Ebs Sup in the same ratio as they were obtained: 0.9 mg of Ebs Sup per mg of Ebs Res. This combination was incubated and directly freeze dried for WHC measurements.

In addition, Ebs Res (50 mg) was recombined in water with soluble defined polysaccharides in the amounts that they were expected to be present in Ebs Sup (45 mg), based on the carbohydrate composition (w/w %) of Ebs Sup. The polysaccharides used were soluble AG (12 mg) and PGA (6 mg for Ebs Res). Incubation with PGA was performed in a 0.2 M NaOAc buffer of pH 3.6. Samples were centrifuged and a part of the supernatant was withdrawn and quantified. Again, all residues were freeze dried for WHC measurements.

Furthermore, Ebs Res (30 mg) was also incubated with three concentrations of HBSS equivalent to the expected amount of Ebs Sup solids. The amounts combined were 10, 19 and 28 mg of HBSS. After centrifugation (20 min, 19,500 x g, 20°C) and withdrawing a part of the supernatant, the remaining sample was freeze dried for WHC measurements.

3.3.5 Binding of galactan to cellulose

The binding assay of AG to cellulose was performed using a concentration range of AG from 0.05 to 1 mg/mL. AG solutions were added to coarsely powdered filter paper cellulose (Grade 3, Whatman, Buckinghamshire, UK). For this, the filter paper was milled in an MM 2000 Retsch mill (Retsch) at an intensity of 100% for 8 min. Incubations were performed head over tail for 6 h at 40°C. After this, the samples were centrifuged (9000 x g, 10 min, 20°C) and the content of total sugars in the supernatants (about 1.2 mL) were

analysed using galactose as the calibrant (see 3.3.6.1). The binding of AG to cellulose (Qe) was expressed as the µg of AG bound per mg of cellulose and calculated as $Qe = \frac{(Ce-Co)*V}{m}$, where Ce is the concentration of AG in the supernatant after inubation and centrifugation, Co, the concentration of AG before incubation, V, the volume (1.5 mL) of AG solution applied and m, the amount of cellulose used (50 mg) for the assay. The cellulose AG residues were freeze dried prior to WHC measurement. The incubations were done in duplicate.

3.3.6 Analytical methods

All analyses were performed in duplicate.

3.3.6.1 Carbohydrate content and composition was performed as described elsewhere (Ramaswamy et al. 2013). The total neutral sugar content for the binding assay was determined using the automated orcinol method (Tollier and Robin, 1979). Galactose was used for calibration (Sigma).

3.3.6.2 High performance size exclusion chromatography (HPSEC) was performed as described elsewhere (Ramaswamy et al., 2013).

3.3.6.3 High performance anion exchange chromatography (HPAEC) was performed using an ICS 5000 (HPAEC) system (Dionex, Sunnyvale, CA, USA) with electrochemical detection (ED). About 10 µl of each sample was injected into the system and separation was performed using a CarboPac PA-1 column (2 x 250 mm) proceeded by a CarboPac PA-1 guard column (2 x 50 mm). The flow rate of the eluent(s) was 0.3 mL/min. The gradient used for the elution of mono and oligosaccharides has been described elsewhere (Jonathan et al. 2012).

3.3.6.4 Matrix assisted laser desorption/ionisation time of flight mass spectrometry

Oligosaccharides were profiled using Matrix Assisted Laser Detection Time of Flight Mass Spectrometry (MALDI-TOF MS) as described elsewhere (Ramaswamy et al., 2013).

3.3.6.5 Water holding capacity measurements were performed using the Baumann's apparatus (Baumann, Frankenthal, Germany; Baumann, 1966) as described elsewhere (Ramaswamy et al., 2013). Briefly, a small quantity (minimum 0.010 g) of dried sample (soluble, insoluble) was placed over the filter disc (G2; Duran, Germany) and the volume of water used to hydrate the sample until saturation was recorded. For all samples containing soluble dry matters, the sample was placed on a filter paper (Grade 595, Whatman, Germany) placed over the disc, and water absorbed by the filter paper was corrected accordingly. All measurements were performed in triplicates.

3.4 Results and discussion

3.4.1 Effect of time of hydration and pH on the WHCs of PPF fractions

3.4.1.1 WHC of PPF and its fractions

The WHC and WHC^{P} of PPF were 8.3±0.4 mL/g and 10.7±0.5 mL/g, respectively (Table 1). The latter was calculated based on the fact that 72% of PPF was recovered as PPF Res (Table 3). PPF and PPF Res showed quite similar WHCs (Table 1), although the latter was devoid of any soluble dry matter. Nevertheless, upon prolonged incubation with water the WHC of PPF Res increased from 9.3 to 14. Similarly, upon incubation with water, the WHC of BIS^{mw} increased (from 9.9 to 11.4), but the increase was not as large as for PPF Res. Therefore, the increase in WHC after incubation in water and freeze drying could have resulted from a morphological alteration of the residue.

The WHC of BIS^{mw} (Table 1), which is a starch depleted residue (9.9±0.1), was rather similar to that of PPF Res (9.3±1.2), indicating no substantial influence of starch on the WHC. This was also seen from the rather similar amounts of WHC/g of CWPs for PPF Res and BIS^{mw} (18.6±2.3 and 16.5±0.1; no further data shown), by dividing the WHC of PPF Res and BIS^{mw} by the CWP content ((w/w)% (Table 3)) of PPF Res and BIS^{mw}, respectively. Also, the WHC/g of CWPs of PPF Res was similar to PPF (16.6±0.8) despite the difference in starch content between PPF Res (28 (w/w)%) and PPF (20 (w/w)%; Table 3).

Table 1: WHCs (mL/g dry matter) of PPF; its residues, Ebs PPF and filter paper cellulose, as is and upon incubation with water; WHCs of soluble polysaccharides (below).

	WHC (mL/g)		WHC^P (mL/g)	
Samples:	As is	Incubation with H_2O	As is	Incubation with H_2O
PPF	8.3±0.4	8.2±1.3[a]	10.7±0.5	10.6±1.8[a]
PPF Res (pH 4.8)	9.3±1.2	14.0±0.6	9.3±1.2	14.0±0.6
PPF Res, pH 4.2[b]	9.0±0.3	nd	9.0±0.3	nd
PPF Res, pH 3.6[b]	6.6±0.2	nd	6.6±0.2	nd
BIS^{mw}	9.9±0.1	11.4±0.1	9.9±0.1	11.4±0.1
Ebs PPF	3.3±0.4	3.9±0.1[a]	6.2±0.8[c]	7.4±0.1[a,c]
Ebs Res	6.1±0.2	6.7±0.5	6.1±0.2[c]	6.7±0.5[c]
Filter paper cellulose	5.4±0.4	5.2±0.1	5.4±0.4	5.2±0.1

Soluble polysaccharides:	WHC
HBSS	1.8±0.7
PPF Sup	2.0±0.06
AG	2.1±0.6
PGA	1.6±0.3
Arabinan	3.2±0.6

[a] Incubations for 2.5 h

[b] Incubations in buffer

[c] WHC^P, assumed to be the same as WHC^i

[nd] Not determined

3.4.1.2 Effect of hydration time on the recombination of PPF Res with PPF Sup

The hydration time of recombination of insoluble residues (PPF Res) with soluble solids (PPF Sup) was investigated to understand if prolonged hydration of the insoluble residue by soluble solids was time dependant.

Table 2: Effect of hydration time on the WHC (mL/g) of PPF Res recombined with PPF Sup and their corresponding WHC^P.

PPF Res + PPF Sup	WHC	WHC^P
Hydration (hours)		
0.5	10.3±1.2	13.3±1.6
1	10.4±1.4	13.5±2
2.5	9.5±0.9	12.3±1.2
6	10.9±0.2	14.2±0.3
12	9.7±0.3	12.5±0.4
24	9.8±1.3	12.0±1.3

The results in table 2 indicated no substantial differences in the WHC for samples subjected to the different incubation times tested in a range from 0.5 to 24 h.

3.4.1.3 Effect of pH

Lowering the pH of PPF Res from 4.8 to 3.6 lowered the WHC from 9.3 to 6.6 mL/g (Table 1). At the higher pH values, carboxyl groups, e.g. in homogalacturonans (HGs) are more ionized, due to which repulsion between the groups extended the cell wall matrix, allowing more water to be embedded. Although a high buffer concentration was used (0.2 M) for pH stability, the buffer strength was found to have a negligible effect (data not shown) and is overruled by the effect of ionization of the carboxyl groups. Similar findings have also been reported for sugar beet fibre (Michel et al., 1988; Renard et al., 1994).

3.4.2 Effect of BIS^{mw} and PPF Res on the WHC of PPF

3.4.2.1 Water holding capacities of combinations of BIS^{mw} or PPF Res with soluble CWPs

The de-starched insoluble residue, BIS^{mw} was combined with diffrerent concentrations of HBSS and with a combination of soluble galactan and PGA, to

understand the contribution of soluble CWPs to the WHC of the total sample. The WHC of HBSS was 1.8 mL/g (Table 1), which was much lower than the WHC of BIS^{mw} (Table 1). In figure 2A, it can be seen that increasing the proportion of HBSS in the HBSS/BIS^{mw} mixture lowered the WHC of the sample. The theoretical WHCs, calculated from the sum of the separately measured WHCs of BIS^{mw} and HBSS, were close to the measured WHCs. This indicated that the water held in the sample was an additive contribution of the water held by HBSS and BIS^{mw}.

Although upon addition of HBSS, the WHCs decreased, the WHC^{i} increased. At the maximum ratio of HBSS/BIS^{mw}, the WHC^{i} was about 8% higher than the incubation of BIS^{mw} alone. However, the water held was also contributed slightly by the soluble solids present in the sample. Therefore, to understand how much water was held only by the insoluble residue, the WHC^{P} was calculated. In figure 2A, at the highest ratio of HBSS/BIS^{mw}, the value of WHC^{P} was still comparable to that of BIS^{mw} alone in water. These results again indicated that the water held by the insoluble part did not differ much upon increased amounts of HBSS added. Apparently, no interaction between HBSS and BIS^{mw}, expected to influence the WHC (Ramaswamy et al., 2013), was found. Therefore, WHC^{P} indicated that all water was mainly held by the insoluble residue.

Similarly, increasing the amounts of a combination of AG and PGA, in the proportion in which they were present in HBSS (Table 3; see 3.3.4.2), did not increase the WHC^{i} and WHC^{P} compared to the incubation of BIS^{mw} alone in water; Figure 2B).

In figure 3A, the effect of combining BIS^{mw} with PGA, AG and arabinan alone on the WHC is shown. Surprisingly, for AG with BIS^{mw}, the measured WHC was higher than the theoretical WHC. This pointed at an interaction between AG and BIS^{mw}, while no interaction was shown for AG with BIS^{mw} when the AG was mixed with PGA (Figure 2B). Interestingly, addition of PGA to BIS^{mw} seemed to lower the WHC, while addition of arabinan seemed to lower the WHC^{P} compared to galactan with BIS^{mw}. Also, the measured WHC of arabinan with BIS^{mw} was lower than the theoretical WHC. This indicated that probably the presence of these components in HBSS, counteracted the interaction of the AG in HBSS with BIS^{mw}. No explanation could be given for this phenomenon.

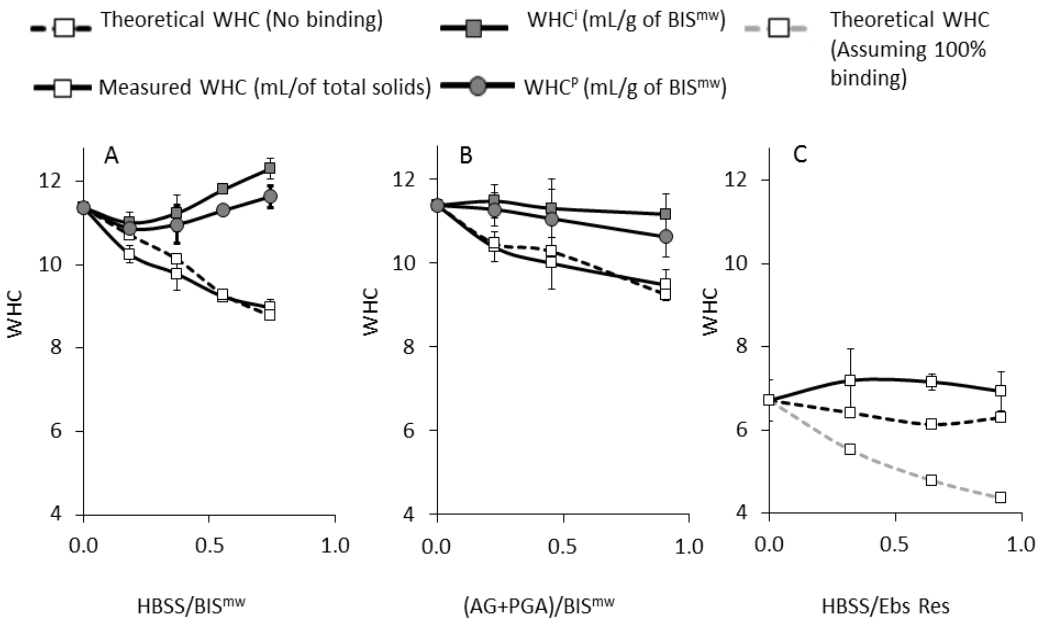


Figure 2: Measured and calculated WHCs of BIS^{mw} combined with increasing proportions of HBSS (A), and arabinogalactan(AG) and PGA (B). Measured and calculated WHCs of Ebs Res combined with increasing proportions of HBSS (C).

Like BIS^{mw}, PPF Res, a starch containing residue, was also recombined with PPF Sup in the amounts expected to be present in PPF. Like for the combination of BIS^{mw} with HBSS, no effects in the WHCs, WHC^{i} and WHC^{P} were observed compared to PPF alone (Figure 3D). For this reason, PPF Res was not combined with HBSS. PPF Res was combined with AG, PGA and arabinan (Figure 3B), the most abundant CWPs in PPF Sup (Table 3). The WHCs ranged from 8.2-14 mL/g (Figure 3B). In contrast to the addition of AG to BIS^{mw}, the theoretical and measured WHCs were similar for AG combined with PPF Res. This indicated that no interactions influencing the WHC were observed between AG and PPF Res. Similar to BIS^{mw}, the measured WHC, WHC^{i} and WHC^{P} of PPF Res combined with PGA and arabinan were lower than the WHCs of combinations of PPF Res with AG. This contrasted with the findings of Larsen et al. (2011) where arabinan was found to hydrate RG-I more than AG, possibly because it was linked to RG-I. Our findings indicated that the

presence of arabinan and PGA as separate entities slightly decreased the amount of water held by BIS^{mw} and PPF Res.

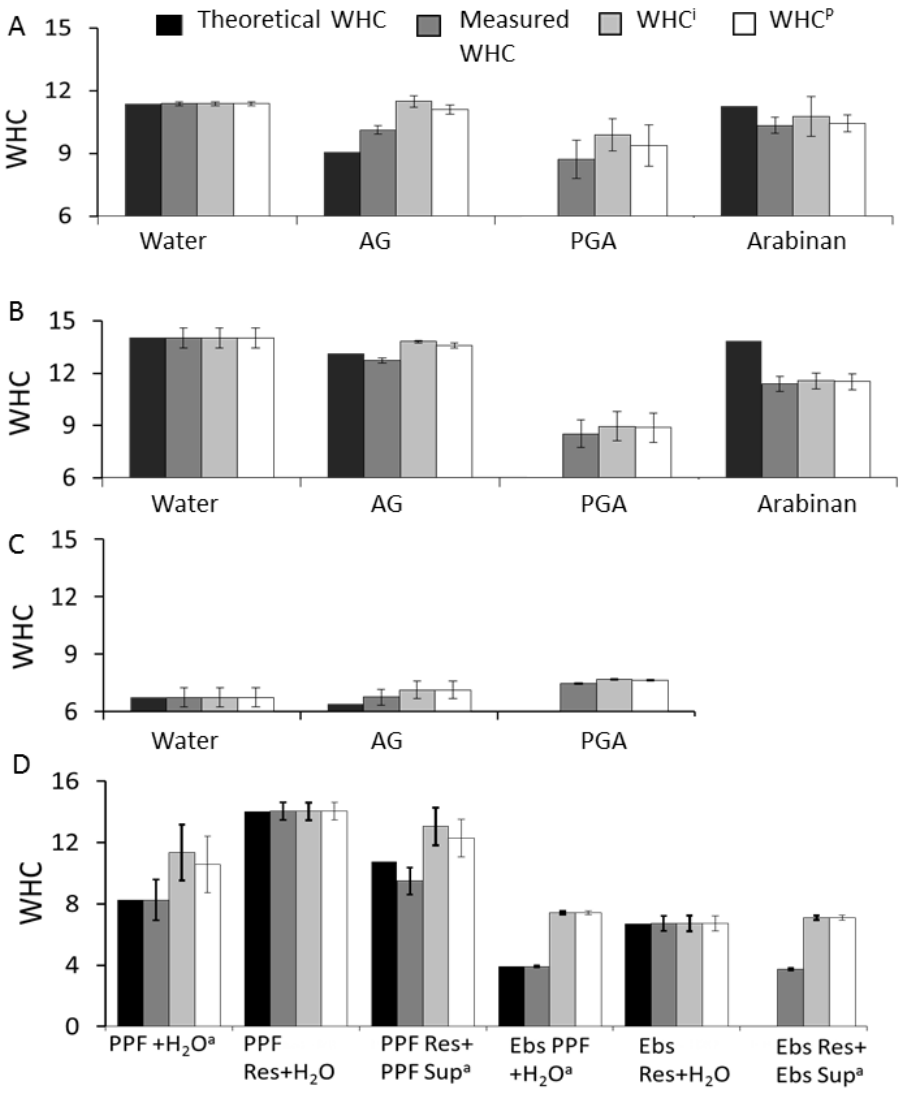


Figure 3: Measured and calculated WHCs of BIS^{mw} (A), PPF Res (C) and Ebs Res (D) recombined with defined CWPs. Measured and calculated WHCs of PPF Res and Ebs Res combined with PPF Sup and Ebs Sup, respectively (B). [a] is incubation for 2.5 hours.

3.4.2.2 Carbohydrate composition of PPF and PPF fractions

The carbohydrate compositions of HBSS, BIS, PPF Res and PPF Sup are shown in table 3. PPF CWPs consisted mainly of Glc (37 mol%), Gal (30 mol%), UA (18 mol%) and Ara (8 mol%) residues.

In HBSS, 39% (w/w) of all CWPs from PPF were recovered, while 54% were recovered in the BIS fraction. The remaining CWPs were removed upon dialysis, as observed previously (Ramaswamy et al., 2013). Mostly pectins (60% (w/w) as the sum of rhamnosyl (Rha), uronyl (UA), galactosyl (Gal) and arabinosyl (Ara) residues) were present in HBSS. Almost all of the non-pectic CWPs (total of xylosyl (Xyl), mannosyl (Man) and glucosyl (Glc)) remained in BIS. These results confirm previous data (Ramaswamy et al., 2013).

The constituent monosaccharide composition of BIS^{mw} was expected to be the same as BIS, since upon washing, nearly the same amount of dry matter was recovered in BIS^{mw}. The higher ratio of Gal:Rha observed in HBSS (23) compared to BIS (9), and the recovery of more than 60% Gal from PPF in HBSS, indicated a higher abundance of AG per Rha in HBSS than in BIS. This implicated that in HBSS, RG-I linked galactan was present, which could contribute to the hydrating effect exerted by HBSS on Ebs Res (see 3.4.4). BIS^{mw} and PPF Res showed similar CWP compositions (Table 3).

The absence of starch in BIS^{mw} resulted in a 38% higher CWP content (w/w %) compared to PPF Res. The results implicated that the higher amount of CWPs contributed to the increased measured WHC of BIS^{mw} after addition of AG, while this was not the case for PPF Res.

3.4.3 Effect of enzyme addition on the WHC of PPF

After incubating PPF with the enzyme Ebs, the WHC reduced from 8.3 to 3.3 mL/g (Table 1), while the WHC^{P} reduced from 10.6 to 7.4 mL/g (Table 1). The WHC (and WHC^{P}) of the remaining insoluble solids (Ebs Res) was 6.1±0.2 mL/g compared to 9.3±1.2 mL/g for PPF Res (Table 1).

Table 3: Carbohydrate composition of PPF, its fractions, including CWP yield over insoluble residue and supernatants

Fractions from PPF	Yield % CWPs (dry matter of CWPs)	w/w %		Sugar composition (mol %) and yield % CWP residues[a,b]							
		Starch	CWP	Rha	Fuc	Ara	Xyl	Man	Gal	Glc[c]	UA
PPF	100	20	50	2	<1	8	3	2	30	37	18
				(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)
HBSS	39	<1[a]	68	2	<1	7	1	<1	48	<1	42
				(52)	(45)	(37)	(8)	(<1)	(63)	(<1)	(65)
BIS	54	3[a]	69	1	<1	5	6	2	8	69	8
				(31)	(64)	(40)	(112)	(94)	(14)	(119)	(19)
PPF Sup	34	<1[b]	64	3	<1	12	1	<1	53	2	29
				(48)	(12)	(52)	(8)	(<1)	(60)	(1)	(55)
PPF Res	72	28[b]	50	1	<1	6	5	3	16	58	11
				(47)	(84)	(59)	(102)	(120)	(40)	(112)	(43)
Ebs Sup	80	<1[b]	70	2	<1	8	4	2	39	28	18
				(74)	(35)	(84)	(86)	(84)	(103)	(60)	(79)
Ebs Res	39	33[b]	32	2	<1	8	3	2	6	57	23
				(29)	(93)	(36)	(34)	(37)	(9)	(57)	(53)

[a] Values in brackets indicate yields of CWP residues (g CWP residue/100 g CWP residue in PPF).

[b] Rha, rhamnose; Fuc, fucose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; UA,uronic acid.

[c] Non-starch Glc

Prolonged incubation of Ebs Res with water showed a similar WHC^P (6.7±0.5; Figure 3D) to that of Ebs Res (6.1±0.2), unlike the increase in the WHC^P found for BIS^{mw} and PPF Res upon incubation with water (Table 1). Therefore, the absence of an increase in the WHC^P of Ebs Res after prolonged incubation in water and freeze drying pointed to negligible morphological alteration of the residue. Furthermore, the WHC^P of Ebs Res (6.1±0.2) was close to the value of filter paper (FP) with water (5.4±0.4). This was in agreement with the fact that Ebs Res mostly consisted of cellulose, which is discussed in section 3.4.4.2. Therefore, the WHC^P values indicated that the hydration of Ebs Res was primarily due to

the remaining cellulose after liquefaction. It should be noted that after Ebs treatment, the pH of Ebs Res decreased to pH 3.8, compared to PPF Res (pH 4.8) and BIS^{mw} (pH 5.0) probably due to de-esterification of pectins.

3.4.4 Water holding capacities of combinations of Ebs Res with soluble CWPs

The effect of combining soluble polymeric CWPs (HBSS) with Ebs Res on the WHC was investigated to understand if degradation of soluble CWPs was important to lower the WHC. Ebs Res was combined with HBSS and the WHCs of HBSS/Ebs Res mixtures were measured (Figure 2C). However, opposed to the absence of binding of CWPs with other residues (BIS^{mw} and PPF Res), the HPSEC profiles of the supernatants of HBSS combined with Ebs Res (figure 4), indicated binding of high molecular weight material in HBSS to Ebs Res, as shown by their absence in the corresponding supernatants. Since the actual amount of HBSS bound to Ebs Res was not known, no theoretical WHC could be calculated. Instead, two theoretical WHCs were calculated: one assuming no binding of HBSS, and the other, assuming 100% binding of HBSS. The results (Figure 2C) showed that the measured WHCs were higher than the theoretical WHCs. This indicated that, most likely due to adsorption of HBSS to Ebs Res, an increase in the measured WHC was observed. No effects of separate addition of AG and PGA on the WHC of Ebs Res was found (Figure 3C). The linkages between AG and RG-I probably improved the hydration of RG-I in HBSS, as found previously (Larsen et al., 2011).

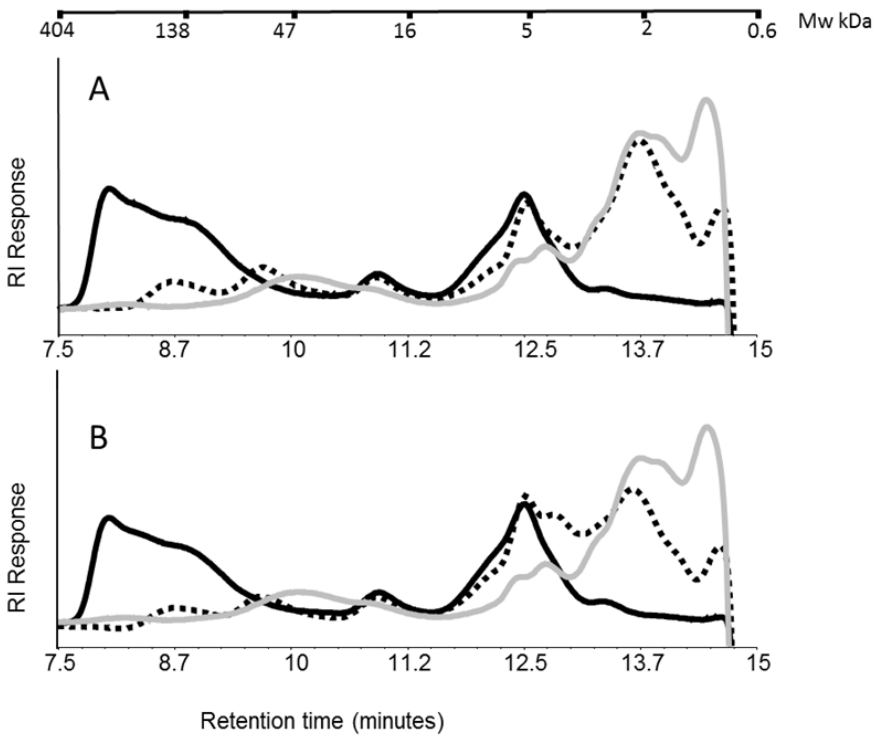


Figure 4: HPSEC profiles of supernatants of HBSS (▬), Ebs Res (▬), HBSS combined with Ebs Res (▪ ▪ ▪) for 10 mg (A) and 19 mg (B) of HBSS added to Ebs Res.

This apparently also increased the WHC compared to the theoretical values (Figure 2C). Also, the adsorption of AG to cellulose has been previously indicated in a XG poor environment (Zykwinska et al., 2005). The lower mol% of Xyl in Ebs Res than that in BIS^{mw} (Table 3) indicated a XG poor environment, due to which AG-RG-I in HBSS was capable of adsorbing to cellulose in Ebs Res, but not to BIS^{mw}. Our results suggested that the degradation of the crosslinks between RG-I and AG could be essential to lower the WHC.

3.4.4.1 HBSS oligomer profiling with Ebs

The presence of linkages between HG-RG-I AG in HBSS was shown upon degrading HBSS with Ebs. In supplementary figure 2, a MALDI-TOF mass spectrum of this

HBSS digest showed masses of hexoses [H] and pentose-hexose [P.H] combinations. Since HBSS was composed of Gal, Ara and UA residues (Table 3), it was confirmed then that these masses represent galactan-oligosaccharides and galactan-arabino oligosaccharides. Also masses representing short fragments of RG-I backbone linked to a few number of Gal/Ara residues were present. This indicated that although RG-I could be linked to AG side chains, the truncation of the side chains could still prevent hydration of RG-I as was observed by Larsen et al., (2011). So, we concluded that HBSS contributed to the WHC when combined with Ebs Res due to the involvement of polymeric RG-I AG (possibly also with HG in HBSS).

3.4.4.2 Carbohydrate composition of soluble and insoluble residues after Ebs treatment

Ebs Sup represented 80% (w/w) of the total constituent carbohydrates from PPF (Table 3). Mostly, Gal (39 mol%), Glc (28 mol%), UA (18 mol%) and Ara (8 mol%) were present pointing at the abundance of AG, glucans and HG. Hardly any AG remained in Ebs Res (Table 3). Ebs Res was mainly composed of cellulose, next to low amounts of hemicellulose. In Ebs Res, less than 60% of cell wall Glc, 40% of Xyl and Man originally present in PPF was recovered, pointing at the substantial solubilization of hemicellulosic CWPs by Ebs. Therefore, the lower pH of Ebs Res and the removal of hemicellulosic CWPs (Xyl, Glc and Man) from the residue probably contributed to the lower WHC^{P} of Ebs Res compared to BIS^{mw} and PPF Res (Table 1). It seems that although AGs have been shown to be more mobile than the rigid XGs, the latter seem to help immobilize water in the CWP network (Ha et al., 2005; Fenwick et al., 1999).

3.4.4.3 Activities in Ebs and profiles of solubles in Ebs Sup

To understand the degradation of CWPs in PPF by Ebs, the enzyme activities in Ebs were studied. The PNP β-galactosidase activtiy was 40 U/mL and α-arabinofuranosidase, 2 U/mL (data not shown), pointing at a larger ability of removing Gal residues than Ara residues, e.g. from AG side chains of RG-I. In supplementary figure 3, the degradation profiles of various defined polysaccharides by Ebs are shown. Ebs completely degraded tamarind XG (soluble part) as much as galactan after 16 h. However, despite a

47 hour incubation, 34% of Xyl originally present in PPF was still recovered in Ebs Res, compared to less than 10% of Gal in Ebs Res. The notion that Xyl in PPF is expected to be present mainly as XG, indicated that Ebs could not degrade XG completely, either due to lack of accessibility or due to the inability to degrade insoluble XG.

After treatment with Ebs, the soluble carbohydrates remaining in Ebs Sup contained oligosaccharides with a Mw less than 4 kDa as shown in the HPSEC chromatogram in supplementary figure 4. As described in 3.4.4.2, Ebs Sup is expected to be abundant in galactan and GalA-rich pectic saccharides. High performance anion exchange chromatography (HPAEC) of Ebs Sup revealed that the monosaccharides Rha, Ara, galacturonic acid (GalA), Gal and Glc were present of which Gal and Glc were the most abundant (Supplementary figure 4). In addition, galactan oligosaccharides also eluted in Ebs Sup, of which MALDI-TOF MS (data not shown) indicated that most of them contained less than eight residues. Next to these galactan oligosaccharides, also masses of hexose [H] combined with pentose [P] oligosaccharides were identified. These masses represented arabinogalactan (AG) or xyloglucan (XG) oligosaccharides. Other masses represented GalA oligos up to eight residues and masses of Rha.$GalA_2$ with (arabino)galactosyl side chains, where the galactosyl residues were not more than five residues long. Therefore, the results confirmed the degradation of RG-I, AG and XG.

3.4.5 Effect of adsorption of CWPs to cellulose on the WHC

The adsorption of polymeric HG-RG-I AG, as present in HBSS to cellulose, and not AG alone, showed an increase in the WHC of Ebs Res combined with HBSS observed earlier in this study. Therefore, the effect of adsorption of much lower (at least 6 times) concentrations of AG (water soluble) to cellulose (insoluble) on the WHC of cellulose was investigated. Figure 5 showed that the AG adsorbed to cellulose over a range of AG concentrations. The trends were consistent with the findings of Zykwinska et al. (2005). However, no increase in the WHC^P of cellulose was shown upon increased binding of AG. Possibly, the concentrations of AG adsorbed were too low to result in an effect on the WHC.

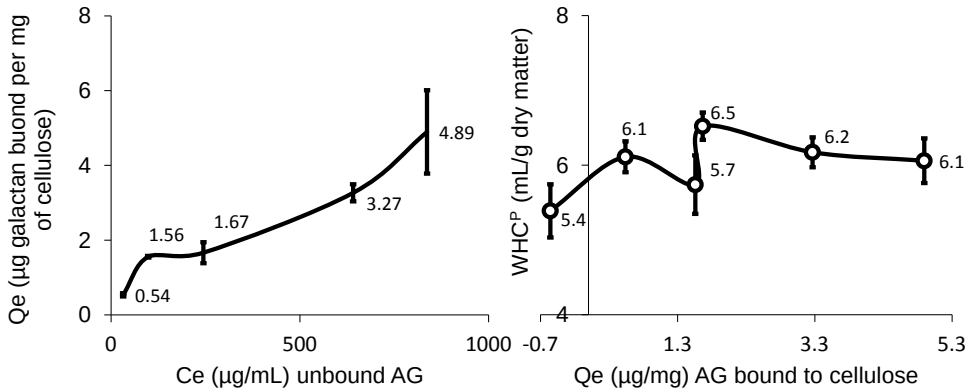


Figure 5: Binding affinity (Qe) Vs concentration (Ce) of unbound arabinogalactan (AG) to cellulose (A); Plot of Qe versus the water holding capacities of insoluble cellulose (WHC^P; mL/g dry cellulose) combined with AG (B).

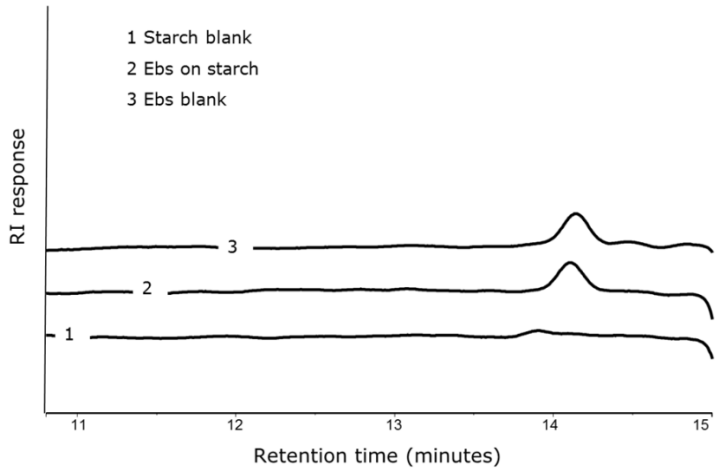
3.5 Conclusion

The water in PPF is held by the insoluble CWP network involving hemicellulosic CWPs (mainly XGs), since the WHC decrease was drastic (61%) after the enzymatic removal of these CWPs. More than just the solubilization of CWPs, the truncation of side chains on RG I-AG and reducing the pH, probably due to de-esterification, was found to be important to achieve a decrease in the WHC.

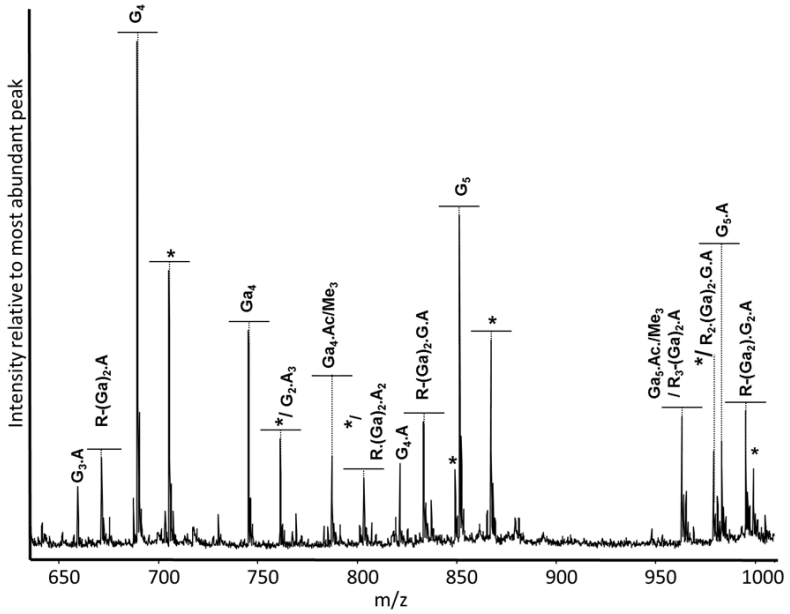
3.6 Acknowledgement

The authors are grateful to Steef Lips and Jelle Dijkstra for their help in the preparation of PPF fractions. This project is jointly financed by the European Union, European Regional Development Fund and The Ministry of Economic Affairs, Agriculture and Innovation, Peaks in the Delta, the municipality of Groningen, the province of Groningen as well as the Dutch Carbohydrate Competence Centre (CCC WP8), Wageningen University, AVEBE, Royal Cosun and Duynie B.V.

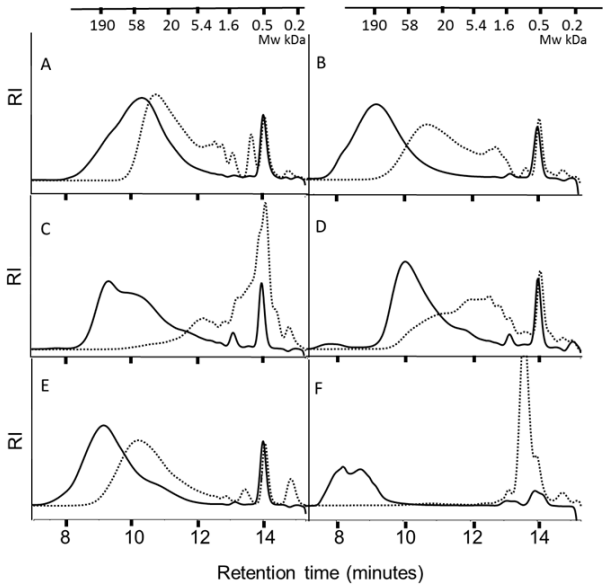
Supplementary figures



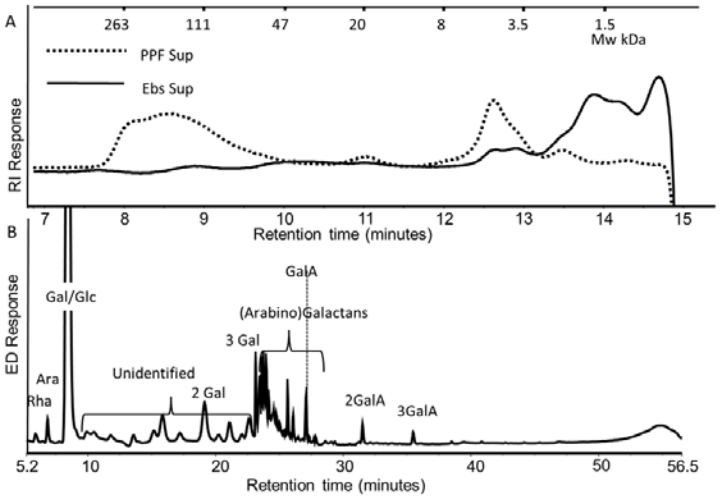
Supplementary figure 1: HPSEC profiles of starch tested with Ebs.



Supplementary figure 2: MALDI-TOF MS spectrum of HBSS treated with Ebs. Masses represent sodium adducts. * represents K^+ adduct. G is Gal, A is Ara, R is Rha, Ga is GalA.



Supplementary figure 3: HPSEC profiles of various defined polysaccharides before (▬) and after (▪▪▪▪) hydrolysis with Ebs treated substrate (Mw standards are based on pullulan). (A) Polygalacturonic acid, (B) High methylesterified pectin, (C) Arabinogalactan, (D) Arabinan, (E) Carboxy methyl cellulose, (F) Xyloglucan



Supplementary figure 4: (A) HPSEC profiles of PPF Sup and Ebs Sup (0.5 mg/mL each); (B) HPAEC profile of Ebs Sup (0.25 mg/mL).

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Chapter 4

Improved recovery of starch assisted by enzymes and reduced water holding of residual fibre on a semi-technical scale

Abstract

During the industrial extraction of starch from potatoes, some starch remains within undisrupted potato cells in the fibrous side-stream. The aim of this study was to investigate if enzymatic degradation of cell wall polysaccharides (CWPs) can enhance starch recovery and lower the water holding capacity (WHC) of the "fibre" fraction. The use of a pectinase-rich preparation removed 58% of the starch present in the "fibre" fraction. Also, the "fibre" fraction retained only 40% of the water present in the non-enzyme treated "fibre". This was caused by the degradation of pectins, in particular arabinogalactan side chains.

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4.1 Introduction

After the industrial extraction of starch from potatoes, some starch still remains in the fibre-rich by-product. This starch is trapped within more or less intact potato cells (Ramaswamy, et al., 2013). Albeit representing a low proportion of the starch present in potato tuber, a major challenge is to extract this residual starch. Another challenge is to decrease the high water holding capacity (WHC) of the fibre fraction. This fibre fraction (dry basis) represents 6.4% (w/w) of the total dry matter of potatoes (Grommers and Krogt, 2009). The high water holding capacity (WHC) of this fraction negatively affects its processing as an animal feed and increases transportation costs.

In the fibre fraction, cell wall polysaccharides (CWPs) constitute 48% (w/w) of the dry matter. They comprise pectins, rich in arabinogalactan side chains, cellulose and hemicelluloses, the latter mainly composed of xyloglucan. Their WHC is affected by interactions between these CWPs, in which water soluble arabinogalactans and water insoluble xyloglucans are important (Ramaswamy et al., 2013, Larsen et al., 2011). Also, it was indicated that long side chains of arabinan and arabinogalactan impart a more hydrophilic character to the rhamnogalacturonan (RG-I) backbone compared to short chains (Larsen et al., 2011). One way to reduce the WHC is by using enzymes that can degrade CWPs. Reduced WHC of pre-treated rape seed meal has been observed upon addition of pectinolytic enzymes (Pustjens et al., 2012). Also, a reduced WHC was found for xylanase-treated wheat cell wall material. However, in that study it was shown that the WHC of the insoluble residue increased by a factor of 2.5 (Gruppen et al., 1993). Therefore, it seems that although enzymes can be used to modify CWPs, the type of CWPs degraded determines the extent of WHC of the residual material.

An additional advantage of using enzymes in the starch extraction process is that by degrading fibres these enzymes can loosen the cell wall network in undisrupted cell walls before separating the fibre fraction from the starch fraction. Lab-scale treatment of potatoes with a cellulase improved starch release by 18%-28% (Sit et al., 2011). However, at production scale, the starch extraction process is different from at lab scale (Grommers and Krogt, 2009; Wischmann et al., 2007). This is illustrated by main differences observed in the particle size distributions of starch granules between lab scale and industrial scale

derived starch (Wischmann et al., 2007). Consequently, only by using a defined *semi-technical* scale set-up, the effects of enzyme addition in the starch recoveries and WHCs are expected to be predictable for industrial application. Therefore, in this study starch was isolated from potatoes at a *semi-technical* scale set-up.

Our objective was to test the hypothesis that cell wall degrading enzymes assist the release starch into the starch fraction and lower the WHC of the corresponding fibre fraction at a semi-technical scale set-up. The effect of two commercial enzymes, namely a pectinase-rich and a galactanase-rich preparation, were studied at different concentrations.

4.2 Materials

Potatoes of the *Seresta* variety and potato starch were provided by AVEBE (Veendam, The Netherlands) as soon as they were harvested in August 2012. The potatoes were stored in a dark, dry and ambient (10°C-15°C) environment and were used within seven weeks of harvesting. Enzymes used were Pectinex Ultra-SP (USP), provided by Novozymes (Bagsvaerd, Denmark), and a glucanase rich enzyme preparation (Ebs), provided by Erbsloh (Geisenheim, Germany). The enzyme activities and doses applied are described in Table 1. PNP-β-galactopyranoside, PNP-β-glucopyranoside and PNP-α-arabinofuranoside were obtained from Sigma-Aldrich. PNP-α-xylopyranoside was obtained from Koch-Light Laboratories (Buckinghamshire, UK). To assess the degradation of CWPs by USP and Ebs, linear arabinan (British Sugar, Peterborough, UK), arabinogalactan (Megazyme, Wicklow, Ireland), polygalacturonic acid (PGA, Fluka Biochemika) and tamarind xyloglucan (Dainippon Sumitomo Pharma, Osaka, Japan) were used. Potato starch was obtained from AVEBE.

4.3 Methods

4.3.1 Semi-technical scale preparation of "fibre" fraction and "starch" fraction from potatoes

4.3.1.1 Semi-technical scale set-up

In figure 1, a scheme of the semi-technical scale set-up is shown. This set-up is known to mimic the commercial starch extraction process well (AVEBE). In all trials, 4 Kg of potatoes (fresh weight) were washed, mixed with $NaHSO_3$ (0.07 w/w % of dry weight of potatoes) and rasped to obtain potato pulp. Rasping was performed with a Nivoba Ultra Rasp RU 40-260 (Nivoba Engineering, Veendam, The Netherlands). The pulp was weighed and sieved via a centrifugal sieve GL200 (Larssons, Bromolla, Sweden) of pore size 125 µm. After sieving, the pulp was separated into a fibre-rich fraction and a starch-rich fraction. The fibre-rich fraction was again sieved three times through the same sieve and each time a starch-rich fraction was obtained. Therefore, in the end, a total of four starch-rich fractions were obtained and denoted as "starch 1", "starch 2", "starch 3" and "starch 4". One fibre-rich fraction was obtained, which was denoted as "fibre" (Figure 1).

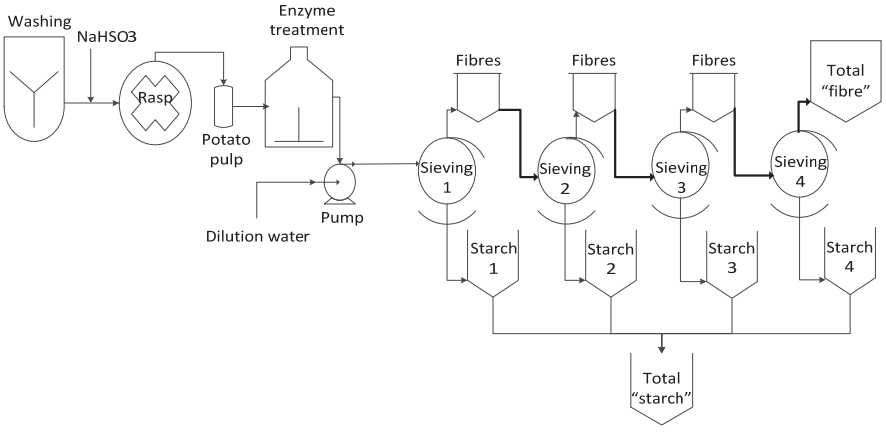

Figure 1: Flow scheme of the semi-technical scale starch separation process.

"Starch1-4" and "fibre" fractions were weighed. For enzyme treatments, the potato pulp was incubated with enzymes at 40°C for 2 h before sieving. All incubations were performed in stirred tank kettles of a capacity of 12 L (model 88.460, Terlet, Zutphen, The Netherlands) and were stirred at a speed of 24 rotations per min. The trial in which pulp was incubated without enzymes was denoted as "blank trial". The trial in which the pulp was not incubated and sieved directly after rasping was denoted as "standard trial".

Table 1: Enzymes characterized and doses applied.

	USP	**Ebs**
Full name	Pectinex Ultra-SP	Acetylesterase comprising β-glucanase complex
Supplier	Novozymes	Erbsloh Geisenheim
Source	*Aspergillus aculeatus*	*Penicillium funicolusum*
Protein content (w/w %)[a]	4.0 ± 0.01	12.9 ± 0.4
Activity [b]	**(U/mL)**	
α-1,5 Arabinofuranosidase	17	2
β-Galactosidase	67	40
β-Glucosidase	135	432
α-Xylosidase	0.15	0.006
Doses applied	**g protein per Kg potatoes (dry matter), Code**	
	0.01, USP1	0.43, Ebs1
	0.03, USP2	0.86, Ebs2
	0.08, USP3	USP+Ebs = 0.01 USP1+0.43 Ebs1

[a] Gram of protein (N%*6.25) per 100 gram of enzyme liquid

[b] Enzyme activity determined by the PNP assays per mL of enzyme liquid

Three trials were performed with increasing doses of the USP enzyme and denoted as USP1, USP2 and USP3. Two trials were performed with increasing doses of the Ebs enzyme and denoted as Ebs1 and Ebs2. Table 1 shows the properties of enzymes used as well as the doses applied. In addition, it was assured that the enzymes were unable to degrade starch. For this, the enzymes were incubated at a dose of 1% (w/w) of enzyme liquid on dry matter of potato starch (6 % (w/w) in milliQ water) at 40°C for 24 h with head over tail

mixing. Samples were centrifuged (30 min, 18,000 x g, 20°C) before analysis by HPSEC. No differences in the HPSEC profiles were seen upon incubating starch with the enzymes.

Fractions were stored at -20°C until further analysis. For analysing dry matter contents, the potato pulp, "fibre" and the four "starch" fractions were immediately homogenized and sampled. For other analyses, amounts from 100 to 500 g of each of "starch1-4" fractions were homogenized and pooled in the ratios at which they were obtained. The pooled sample was denoted as "starch". In all experiments, samples of "fibre" and "starch" fractions were shortly inactivated by heating at 100°C for 5 min before freeze drying prior to analyses. For particle size measurements, samples of "fibre" fractions were immediately frozen. Losses of solids in our semi-technical scale experiments were unavoidable and measured. These losses were lower than 2% of the dry matter of potatoes and not included in the mass balance.

4.3.1.2 Separation of the "starch" fraction into soluble and insoluble parts

Soluble and insoluble parts of the "starch" fraction were prepared by washing the "starch" sample (about 50 to 75 mg) with 2 mL water. After incubating via head over tail for 2 h at room temperature, the samples were centrifuged (21,320 g, 30 min, 20°C) and the supernatant was collected. The washing was performed thrice and all supernatants were pooled together and denoted as "soluble starch". Samples of the residue ("insoluble starch") and the supernatant ("soluble starch") were freeze dried for analysis of carbohydrate and protein contents. About 10-16% of the "starch" originally present was recovered as "soluble starch".

4.3.2 Analyses of enzyme activities

4.3.2.1 Characterization of exo-enzyme activities

To measure exo-activities of glycosidases in the two enzyme preparations, p-nitrophenyl (PNP) glycosides were used. About 25 µl of USP or Ebs or diluted solutions thereof was mixed with 25 µl of 0.1 (w/w)% of the PNP-glycoside solution. The mixture was incubated with 75 µl of 0.05 M NaOAc buffer, pH 5.0 for 1 h at 40°C. After this, 125 µl

of 0.5 M glycine NaOH buffer, pH 9.0 containing 0.002 M EDTA was added and the absorbance of the PNP released was measured at 405 nm. The activity of the enzymes was expressed as units (U) of PNP released per mL of enzyme solution, where 1U corresponds to 1µmol of PNP released per min.

4.3.2.2 Characterization of endo-enzyme activities

The ability of USP and Ebs to degrade CWPs was tested by applying them on defined polymeric substrates under the same conditions (40°C, 2 h, ($NaHSO_3$ (0.07 w/w % of dry potato)), same substrate dilution and enzyme to substrate ratio) that were used in the semi-technical scale treatments. The enzymes were diluted and applied such that 6 µg protein of USP and 70 µg protein of Ebs were dosed per 1 mg of soluble PGA, 2 mg of soluble arabinogalactan, 0.5 mg of soluble arabinan and 0.6 mg of tamarind xyloglucan in a final volume of 1 mL. Soluble fractions of arabinan, arabinogalactan and polygalacturonic acid were prepared by stirring (30 min, 40°C; head over tail) and centrifuging a suspension (2 mg/mL; 21,320 g x 30 min, 20°C) after which the supernatant was freeze dried. Incubations were performed head over tail in 100 mM NaOAc buffer, pH 5.9 (same pH as that of potato juice) and separately in 40 (v/v) % potato juice as a medium. Potato juice was collected by blending potatoes through a Sanamat juice extractor (RSA, Uetendorf, Switzerland) and filtering the juice through a 0.2 µm cut off filter (FP30/0.2 CA-S, GE Healthcare, Dassel, Germany). The samples were centrifuged (21,320 g x 20 min, 20°C) and the supernatant was collected for further analyses.

4.3.3 Analytical methods

All analyses, except for particle size, were performed twice. For particle size, five measurements were taken for each sample.

4.3.3.1 Dry matter content Samples were weighed in aluminium cups and dried at 105°C in a pre-heated oven overnight (16 h) or to more than a day (26 to 40 h) for some samples until the weight was constant.

4.3.3.2 Starch content: The total starch and bound starch content (including resistant starch) was determined enzymatically using the Megazyme total starch kit (Megazyme, Wicklow, Ireland). For determining the bound starch content of the "fibre" fraction, 1 g of dried "fibre" was washed with 10 L of water over a sieve of 106 µm (Retsch, Haan, Germany). The residual "fibre" fraction on the sieve was dried and starch content was determined, which was considered to be bound starch.

4.3.3.3 Constituent monosaccharide composition and content were analysed as described previously (Ramaswamy et al., 2013). From the content of Glc analyzed by gas chromatography, the content of starch glucose (Glc) was subtracted to obtain the content of cell wall Glc.

4.3.3.4 Protein content (N% x 6.25) of the "starch" fractions was determined using a Thermo Quest NA 2100 Nitrogen analyzer (Interscience, Breda, The Netherlands). D-Methionine (Acros organics) was used as an external standard. The protein content of the "soluble starch" fractions was determined after the fractions were re-dissolved in water (1 mg/mL). About 20 µl of the sample or diluted sample was mixed with 200 µl of Bradford reagent (Sigma-Aldrich). A calibration curve of bovine serum albumin (Sigma-Aldrich) was used in the concentration range of 0.25 to 1.4 mg/mL. After 15 min of incubation at room temperature, the absorbance was measured at 595 nm.

4.3.3.5 High Performance Size Exclusion Chromatography (HPSEC): An Ultimate 3000 HPLC (Thermo Scientific, Sunnyvale, CA, USA) was used with three TosoHaas TSK-gel columns in series (4000, 3000 and 2500 SuperAW; each 150 x 6 mm; TosoHaas, Tokyo, Japan) preceded by a TSK Super AW-L guard column (3,5 cm x 4.6 mm; TosoHaas). 20 µL of sample (0.5 mg/mL) was injected and eluted with 0.2 M sodium nitrate at a flow rate of 0.6 mL/min at 55°C. Detection was performed with a refractive index detector Shodex R101 (Showa Denko, Kawasaki, Japan). The system was controlled using Chromeleon (version 7) software (Dionex, now ThermoScientific). For calibration, pullulan standards (Sigma; mass range of 180 Da to 790 kDa) were used.

4.3.3.6 Particle size distribution was determined by laser diffraction using a Malvern Mastersizer 2000 (Malvern, UK). Samples were homogenized and particle size was measured in five replicates. The mean particle size of the volume-weighted ($d_{4,3}$) distributions was calculated as $d4{,}3$. For the % volume distribution, the size range measured was from 10 µm to 2000 µm. For confirming whether particle sizes exceeding 2000 µm exist, a number of samples of the freeze dried "fibre" fraction were sieved over a 710 µm sieve and the weight % of particles over the sieve was calculated after freeze drying. Small amounts (about 1 mg) of these particles were wetted with water and analyzed by light microscopy using an Axio Scope A1 polarized light microscope (Carl Zeiss; Gottingen, Germany) at a total magnification of 100. Differences in size ranges of cell wall thickness were observed using the Image J software (version 1.44n, National Institutes of Health, Bethesda, MD, USA) after calibration with the slide micrometer.

4.3.3.7 Water Holding Capacity measurements were performed using the Baumann's apparatus as described previously (Ramaswamy et al., 2013). The WHC was expressed as the mL of water held per g of dry matter of "fibre" fraction. In addition, the term "retained WHC" was used to relate the amount of water held by the enzyme treated "fibre" fraction $\left(\left(\frac{mL}{g}\right)^E\right)$ to the amount of water held by the blank "fibre" fraction $\left(\left(\frac{mL}{g}\right)^B\right)$ and denoted as WHC^R. $WHC^R = \left(\frac{\left(\frac{mL}{g}\right)^E \times (g)^E}{\left(\frac{mL}{g}\right)^B \times (g)^B}\right) \times 100$, where $(g)^E$ and $(g)^B$ represent the amount of enzyme treated "fibre" and blank "fibre" recovered from potato (on dry matter), respectively.

4.4 Results and discussion

4.4.1 Recoveries of total solids, starch and CWPs in "starch" and "fibre" fraction

The potatoes used contained 28.5 % (w/w) dry matter. Based on dry matter, the starch and cell wall polysaccharide (CWP) contents were 70% (w/w) and 9% (w/w), respectively (Table 2). These values are common for potatoes.

Recoveries of solids (dry matter), starch and CWPs in the "fibre" and "starch" fraction are shown in figure 2. The recovery of solids from potato ranged from 87% to 100% (w/w) for all the trials (Figure 2). The recoveries of CWPs ranged between 96% and 100% (Figure 2). In addition, the total starch recovered in the "standard trial" (see 2.2.1) was 96% (w/w) on dry matter, of which 94% was recovered in the "starch" fraction and 2% in the "fibre" fraction.

These results are in close agreement with the starch recovery on commercial scale (Grommers and Krogt, 2009).

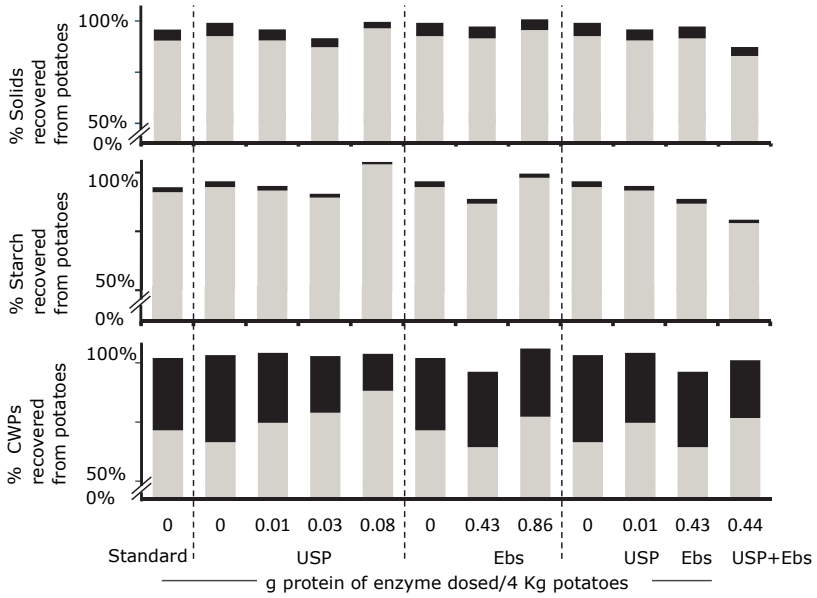

Figure 2: Recovery and distribution of total solids, starch and CWPs from potatoes over "starch" and "fibre" fractions. ■ :"fibre" and ■ :"starch" fraction.

For the "standard" and "blank" trials, about one-third of all CWPs were recovered in the "fibre" fraction and the rest of CWPs was present in the "starch" fraction. Findings on such distributions of CWPs over "starch" and "fibre" fractions for the industrial process have not been reported. The amount of CWPs recovered in the "fibre" fractions decreased at higher enzyme doses applied. This decrease was more obvious for USP than for Ebs.

Starch in the "fibre" fraction can be distinguished as free starch, which is easily removable from partly intact cells, and as bound starch, which is enclosed within intact or partly intact cells (Leszczynski, 1989). Since the amount of starch recovered in the "fibre" fraction decreased upon enzyme treatments (USP2, USP3 and Ebs2; Figure 2), we determined whether the decrease of starch was due to removal of bound starch. Our results showed no difference between the contents of total and bound starch in the "fibre" fraction. This implied that all free starch was already washed out during the sieving process and recovered in the "starch" fraction (data not shown). The recoveries of starch from potatoes ranged between 87% and 104%.

Overall, considering the scale of the experimental set-up, it was concluded that the mass balances of starch, CWPs and dry matter of these *semi-technical* scale trials were good to enable investigation of the effect of enzymes.

4.4.2 Relationship between enzymatic degradation of cell wall polysaccharides, starch removal from "fibre" and resulting water holding capacity

The proportions of CWPs and starch removed from "fibre" fractions and their effects on the WHC are presented in figure 3. In this figure, the removal of CWPs and starch is related to that of the blank "fibre" fraction (treatment without enzymes). For both USP and Ebs, the release of starch linearly correlated with the removal of CWPs (r^2 = 0.98 for USP and 1.00 for Ebs). The removal of CWPs and starch by the combined action of USP and Ebs was almost equivalent to the sum of the individual treatments showing an additive effect. These results indicate that USP and Ebs degraded cell walls in potato pulp, which increased the starch recovery in the "starch" fraction.

In addition, the WHC of the "fibre" fractions was investigated in relation with the removal of CWPs from these "fibre". Figure 3 shows WHC^R, which presented the percentage of water held by the total amount of enzyme treated "fibre" related to the blank "fibre" (see 4.3.3.7). The 0.08 g of USP treated "fibre" showed the lowest amount of water held, which was only 40% of the water held by the blank "fibre" fraction. The WHC^R reduced more by the action of USP than by Ebs, mainly because USP removed more CWPs

than Ebs. Since starch is not expected to contribute substantially to the WHC (Pinnavaia and Pizzirani, 1998), it was concluded that an increased removal of CWPs decreased the WHC^R of "fibre" fractions. Enzyme aided reduction in WHC has been reported also for wheat bran and rye bran (Petersson et al., 2012).

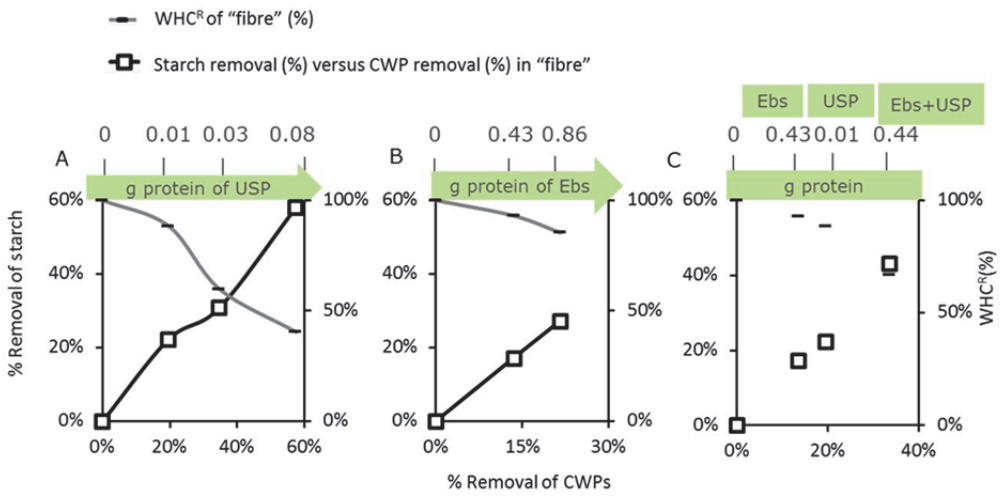

Figure 3: Correlation between the removal of starch (% relative to the blank), removal of CWPs (% relative to the blank) from the "fibre" fraction relative to the blank "fibre" fraction and WHC^R of "fibres" (%).

To understand the effect of the type of CWPs present on the amount of water held, the CWPs in the various fractions are described in more in detail.

4.4.3 Relationship between cell wall polysaccharide composition and water holding capacity

4.4.3.1 Cell wall polysaccharide yield and composition of "fibre" fractions

The most abundant carbohydrate residues in potato were cell wall glucosyl (Glc - 48 mol%), galactosyl (Gal-19 mol%) and uronyl (UA-15 mol%) residues, indicating that

CWPs mainly comprised cellulose, arabinogalactans and homogalacturonans (HG)(table 2A). The presence of rhamnosyl (Rha (7 mol%)) residues pointed out that pectins comprised rhamnogalacturonan-I (RG-1) and homogalacturonan (HG) (Meyer et al., 2009). The 6 mol% of arabinosyl residues indicated that the side chains on RG-I contained Ara residues.

Table 2A: Composition and WHCs of the "fibre" fractions

Trial [a]	WHC of "fibre" fraction (mL/g)	w/w %			CWPs in "fibre" fraction: Molar composition (mol %)[d]							
		Protein	Starch	CWP	Rha	Fuc	Ara	Xyl	Man	Gal	Glc[e]	UA
Potatoes		9	70	9	7	1	6	2	2	19	48	15
Standard[b]	14.2	6	28	50	3	<1	8	4	2	35	25	23
Blank[c]	15.5	6	27	50	2	<1	8	4	2	36	27	22
USP1	16.8	8	25	48	3	<1	8	4	2	32	31	21
USP2	13.9	9	27	48	2	<1	8	5	3	23	39	21
USP3	13.3	10	23	44	2	<1	8	6	4	15	44	21
Ebs1	16.3	8	25	48	2	<1	8	4	2	30	32	21
Ebs2	16.3	8	25	51	2	<1	8	4	2	27	33	23
USP+Ebs	15.5	10	22	49	2	<1	8	4	3	26	35	23

[a] Refer to Table 1 for details of trials;
USP1, USP2 and USP3 are 0.01, 0.03 and 0.08 g protein of enzyme liquid, respectively, per Kg dry potatoes
Ebs1 and Ebs 2 are 0.43 and 0.86 g protein of enzyme liquid, respectively per Kg dry potatoes
[b] Trial in which potato pulp is not incubated
[c] Trial in which potato pulp is incubated without enzymes
[d] Rha, rhamnose; Fuc, fucose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; UA, uronic acid
[e] Non starch Glc

In the "fibre" fraction from the standard trial, 31% of CWPs were recovered (Table 2B), of which Gal (35 mol %), Glc (25 mol %) and UA (23 mol %) were the most abundant constituent residues (Table 2A). This indicated that the "fibre" fraction was abundant in arabinogalactan, cellulose and HG. This fraction represented about 35% of a total of Rha and UA residues, 49% of a total of Ara and Gal residues, and 21% of other CWPs (total of xylosyl (Xyl), mannosyl (Man) and non-starch (Glc)) residues from potato. The galactosyl units analysed are considered to belong mostly to polysaccharides originating from cell walls. Small amounts of galactolipids may be present (less than 1.85% (w/w) in the "fibre" fraction (Prescha et al., 2009).

With increasing doses of USP or Ebs, the carbohydrate composition of the resulting "fibre" fractions changed. Mostly, Gal decreased in the "fibre" fraction, while Glc increased (Table 2A). Modifications in the CWP network influence the WHC of "fibre" (Ramaswamy et al., 2013). Therefore, the amount of CWP residues recovered in the "fibre" fractions were used to understand the effect of the removal of CWPs on the WHC of the "fibre" fraction.

Table 2B: Yield of carbohydrate residues in the "fibre" fractions

Trial [a]	Yield in "fibre" fraction (w/w % based on dry matter of potatoes)									
	Dry matter	CWPs	Rha[d]	Fuc[d]	Ara[d]	Xyl[d]	Man[d]	Gal[d]	Glc[d,e]	UA[d]
Potatoes	100	100	100	100	100	100	100	100	100	100
Standard[b]	5.4	31	11	15	36	45	29	52	20	44
Blank[c]	6.5	37	10	8	41	48	25	61	27	49
USP1	5.3	30	10	10	33	42	25	44	24	37
USP2	4.4	24	7	6	24	39	26	25	24	29
USP3	3.1	16	4	2	15	30	22	10	18	18
Ebs1	5.8	32	8	11	33	42	26	43	27	42
Ebs2	5.0	29	8	8	31	41	21	36	25	39
USP+Ebs	4.4	24	7	7	25	35	25	29	23	32

[a] Refer to Table 1 for details of trials;
USP1, USP2 and USP3 are 0.01, 0.03 and 0.08 g protein of enzyme liquid, respectively, per Kg of dry potatoes
Ebs1 and Ebs 2 are 0.43 and 0.86 g protein of enzyme liquid, respectively, per Kg of dry potatoes
[b] Trial in which potato pulp is not incubated
[c] Trial in which potato pulp is incubated without enzymes
[d] Rha, rhamnose; Fuc, fucose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; UA, uronic acid
[e] Non starch glucose

The yield of Gal (Table 2B) in "fibre" fractions decreased substantially upon enzyme treatment indicating substantial removal of galactans. In view of the lowered WHC (Table 2A) of the fractions obtained at increasing doses of USP, it was concluded that arabinogalactans largely affect the WHC of the "fibre" fraction. The recoveries of Ara decreased from 41% in the blank "fibre" to 15% of Ara remained in USP3 (0.08 g protein) treated "fibre" (Table 2B), indicating at the removal of arabinans and/or Ara substituted

arabinogalactans. For Ebs treated “fibres”, the decrease in the recovery of Ara was much lower. Apparently, it was possible that arabinans also affected the WHC, since they have been shown to hydrate RG-I even more than arabinogalactans (Larsen et al., 2011). Also, USP treatments decreased the recoveries of UA and Rha in the “fibre” fractions more than Ebs treatments (Table 2B) pointing at the removal of HG and RG-I. Although it could not be concluded that the degradation of the pectic backbone also lowered the WHC, previous studies have shown that the degradation of HG by polygalacturonase and pectin methyl esterase lowered the WHC of dehulled lupins (Ali et al., 2005).

Next to pectic CWPs, a small decrease in the recoveries of Xyl and CW Glc was observed upon addition of USP (Table 2B), indicating the removal of xyloglucans (XGs). Although the role of XGs in the WHC of potato fibre has not been conclusively identified before, XGs have been indicated previously to possess a high WHC (Nishinari et al., 2000).

In general, it can be concluded that the removal of RG-I side chains, mainly arabinogalactans, resulted in lower WHCs of the “fibre” fractions.

4.4.3.2 Cell wall polysaccharides and proteins in the “starch” fractions

In the starch separation process, contamination of the insoluble “starch” rich fraction with water insoluble CWPs and proteins should be avoided, since this may necessitate additional cleaning steps (Grommers and Krogt, 2009). The recovery of CWPs and proteins in the “insoluble starch” fraction was calculated from the difference in the content of CWPs and proteins in the “starch” fraction and the “soluble starch” fraction. The “insoluble starch” fraction was not directly analyzed because it is complicated to obtain accurate values for CWP and protein contents. The content of CWPs in the “soluble starch” fraction increased with increasing enzyme doses, from 10% (w/w) for the blank “starch” fraction to 22% (w/w) for USP3 (0.08 g protein) and 16% (w/w) for Ebs2 (0.86 g protein). Corresponding to this increase, is the decreased recovery of CWPs in the “insoluble starch” fraction with increasing enzyme doses, which was more obvious for USP than Ebs treatments (Figure 4). It is beneficial that these CWPs accumulate as soluble solids in the commercial process, because these solubles (in potato juice) are separated from “starch” before the “starch” fraction is purified (Grommers and Krogt, 2009). The

content of proteins in the “soluble starch” fraction also increased slightly, from 16±2.5 for the blank “starch” fraction to 24±3.6 for USP3 (0.08 g protein).

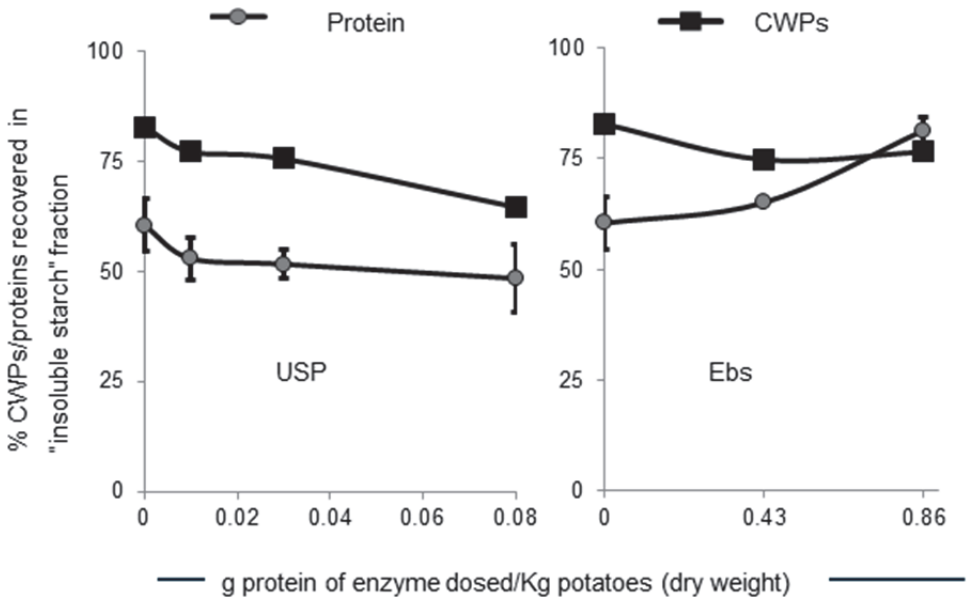

Figure 4: Percentage (w/w) of CWPs and proteins recovered in “insoluble starch” fraction from total CWPs and total proteins originally present in the “starch” fraction.

This was reflected by negligible differences in the recoveries of proteins in the “insoluble starch” fractions (Figure 4), implying that the “insoluble starch” was free from any additional build-up of proteins upon treatment with USP. Only for Ebs2 (0.86 g protein), the amount of protein recovered in the “insoluble starch” was 35% higher compared to the blank “insoluble starch” fraction. We have no explanation for this. Nevertheless, our results indicated that for treatments with USP, the insoluble “starch” fraction was by large not contaminated with CWPs and proteins.

4.4.4 Particle size

The particle size of the "fibre" fraction was studied to determine the effect of the enzyme treatments on the size distribution of all particles in the "fibre" fractions. Figure 5A showed that the % of the volume diameter of most particles measured fell between the size range from 100 µm and 2000 µm and the maximum volume % of particles fell in the size range of about 600 to 850 µm.

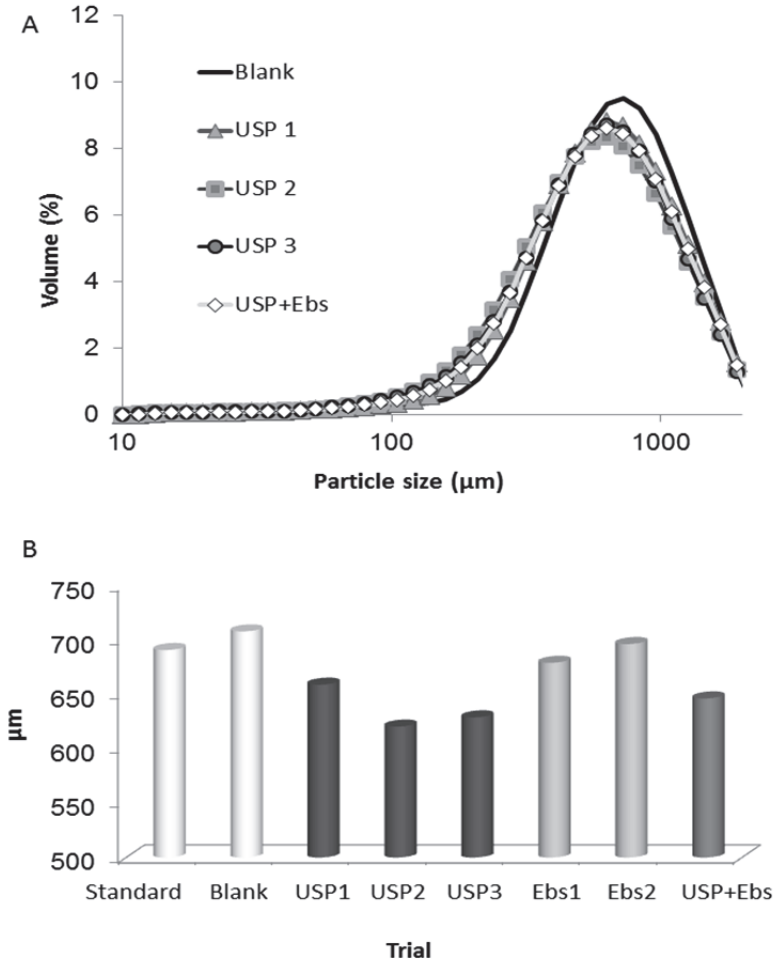

Figure 5: Particle size distribution in volume % of the "fibre" fractions (A); Volume weighed average sizes (*d 4,3*) of the "fibre" fractions (B).

After treatment with USP, the volume % of particles decreased only slightly compared to the blank and hardly any effect was observed for Ebs treatments (data not shown). However, since calculations on volume distribution are not absolute and could also include agglomerated particles, the weight distribution based on actual recoveries of fibre particles larger than 710 µm was determined. Only 5% of the total dry matter of the blank "fibre" was larger than 710 µm and was comparable to the 3% of the USP3 treated "fibre". These results confirmed that most particles were less than 710 µm and that enzyme addition did not cause substantial differences in the particle weight distribution. The cell wall width of these particles larger than 710 µm was determined microscopically (Supplementary figure) to observe if morphological differences were substantial before and after treatment with USP3. The thickness of the cell wall varied from about 3 µm to 30 µm (supplementary data) and not much difference was observed between treatments. This pointed out that the removal of about 58% of CWPs compared to the blank did not modify the morphology of the cell walls substantially, concluded by the microscopic analyses performed. Despite the similar morphologies observed, in figure 5B it is shown that the volume weighed average size ($d_{4,3}$) of fibres decreased more with USP than with Ebs. Reduced particle size was only observed for USP treated "fibre" fractions, USP2 (0.03 g protein) and USP3 (0.08 g protein). In view with the reduced WHC of USP2 (0.03 g protein) "fibre" compared to USP1 (0.01 g protein) "fibre", it is possible that a decrease in particle size lowers the WHC of "fibre". The results are supported by observations with sugar beet fibre where a decrease in particle size from 540 um to 205 um lowered the WHC from 8.5 to 7.3 (Thibault et al., 2001).

4.4.5 Enzyme activities in presence of potato juice

The activities of USP and Ebs on model substrates were tested. The exo-activities of the enzymes (Table 1) indicated that USP was enriched in arabinofuranosidase, xylosidase and to a lesser extent in β-galactosidase compared to Ebs.

The degradation of purified CWPs (arabinogalactans, arabinans, polygalacturonic acid and tamarind xyloglucan, see 4.3.2.2) by USP and Ebs at their highest doses (USP3 and Ebs2) showed degradation of high molecular weight (Mw) material to low Mw

irrespective of the presence of potato juice (PJ). However, in the presence of PJ, the profiles were interfered by components in PJ (data not shown).

Only for arabinan degradation, representative HPSEC profiles were obtained (Figure 6). In buffer, the profiles of both USP and Ebs degraded arabinan were comparable, showing similar abilities to degrade arabinan. However, in 40% PJ, substantial high Mw material remained after enzyme digestion. This was especially the case with Ebs (Figure 6). This indicated that in PJ the enzymes showed lower abilities to degrade high Mw arabinan than in buffer (absence of PJ). This was more obvious for Ebs than for USP. Since the pH of PJ and buffer were the same, these results suggested that both USP and Ebs were suppressed by components in PJ.

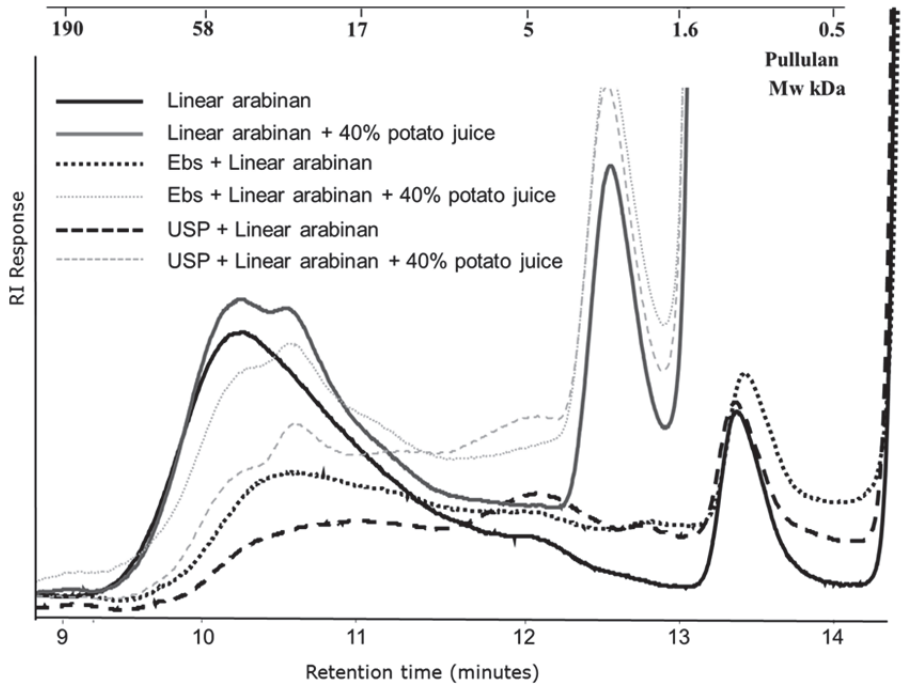

Figure 6: HPSEC profiles of arabinan treated with Ebs and USP in the presence and absence of potato juice (40%).

This could be explained either due to the lack of activities to degrade arabinan in the presence of PJ and/or to the lack of accessibility of the enzymes to arabinan in PJ.

Possibilities of inhibition of cell wall degrading enzymes by components in potato juice, such as phenolic acids or proteins have been mentioned in previous studies (Lyon and McGill, 1989; Bulantseva et al., 2005).

4.5 Conclusion

The study showed that starch recovery from “fibre” increased significantly (58%) upon enzyme treatment (0.08 g protein of USP) by solubilizing arabinogalactan (80% (w/w)) and homogalacturonan (HG) and arabinan (63% (w/w) each) from “fibre”. The absence of these CWPs also caused the “fibre” fraction to retain only 40% of the total amount of water originally present. This pointed at the importance of pectic arabinogalactan side chains, in contributing to the WHC of the “fibre” fraction. It can be expected that at industrial scale, USP or enzymes similar to USP can help to release starch from potatoes and lower the WHC of the “fibre” fraction.

4.6 Acknowledgement

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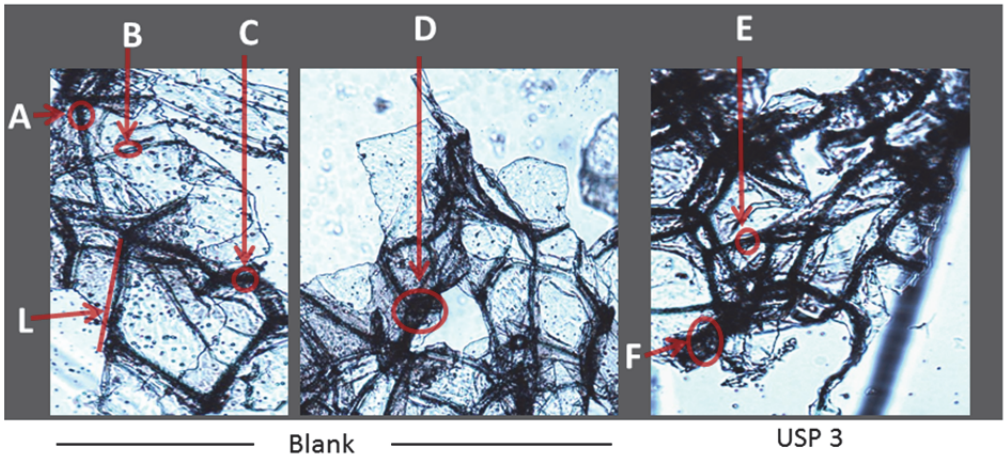
Supplementary figures



Fibre > 710 µm	Blank					USP 3	
Code	A	B	C	D	L (Length)	E	F
Width (µm)	7	3	15	28	96	8	23

Supplementary figure 1: Microscopic pictures of fibre fraction > 710 µm obtained for the blank and for the USP3 trial.

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Chapter 5

Inhibition features of enzymatic hydrolysis of arabinan in potato juice

Abstract

The effect of potato juice (PJ) on the enzymatic degradation of soluble cell wall polysaccharides (CWPs) was tested to investigate if enzyme performance was affected by components present in PJ. Among the CWPs tested, the hydrolysis of arabinan by commercial pectinase preparations showed the highest inhibition by components in PJ. This was shown by the decreased release of the most abundant oligosaccharide, arabinotriose from arabinan, which was only about 11% of that released in the absence of PJ. Nevertheless, after 24 h of arabinan hydrolysis, a similar amount of arabinotriose released (≥ 85%) compared to that in the absence of PJ was observed, indicating that the hydrolysis was delayed. The PJ mediated inhibition was caused by low molecular weight components, smaller than 3 kDa, without affinity for a reversed phase (C18) column. These components did not include glucose, sucrose, phenolic acids.

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5.1 Introduction

The cell wall polysaccharides (CWPs) in potato comprise arabinogalactan, homogalacturonan, rhamnogalacturonan-I, xyloglucan and cellulose (Vincken et al., 2000). The enzymatic degradation of cell wall polysaccharides (CWPs) showed to improve starch recovery from potatoes (Sit et al., 2011), and to de-water the fibre-rich by-product (Slomińska and Starogardzka, 1987). So, the industrial starch extraction process (Grommers and Krogt, 2009) could benefit from the enzymatic hydrolysis of potato CWPs. However, components present in potato juice (PJ) such as phenolic acids, have been reported to inhibit CWP degrading enzymes (Lyon, 1989).

Phenolic acids typically present in potato include chlorogenic acid (ChA) and its isomers, caffeic acid, *p*-coumaric acid, sinapic acid and ferulic acid. Also, their corresponding conjugates like spermines, spermidines, putrescine and octapamine are part of potato juice (Cuenca et al., 2012). In general, these phenolic acids contribute to the resistance of plants against phytopathogens. One of the mechanisms proposed is the ability to inhibit cell wall degrading enzymes. More specifically, increased inhibition of polygalacturonase from the pathogen *Rhizoctonia solani* has been observed at increasing levels of phenolics in the extracts of cotton seedlings, of which oxidized catechin was indicated to play an inhibiting role (Hunter 1974, Lyon and McGill, 1989). Nevertheless, the inhibiting effect of phenolics is not well documented. Different phenolics have shown to inhibit polygalacturonic acid degrading enzymes. For example, polygalacturonic acid lyase from *Erwinia carotovora* was inhibited by syringic acid by 54%, and by synapic acid by 43% at 400 µg/mL, while polygalacturonase from this *Erwinia sp.* was inhibited also by caffeic acid by 57% at only 50 µg/mL (Lyon and McGill, 1989). The possibility that the synergistic action of these phenolics contributes to plant resistance has also been proposed (Ghanekar et al., 1984, Friedman, 1997). In addition, polygalacturonase inhibitory proteins (PGIPs) have been isolated from potato tubers and their activities have been described (Glinka and Protsenko, 1998).

Most attention has been given to the inhibition of polygalacturonase, while only limited information exists about inhibition of other cell wall degrading enzymes.

In potato, we hypothesize that the enzymatic degradation of pectic CWPs such as polygalacturonic acid, arabinogalactan and arabinan (as observed previously (chapter 4) is inhibited by low molecular weight components in potato juice. To study this inhibition, enzymatic hydrolysis of polysaccharides was carried out in PJ and compared with hydrolysis in the absence of PJ. Also, the inhibitory effect of fractions isolated from PJ was analysed.

5.2 Materials

Potatoes of the variety *Annabelle* were purchased from a local supermarket. Enzymes used were "Ebs", an experimental acetyl esterase β-glucanase complex (VP0996/10, 13% w/w protein per liquid weight of enzyme; *Penicillium funiculosum*, Erbsloh, Geisenheim, Germany), and "USP", a commercial pectinase rich preparation Pectinex Ultra-SP (4% w/w protein per liquid weight of enzyme; *Aspergillus aculeatus*, Novozymes, Bagsvaerd, Denmark). Substrates used were arabinogalactan (Megazyme, Wicklow, Ireland), polygalacturonic acid (PGA, Fluka Biochemika) and arabinan (British Sugar, Peterborough, UK). All other chemicals were from Sigma, unless stated otherwise.

5.3 Methods

5.3.1 Potato juice (PJ), oxidized PJ, and fractions of PJ

Fresh potatoes were used for each experiment. Potatoes were washed and blended in a juice extractor (Sanamat rotor RSA, Uetendorf, Switzerland). The juice obtained was centrifuged (4000 g for 20 min, 15°C) to precipitate starch and fibres. The supernatant was filtered through a 0.2 µm cut off filter (FP30/0.2 CA-S, Whatman, Buckinghamshire, UK). This filtered juice was denoted as PJ. In all experiments, unless stated differently, 0.07 (w/w) % of sodium hydrogen sulphite ($NaHSO_3$) was added to the potatoes before blending. Oxidized PJ was obtained by leaving PJ (without $NaHSO_3$) at room temperature for 2 h to allow sufficient oxidation until the solution was completely brown (denoted as OX PJ). PJ was fractionated by using regenerated cellulose centrifugal

filter units (Millipore, Amicon, Ultracel YM-3, 15 mL, cut-off 3 kDa and YM-10, 4 mL, cut-off 10 kDa, Bedford, MA, USA). The fraction obtained after filtering PJ through the 10 kDa filter was denoted as “PJ<10”. Similarly, the fraction obtained after filtering PJ through the 3 kDa filter was denoted as “PJ<3”. The materials remaining on the filter were recovered in buffer and denoted as “PJ>3” or “PJ>10”. Also, OX PJ was fractionated to <10 kDa in size, and denoted as “OX PJ<10”, as described above.

The PJ<3 fraction was further separated by applying 5 mL of PJ<3 onto an activated Sep-Pak cartridge (C18, 10 g, Waters, Milford, CT, USA). Fractions of PJ<3 were collected from the cartridge in ten steps, in which for every step, the cartridge was eluted with one column volume of solvent. For the first step, water was used as a solvent. For step 2 until 9, a methanol:water ratio was used in which the methanol increased from 1:9 for the second step until the ratio was 9:1 for the ninth step. For the tenth step, only methanol was used as a solvent. The liquid in the fractions was evaporated using a Syncore evaporator (Buchi Syncore, Type Q11, Flawil, Switzerland) under reduced pressure at 40°C, and the remaining traces of methanol were removed by freeze drying. The freeze dried material was solubilized in 1.5 mL millipore water and denoted as “PJ<3-1”, “PJ<3-2”, etc.

5.3.2 Enzyme incubations

All enzyme incubations were performed at 40°C for 2 h using a head over tail rotor. 6 µg protein of USP and 70 µg protein of Ebs were dosed per 1 mg of soluble PGA, 2 mg of soluble arabinogalactan (AG) and 0.5 mg of arabinan in an end volume of 1 mL, respectively. Soluble substrates of PGA, AG and arabinan were prepared by freeze drying the soluble part of a solution (2 mg/mL) obtained after centrifuging the solution (21,320 g for 30 min at 20°C). Enzyme incubations in the absence of potato juice were performed in 50 mM NaOAc buffer, pH 5.9.

5.3.2.1 Enzyme incubations in the presence of PJ, fractions of PJ, and oxidized PJ

Arabinan degradation by USP and Ebs was performed in the presence of various concentrations of PJ. The PJ concentrations applied were 0% (buffer only), 20%, 40% and

60% v/v PJ in buffer in a total volume of 1 mL. Incubations with PJ fractions were performed by adding PJ<3, PJ>3, PJ<10, OX PJ<10 and PJ>10, all at a final concentration of 40% (v/v in buffer) in a total volume of 1 mL. Further, arabinan degradation by Ebs was performed in the presence of various concentrations of PJ<3. The PJ<3 concentrations applied were 0% (buffer only), 20%, 40% and 60% (v/v) PJ in buffer in a total volume of 1 mL. Incubations were performed for 2 h and 24 h. Enzyme doses and hydrolysis conditions were applied as described for arabinan (see 5.3.2 above).

5.3.2.2 Arabinan degradation by Ebs in the presence of saccharides and phenolic acids

Arabinan degradation by Ebs was tested in buffer containing 3.2, 6.5 and 9.7 mg/mL of glucose, and 8.2 mg/mL of sucrose. In addition, incubations were performed in the presence of 400 µg/mL of chlorogenic acid, caffeic acid or p-coumaric acid. Also, a mixture of these phenolic acids (400 µg/mL each) in buffer was used. Enzyme doses and hydrolysis conditions were applied as described above for arabinan.

5.3.2.3 Incubations with fractions from < 3 kDa potato juice

Arabinan degradation by Ebs was tested using the C18 cartridge fractions obtained from PJ<3. Incubations were performed by adding the fractions at a final concentration of 40% ((v/v) in buffer) in a total volume of 1 mL. Enzyme doses and hydrolysis conditions were applied as described for arabinan.

After all incubations, except for the 24 h incubations (to prevent heat induced oxidation and degradation of phenolic acids under investigation), enzymes were briefly inactivated at 100°C for 5 min. Samples were then centrifuged (21,320 g x 20 min, 20°C) before analysing the degradation of high molecular weight arabinan (HPSEC) and monitoring the formation of arabinan oligosaccharides (HPAEC).

5.3.3 Analytical methods

All analyses were performed in duplicates.

5.3.3.1 Nitrogen content was analysed as described elsewhere (Ramaswamy et al., 2013).

5.3.3.2 Polyphenol content: Samples of PJ, PJ>3, PJ>10 were extracted with ethanol (100% (v/v)) for the assay. Other samples were directly used. Samples were mixed with two times their volume with ethanol to precipitate proteins, kept at 4°C for 30 min and then centrifuged (4000 g for 30 min, 4°C). The clear supernatant was assayed for total phenolic content using the Folin-Ciaocalteau assay. In this assay, about 20 µL of supernatant or sample was mixed with 1.5 mL of water and 100 µL of the Folin Ciaocalteau's phenol reagent and left at room temperature for 20 min. Finally, 200 µL of saturated Na_2CO3 solution was added, and samples were incubated at room temperature for 2 h. The absorbance was measured at 725 nm. Calibration was performed using chlorogenic acid as a standard.

5.3.3.3 High performance size exclusion chromatography (HPSEC) was performed as described elsewhere (Ramaswamy et al., 2013).

5.3.3.4 High performance anion exchange chromatography (HPAEC) was performed using an ICS 5000 (HPAEC) system (Dionex, Sunnyvale, CA, USA) with electrochemical detection (ED). About 10 µl of each sample was injected into the system and separation was performed using a CarboPac PA-1 column (2 x 250 mm) preceded by a CarboPac PA-1 guard column (2 x 50 mm), both from Dionex. The monosaccharides were eluted in a linear gradient from 0.02 to 0.05 M NaOH in 3 min, following a linear gradient of 0.05 – 0.075 M NaOH in 10 min, and an isocratic elution of 0.1 M NaOH for 2 min. The oligosaccharides were eluted with a linear gradient of 0 to 1 M NaOAc in 0.1 M NaOH within 50 min. The column was washed with 1 M NaOAc in 0.1 M NaOH for 7 min, followed by 0.1 M NaOH for 3 min. After each run, the column was equilibrated by eluting with 0.02 M NaOH for 20 min. The flow rate of the eluent was 0.3 mL/min. The gradient used was the same for all samples to enable comparison of HPAEC profiles for all enzyme incubations.

For quantifying the amount of glucose (Glc) and sucrose (Suc), a different system was used for HPAEC (ICS 3000 system), but the same columns were used as described above. The saccharides were eluted in a gradient of 0 to 0.02 M NaOH for the first 15 min, followed by an isocratic elution of 0.1 M NaOH for 23 min. Before washing, the gradient

was changed to 0.07 M NaOH and 0.3 M NaOAc in 0.03 M NaOH within 0.1 min. Washing was performed with 1M NaOAc in 0.1 M NaOH for 3 min and 0.1 M NaOH for the next 9 min. Equilibration of the column was done by eluting 0.02 M NaOH for 9.5 min. The flow rate of the eluent was 0.3 mL/min.

5.3.3.5 Reverse Phase Ultra Performance liquid chromatography (RP-UPLC) mass spectrometry: Samples were analyzed either directly or after dilution on an Acquity UPLC System (Waters, Milford, MA, USA) using an Acquity UPLC BEH 300 C18 column (2.1 x 150 mm, 1.7 µm particle size) with an Acquity BEH C18 Vanguard pre-column (2.1 x 50 mm, 1.7 µm particle size). Eluent A was 1 % (v/v) acetonitrile (ACN) containing 0.1 % (v/v) trifluoroacetic acid (TFA) in millipore water and eluent B was 100 % ACN containing 0.1 % (v/v) TFA. Samples (2 µL) were injected onto the column thermally regulated at 40 °C. The elution profile was as follows: 0-2 min isocratic on 3 % B; 2-10 min linear gradient from 3 % to 22 % B; 10-16 min linear gradient 22 - 30 % B; 16-19 min linear gradient 30-100 % B; 19-24 min isocratic on 100 % B; 24-26 min linear gradient 100-3 % B and 26-30 min isocratic on 3 % B. The flow rate was 350 µL min-1. Detection was done using a PDA, which was scanning the absorbance from 200-400 nm. The mass spectra of the samples were determined with an online Synapt High Definition Mass Spectrometer (Waters), coupled to the RP-UPLC system, equipped with a z-spray electrospray ionization (ESI) source, a hybrid quadrupole and an orthogonal time-of-flight (Q-TOF). MS was performed between m/z 100-2000 in positive ion mode with the capillary voltage set to 3 kV and the source temperature at 120 °C. UV and MS data were acquired using MassLynx software (Waters).

5.3.3.6 Ultra High Performance liquid chromatography (UHPLC) mass spectrometry: Samples were diluted and analyzed as described elsewhere (Narváez-Cuenca, et al., 2012).

5.4 Results and discussion

5.4.1 Enzymatic hydrolysis of defined polysaccharides in the presence and absence of potato juice

The enzymatic hydrolysis of arabinogalactan (AG), polygalacturonic acid (PGA), and arabinan by Ebs was investigated in the presence and absence of potato juice (PJ, 40% (v/v)). The pH for both the buffer and PJ incubations were kept the same (pH 5.9). The HPAEC profiles of PGA and AG, both incubated (for 2 h) with Ebs in the presence of PJ, were similar to those of Ebs incubations of these polysaccharides in buffer (Figure 1).

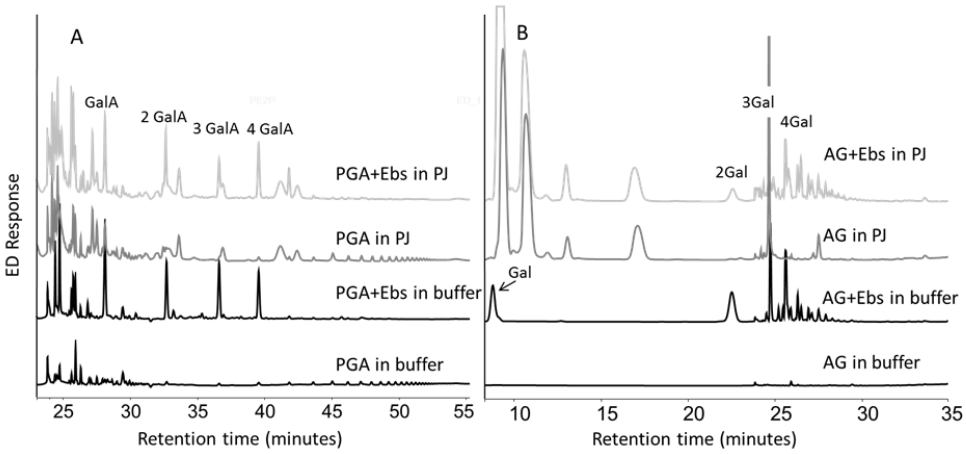

Figure 1: HPAEC profiles of polygalacturonic acid (PGA)(A) and arabinogalactan (AG) (B) before and after hydrolysis (2 h) by Ebs in buffer and in PJ (40% (v/v)). Oligosaccharides coded as: GalA: Galacturonyl (DP 1-4) and Gal: Galactosyl (DP 2-4) residues.

These results were in contrast to the expected inhibition of polygalacturonic acid by inhibitory phenolic acids or proteins in PJ (Lyon and McGill, 1989; Glinka and Protsenko, 1998).

For arabinan hydrolyzed by Ebs, the HPSEC profiles showed lower extents of hydrolysis in PJ compared to the hydrolysis in buffer alone (Figure 2B). Also, the HPAEC profiles showed a substantially lower release of oligosaccharides by Ebs (2 h) in the

presence of PJ compared to incubation in buffer (Figure 2D). This indicated that Ebs was inhibited by PJ components in hydrolyzing arabinan. This inhibition was also observed for USP (Figures 2A and 2C). The HPSEC profiles showed that the hydrolysis of arabinan in PJ was greater for USP (Figure 2A) than for Ebs (Figure 2B), pointing to a lower inhibition of USP than Ebs.

To confirm the inhibition of Ebs and USP on the enzymatic hydrolysis of arabinan by PJ components, arabinan was incubated with different doses of PJ, ranging from 20% to 60% (v/v). For incubations in PJ, the elution of components after the hydrolysis of arabinan in HPSEC and HPAEC was interfered with co-elution of components in PJ.

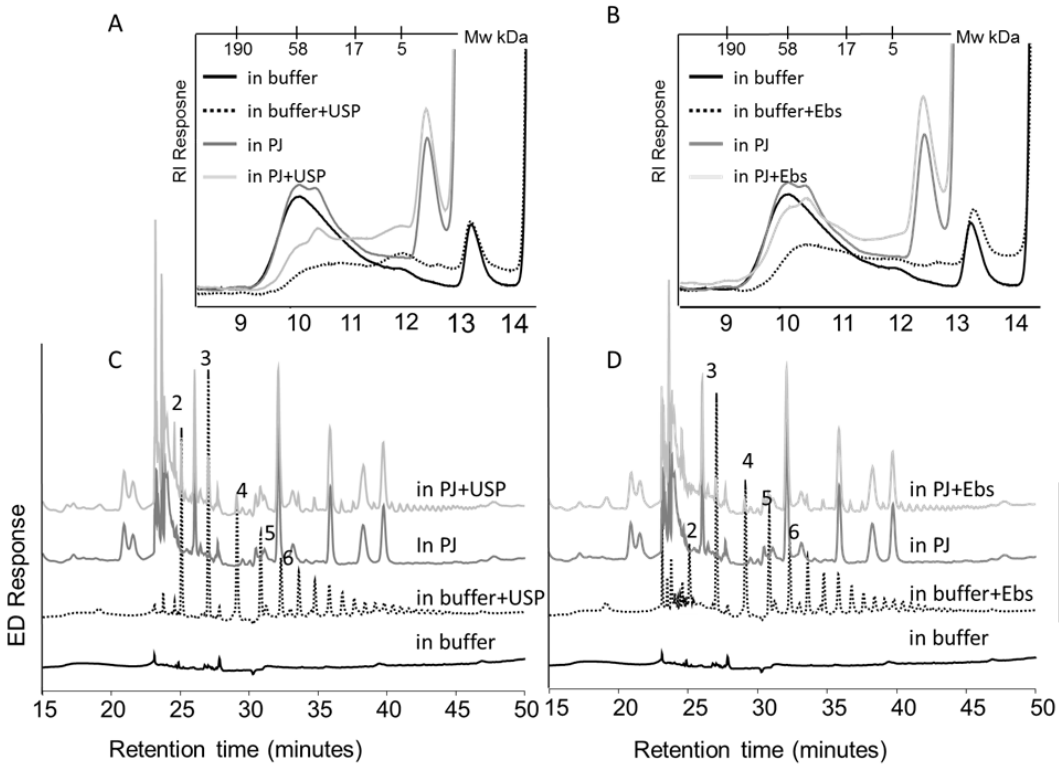

Figure 2: HPSEC profiles of arabinan before and after hydrolysis (2 h) in buffer and in PJ (40% (v/v)) by USP (A) or Ebs (B), and corresponding HPAEC profiles (Ebs (C), and USP (D)). Numbers indicate the degree of polymerization of the arabino-oligosaccharides.

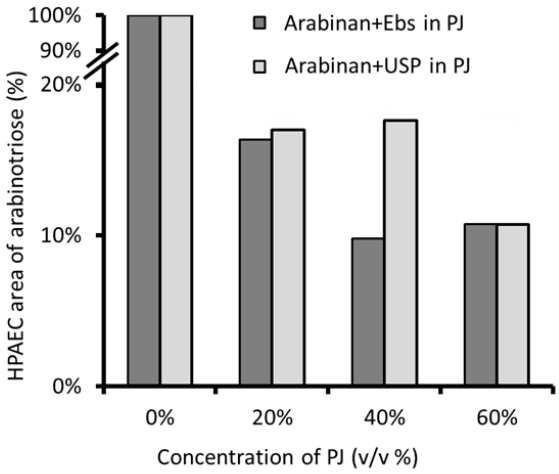

Figure 3: Effect of increasing concentrations of PJ on the formation of arabinotriose by USP and Ebs.

Therefore, the effect of PJ on arabinan hydrolysis was quantified by comparing the area of the most abundant hydrolysis product, arabinotriose, with the area of the arabinotriose released in buffer incubation. The results (Figure 3) showed that already at a PJ concentration of 20% (v/v), the release of arabinotriose from arabinan decreased, from 100% in buffer to 16-17 % in 20% (v/v) PJ for Ebs and USP. Increasing the PJ concentration to 60% (v/v) only lowered the release of arabinotriose from arabinan to 11%, by both USP and Ebs.

5.4.2 Ebs hydrolysis of arabinan inhibited by PJ components smaller than 3 kDa

The size of inhibitory components in PJ was analysed by incubating arabinan (for 2 h) with enzymes in the presence of PJ fractions differing in size (PJ<10, PJ<3, see 5.3.1). Incubation of arabinan in the presence of PJ<10 decreased the release of arabinotriose by Ebs and USP to 11% and 18%, respectively, compared to the incubation in buffer (no further data shown). Hydrolysis of arabinan by Ebs or USP in the presence of PJ>10 showed a release of about 90% of the arabinotriose compared to the hydrolysis in buffer

(data not shown). Also, the corresponding HPSEC and HPAEC profiles of arabinan digest in PJ>10 were comparable to the arabinan digest in buffer (data not shown).

PJ was also separated into a PJ fraction smaller than 3 kDa (PJ<3). Increasing amounts of PJ<3 were added to arabinan and hydrolyzed by Ebs. Again, the arabinan hydrolysis (for 2 h) was represented as the amount of arabinotriose released (Figure 4). At increasing concentrations of PJ<3 in a range of 0% (incubation in buffer) to 60% v/v, an increased inhibition of the enzymatic hydrolysis of arabinan was observed.

Incubation of arabinan in PJ>3 released the same amount of arabinotriose as incubations in buffer (no further data shown). These results confirmed that mainly low molecular weight components (<3 kDa) in PJ inhibited arabinan hydrolysis by Ebs and USP. Upon prolonging the incubations to 24 h, the amount of arabinotriose formed, equalled 85% of that in the absence of PJ; data not shown). This implied that only a part of the enzyme protein was inhibited.

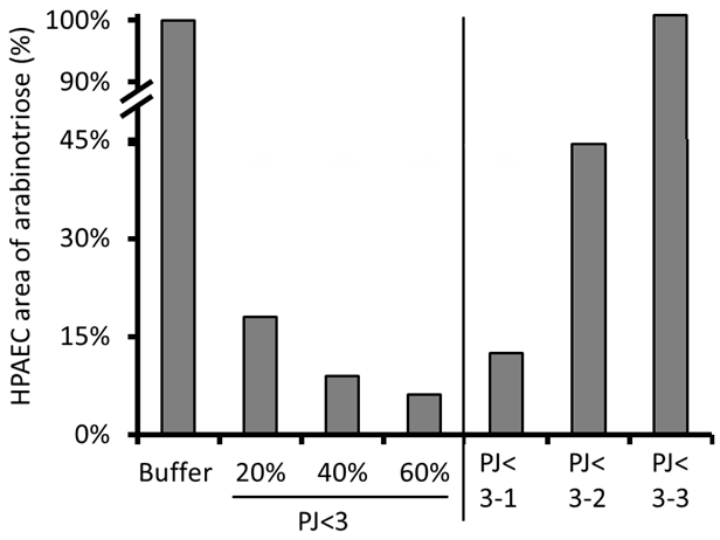

Figure 4: Effect of increasing concentrations of PJ<3 and 40% (v/v) each of PJ<3-1, PJ<3-2 and PJ<3-3 on the degradation of arabinan by Ebs.

5.4.3 Ebs hydrolysis of arabinan in the presence of phenolic acids, glucose and sucrose

Ebs hydrolysis of arabinan was analyzed in the presence and absence of chlorogenic acid (ChA), caffeic acid (Ca), and coumaric acid (CuA), and in the presence or absence of glucose (Glc) and sucrose (Suc). In figure 5, it was observed that the release of arabino-oligosaccharides in the presence of phenolic acids, alone or combined, was comparable to that of buffer incubation shown in figure 2.

The amount of ChA dosed was about ten times higher than the amount analyzed earlier in potato (Narvaez-Cuenca et al., 2012). These results indicated that phenolic acids did not inhibit arabinan hydrolysis, also when combined together, opposite to what has been proposed before (Ghanekar et al., 1984). Similarly, in the presence of different amounts of Glc, representing the concentrations present in 20%, 40% and 60% PJ<3 (see 5.3.2.2), and Suc, representing the concentration present in 60% PJ<3, no inhibition of arabinan hydrolysis was observed (data not shown).

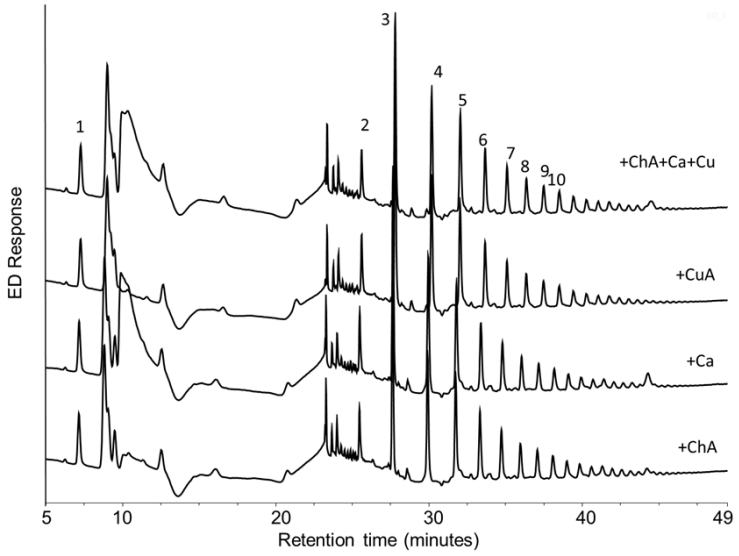

Figure 5: HPAEC profiles of arabinan hydrolyzed by Ebs (2 h) in the presence of chlorogenic acid (ChA), caffeic acid (Ca) and coumaric acid (CuA), and a mixture of these phenolic acids. Numbers indicate the degree of polymerization of the arabino-oligosaccharides.

5.4.4 Ebs hydrolysis of arabinan in the presence of PJ<3 fractions

PJ<3 was loaded onto a C18 cartridge, which was eluted with water first (PJ<3-1), followed by elution in methanol (PJ<3-2 to PJ<3-10). Hydrophobic components, like p-coumaric acid, will bind to the C18 cartridge (Qualls and Haines, 1991; Merken and Beecher, 2000). Testing these fractions on arabinan hydrolysis by Ebs showed that the water extract (PJ<3-1) inhibited the enzymatic degradation of arabinan the most (Figure 4). RP-UPLC-MS of the water extract showed to contain a component with an m/z of 433 (data not shown). A phenolic acid identical to this m/z was discovered to be sulphonated ChA by Narvaez-Cuenca et al., (2011), upon the addition of $NaHSO_3$ to potatoes. Since PJ<3 contained $NaHSO_3$, it confirmed the presence of sulphonated ChA (S-ChA) in the water extract. But, sulphonated ChA was also found in the PJ<3-3 fraction. From these results, it was concluded that most of the inhibitory components were not only low in molecular weight, but also highly hydrophilic without affinity for the hydrophobic C18 column.

5.4.5 Ebs hydrolysis of arabinan in the presence of oxidized potato juice

The fact that ChA did not inhibit Ebs in hydrolyzing arabinan and that S-ChA was present in PJ<3-1, raised the question if sulphonated phenolics inhibit Ebs. In the event that S-ChA inhibits Ebs, then it is expected that oxidized PJ should not inhibit Ebs in hydrolyzing arabinan. However, the results of applying an oxidized PJ fraction, OX PJ<10, showed HPSEC and HPAEC profiles of Ebs hydrolyzed arabinan comparable to incubations in PJ+$NaHSO_3$ (data not shown). This eliminated the possibility that sulphonated phenolics or oxidized phenolics (quinones) inhibit Ebs in hydrolyzing arabinan. Our results were different from the observations presented by Hunter et al., (1974), who showed that oxidized phenolics, such as oxidized catechin, inhibit the activity of polygalacturonase.

5.4.6 Polyphenol, nitrogen and mono- and disaccharide contents in the different PJ fractions

The polyphenol, nitrogen (N), mono-and disaccharide contents of PJ and its fractions are presented in table 1.

Table 1: Nitrogen, polyphenol and mono- and disaccharide contents in PJ and its fractions

		w/w % (dry matter)					
PJ Sample	pH	N	Polyphenol	Glc[a]	Suc[a]	Gal[a]	Fruc[a]
PJ	5.9	8	2	2	1	<0.5	<0.5
PJ>10	5.7	12	0.6	0	0	<0.5	<0.5
PJ<10	6	6	3	0.7	<0.5	<0.5	<0.5
PJ>3	6	12	2	0	0	<0.5	<0.5
PJ<3	6	6	3	2	1	<0.5	<0.5
PJ<3-1	6.1	7	0.6	5	5	<0.5	<0.5

[a] is free glucose (Glc), free sucrose (Suc), free galactose (Gal), free fructose (fruc)

In general, the polyphenol content ranged from 1 to 3 w/w %, and was higher in PJ<3 and PJ<10. Still, the lowest content was noticed for PJ<3-1. Since PJ<3-1 inhibited arabinan hydrolysis by Ebs, it indicated that phenolic acids probably did not play a substantial role in inhibiting the enzymatic hydrolysis of arabinan. The w/w % of N was lower in PJ<3 and PJ<10 than their corresponding larger PJ fractions. The N in PJ<3 and PJ<10 could be represented by small peptides, amino compounds or N containing salts. Alternatively, they could also comprise peptide-polyphenol complexes (<3 kDa). However, peptides in PJ<3-1 were absent, as seen from the absence of peptides in UPLC-MS-analysis (results not shown).

The saccharides present in PJ and its fractions included Glc, Suc, Gal and Fruc (Table 1). The most abundant saccharide was Glc, next to Suc and were observed to be slightly higher in PJ<3-1 compared to other fractions. Nevertheless, Glc and Suc did not inhibit the enzymatic hydrolysis of arabinan. Apparently, other components were present in PJ that inhibited the enzymatic hydrolysis of arabinan.

5.5 Conclusion

For the first time inhibition of enzyme hydrolysis of arabinan by PJ was shown. The components responsible for the observed inhibition could be defined as water soluble small molecules (<3 kDa), without affinity for C18-column material. Glucose, sucrose, phenolic acids (chlorogenic acid, caffeic acid and coumaric acid) and oxidized PJ-phenolics were excluded from the list of potential inhibitory components. The observed inhibition delays the enzymatic hydrolysis of potato CWPs, if the enzymes are to be applied in the potato starch separation process.

5.6 Acknowledgement

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Chapter 6

General Discussion

6.1 Motivation of the research

Cell wall polysaccharides (CWPs) contribute to the water holding capacity (WHC) of agricultural feeds, like PPF. Information on the structural composition of CWPs in PPF and their effect on the WHC was unavailable. As part of a project entitled “Pre-treatment of agricultural by-products”, the research in this thesis aimed at investigating the constituent CWPs and how the enzymatic hydrolysis of CWPs modified the WHC of PPF. Therefore, the approach used was to characterize the CWPs in PPF and the effect of the CWP network on the WHC with the help of enzymes. Simultaneously, the research also aimed at studying how enzymatic hydrolysis of CWPs in potato cell walls could improve the release of starch from potato fibres and how it affects the WHC of the constituent fibre fractions on pilot scale. For this, two enzyme cocktails, Ebs and USP, were used. Subsequently, the enzyme activity on various polysaccharides in the presence of potato juice was studied.

6.2 Polysaccharides in PPF cell walls

The cell wall polysaccharides (CWPs) in PPF comprised mainly cellulose, arabinogalactan and galacturonan (chapter 2). Between the two batches of PPF used in this thesis, the CWP compositions (mol%) were comparable (Table 1). Compared to the 2010 batch, the starch content in the 2012 batch was 33% lower and the cell wall polysaccharide (CWP) content ((w/w)%) was 23% higher (Table 1). Also, the recovery of CWP residues was slightly higher in the 2010 batch. The differences could arise due to seasonal variations of potatoes as observed elsewhere (Grommers and Krogt, 2009). The CWP composition of the “fibre” fraction obtained at the pilot scale (chapter 4), was quite similar to those of the batches of PPF (chapters 2 and 3; Table 1). Furthermore, the observations that pectic CWPs were solubilized in HBSS (chapters 2 and 3) and PPF Sup (chapter 3) from PPF, indicated that by rasping, a less rigid CWP network remained, both in PPF and in “fibre” compared to that in potato.

Table 1: Composition of CWPs in potatoes, PPF and "fibre" fractions.

	Yield %		Molar (%) CWP composition and yield of CWP residues [a] (%)								w/w % dry matter	
Material	Dry matter	CWPs	Rha	Fuc	Ara	Xyl	Man	Gal	Glc[b]	UA	CWPs	Starch
Potatoes	100	100	7	1	6	2	2	19	48	15	7	70
			(100)	(100)	(100)	(100)	(100)	(100)	(100)[c]	(100)		
PPF 2010[c]	6[d]	40	4	<1	8	3	2	27	32	24	44	30
			(18)	(4)	(40)	(43)	(25)	(46)	(35)[c]	(53)		
PPF 2012[c]	6[d]	45	2	<1	7	3	1	30	32	25	54	20
			(10)	(1)	(44)	(52)	(27)	(58)	(48)[c]	(45)		
"Fibre"[c]	5	38	3	<1	7	3	2	32	31	21	50	28
			(11)	(15)	(36)	(45)	(29)	(52)	(33)[c]	(44)		

[a] Values in brackets indicate g of CWP residue recovered per 100 g of residue in potatoes (dry matter) , Rha, rhamnose; Fuc, fucose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; UA, uronic acid

[b] Non-starch Glc

[c] Yield of CWPs calculated after correction of starch and free Glc (Grommers and Krogt, 2009)

[d] Yield of PPF based on Grommers and Krogt, 2009

6.3 Enzymatic CWP degradation of potato and PPF

6.3.1 Enzyme hydrolysis of CWPs in potato pieces

In chapter 4, the application of enzymes on potato pulp was tested. As a follow up, we hereby report on the action of enzymes on potato pieces in the same semi-technical starch separation process. This was performed to investigate if enzymes had an effect on tissue liquefaction prior to rasping. The set-up, the enzymes and their doses applied to potato pieces are shown in figure 1. Analyses of dry matter, starch and CWP distribution were performed as described in chapter 4.

The starch content of the "fibre" fraction of the non-enzyme treated "potato pieces" trial was 1.5 times that of the standard process, in which the potatoes are directly rasped and sieved (chapter 4). The starch content even increased further upon treatment of potato pieces with enzymes. Probably, this increase was due to the fact that incubation

of potato pieces (in water) resulted in softer tissues. Rasping of these softer tissues was not as efficient to break open cells as was rasping of non-incubated potatoes.

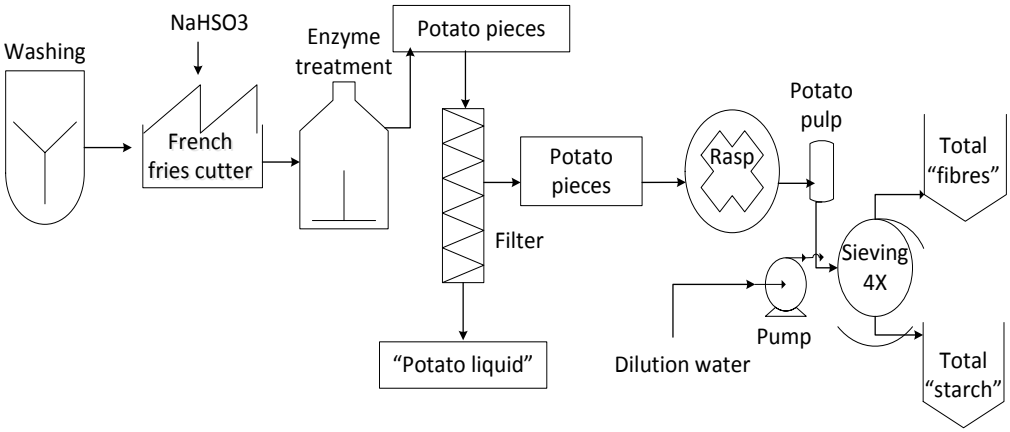

Figure 1: Semi-technical scale of enzyme treatment of potato pieces and starch separation. For detailed sieving process and enzymes used, see scheme in chapter 4. USP was applied at 0.09, 0.14 and 0.29 g protein and Ebs was applied at 0.28 and 0.47 g protein per Kg of potatoes (dry matter). Enzyme combinations included 0.09 g protein of USP with 0.47 g protein of Ebs and 0.09 g protein of USP with 0.17 g protein of Celluclast 1.5 L (Novozymes, CCN030660; *Trichoderma reesei*).

In figure 2, the mass balance of CWPs released in "potato liquid", "fibre" and "starch" fractions is shown. Similar to the enzyme trials performed on incubation of potato pulp (chapter 4), the results showed recoveries between 93% to 108% of CWPs originally present in potato. Visually, the morphology of the enzyme-treated potato pieces was comparable to that of the non-enzyme treated pieces, indicating negligible liquefaction of potato pieces. Nevertheless, upon addition of commercial pectinolytic enzymes, CWPs were released in "potato liquid" (Figure 2). Gal (Gal; 1.3 % (w/w) on dry matter) and UA (0.9% (w/w) on dry matter) were the most abundant sugars released in "potato liquid" for the highest dose of USP (additional data not shown). The amount of CWPs recovered in "potato liquid" for the non-enzyme treated trial, represented 0.8% (w/w) of the total CWPs present in potatoes. In the "fibre" fraction, about 27% (w/w) was found and 66% (w/w) was recovered in the "starch" fraction (data not shown).

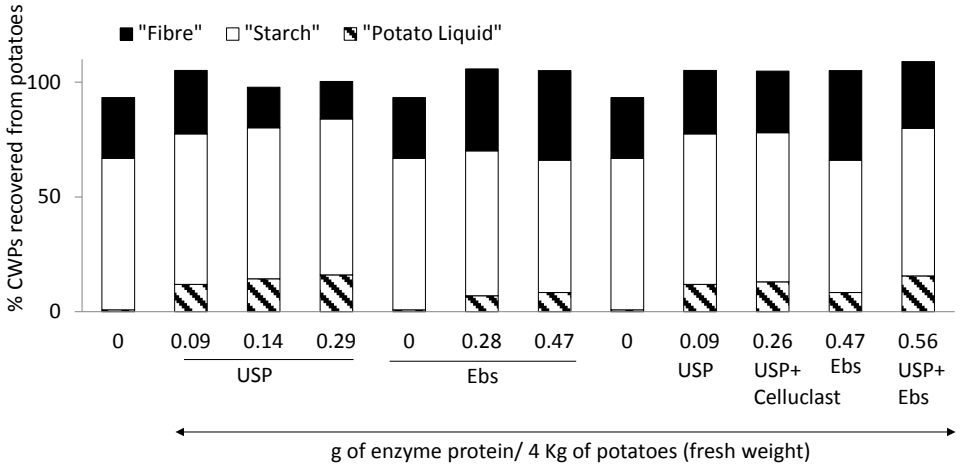

Figure 2: Distribution of CWPs in the "fibre", "starch", and "potato liquid" fractions obtained from enzyme treatment of potato pieces.

The CWPs recovered in the "starch" fraction was slightly lower than that reported for the standard process (72% (w/w); chapter 4). Still, the higher starch content of the "fibre" fractions compared to the standard process indicated that the enzyme addition on potato pieces was not suitable for improving the recovery of starch from the "fibre" fraction. For this, the best moment of enzyme addition in the industrial starch separation process, is directly after the rasping step.

6.3.2 Enzymatic hydrolysis of CWPs in PPF and potato pulp

The application of commercial enzymes on potato fibres has shown to cause significant solubilization of the dry matter (24% to 34%; Meyer, et al., 2009; Norsker et al., 1999). Often these enzymes were endo-glucanase rich preparations with arabinanase- and xylanase- side activities (Meyer, et al., 2009) or pectinase (including of galactanase) rich preparations with hemicellulosic and cellulosic activities (Norsker et al., 1999).

In this thesis two enzymes, an experimental enzyme from Erbsloh, Geisenheim, Germany (Ebs) and a commercial enzyme preparation, USP from Novozymes, Bagsvaerd,

Denmark, were used. The degradation of CWPs was studied by comparing the CWP composition of the non-enzyme treated residue with the enzyme-treated residue resulting from PPF (Table 2). Similarly, the degradation of CWPs of the non-enzyme treated "fibre" was compared to the enzyme treated "fibre" fractions (Table 3).

Table 2: CWP composition of PPF and its residues before and after treatment with Ebs enzyme (chapter 3).

			Dry matter yield	CWPs	Molar (%) CWP composition and yield of CWP residues[b] (%)						
	Material	pH	w/w %		Rha	Ara	Xyl	Man	Gal	Glc[c]	UA
	PPF	5.2	100	54	2	7	3	1	30	32	25
					(100)	(100)	(100)	(100)	(100)	(100)	(100)
Residues	Dose of Ebs (w/w %)[a]	Incubation time									
No enzyme	0.0	47h	72	50	1	6	5	3	16	58	11
					(47)	(59)	(102)	(120)	(39)	(112)	(43)
After Ebs treatment	0.16	47h	61	32	2	8	3	2	6	57	23
					(29)	(36)	(34)	(37)	(9)	(57)	(53)

[a] g protein/g dry fibre.

[b] Values in brackets indicate g of CWP residue recovered per 100 g of residue in PPF (dry matter), Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; UA, uronic acid.

[c] Non-starch Glc.

After enzyme treatments, the "fibres" (Table 3) cannot be completely compared to the PPF residues (Table 2), since "fibres" are not washed and still may contain solubles, while the PPF residues are insolubles remaining after separation of solubles. Nevertheless, some similarities between the residues and the "fibres" resulting after enzyme hydrolysis were found. In general, upon treatment with enzymes rather low amounts of Gal was recovered; 10% (w/w) in the PPF residue (Table 2), 36% in Ebs treated "fibre" (Table 3) and 10% in USP treated "fibre" (Table 3). The recoveries of Ara, UA, Glc and Xyl were between 36 - 57% in Ebs Res, between 31 - 43% in Ebs treated "fibre" and between 15 - 30% in USP treated "fibre". Testing Ebs on defined polysaccharides showed that Ebs was more efficient in hydrolyzing AG and XG than arabinan and polygalacturonic acid, at pH

5.2 (chapter 3). These tests correlated well with the lower decrease in insoluble Ara and UA compared to Gal (Table 2). The lower decrease of Xyl compared to Gal in table 2 indicated that in PPF, AGs were solubilized more than XGs.

Table 3: CWP composition of "fibre" fractions (chapter 4).

			Dry matter yield	CWPs	Molar (%) CWP composition and yield of CWP residues[b] (%)						
	Material	pH	w/w %		Rha	Ara	Xyl	Man	Gal	Glc[c]	UA
	Potatoes	5.9	100	7[e]	7	6	2	2	19	48	15
					(100)	(100)	(100)	(100)	(100)	(100)[d]	(100)
"Fibre" (chapter 4)	Dose (w/w %)[a]	Incubation time									
Non-enzyme	0	2h	6.5	50[e]	3	8	4	2	35	25	23
					(10)	(41)	(48)	(25)	(61)	(45)[d]	(49)
Ebs	0.86	2h	5	51[e]	2	8	4	2	27	33	23
					(8)	(31)	(41)	(21)	(36)	(43)[d]	(39)
USP	0.08	2h	3	44[e]	2	8	6	4	15	44	21
					(4)	(15)	(30)	(22)	(10)	(30)[d]	(18)

[a] g protein of enzyme liquid/Kg dry potatoes

[b] Values in brackets indicate g of CWP residue recovered per 100 g of residue in potatoes (dry matter), Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; UA, uronic acid

[c] Non-starch Glc

[d] Yield of CWPs calculated after correction of starch and free Glc (Grommers and Krogt, 2009)

Similarly, at an increased pH (5.9), for both USP and Ebs, the recovery of Gal in "fibre" from potatoes was much lower than the recoveries of Xyl and CWP Glc (Table 3, chapter 4). The recovery of UA was closer to the recovery of Gal, which could be explained by the comparable degradation profiles of AG and PGA by the enzymes at pH 5.9 (Figure 3). The comparable degradation profiles of the enzymes on AG and XG (Figure 3) and the lower decrease of Xyl than Gal (Table 3) indicated that more AGs were degraded than XG when these CWPs were present in the cell wall. The higher degradation of AG than XG could be either due to lack of accessibility of XG for enzymes or due to the inability of the enzymes to degrade insoluble XG, which needs to be investigated.

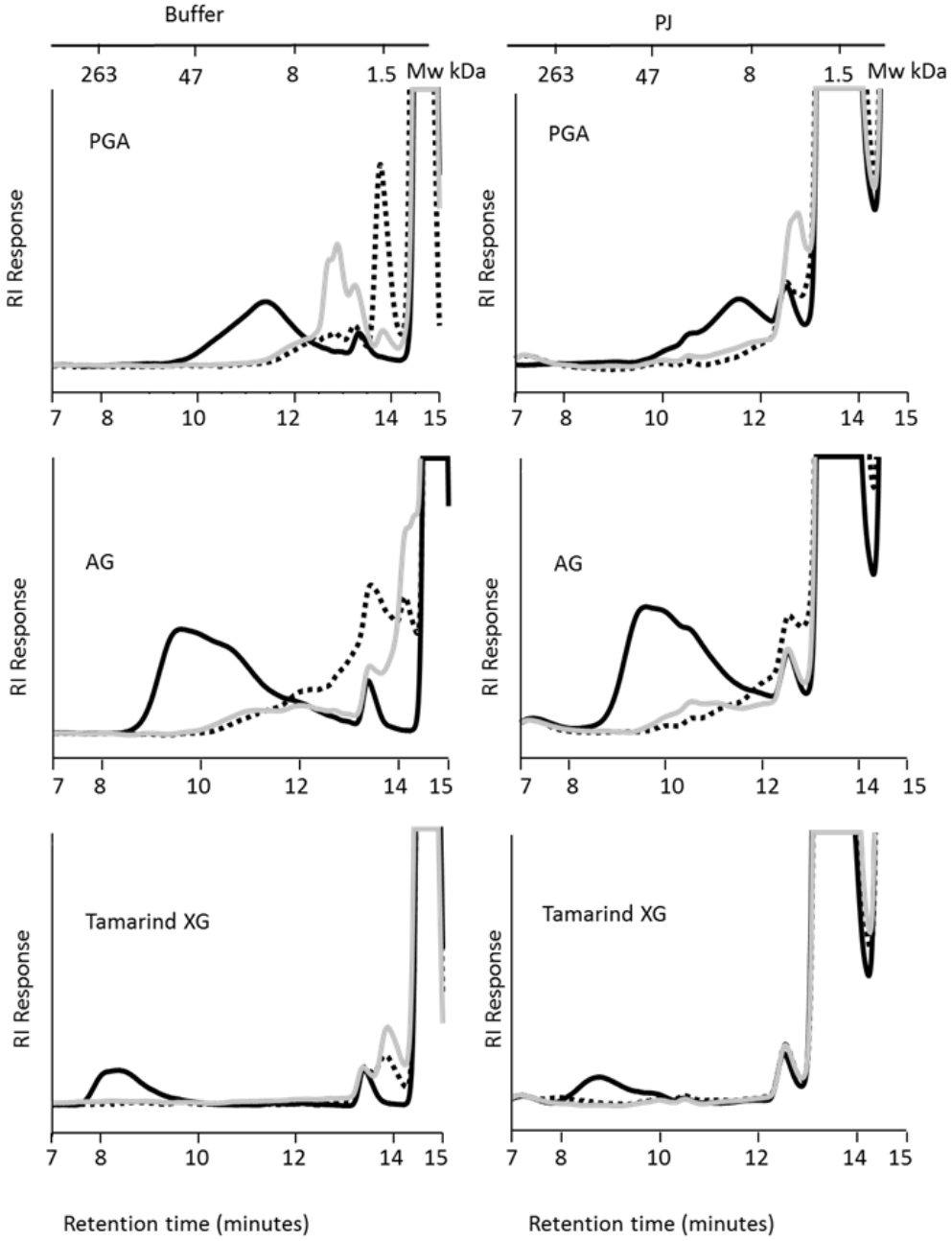

Figure 3: HPSEC profiles of defined substrates before (▬), and after hydrolysis with Ebs (▬) and USP (▪▪▪▪). PGA: Polygalacturonic acid; AG: Arabinogalactan; XG: Xyloglucan; PJ: Potato juice.

In the event that XG was less accessible to enzymes, then the high content of AG present (19 mol% in potatoes and between 27 to 32 mol% in PPF/fibres; Table 1) could play a role in prohibiting accessibility for enzymes to degrade XGs. This is based on the fact that the abundant and porous AG network in potato cell walls (Renard and Jarvis, 1999) could control enzyme diffusion (Leboeuf et al., 2004). Secondly, the fact that the XGs are hydrogen bonded to cellulose (McCann and Roberts, 1991) and confined as separate entities in the cell wall (Moore and Staehelin, 1988) could also contribute to the possibly of limited accessibility of glucanases to XG. However, these explanations are speculative and should be investigated in detail.

6.4 Water Holding Capacity (WHC)

6.4.1 Ensiling of PPF

In comparison to the degradation of CWPs by introducing enzymes, as observed in chapters 3 and 4, and mechanical methods, ensilage is another method to lower the WHC of fibre-rich materials (Okine et al., 2007). This also accounted for PPF. The WHC of ensiled PPF was 60% of that of PPF (Figure 4). Ensilage also facilitated the action of Ebs as the WHC of Ebs treated ensiled PPF was lower than that of Ebs treated PPF (Figure 4).

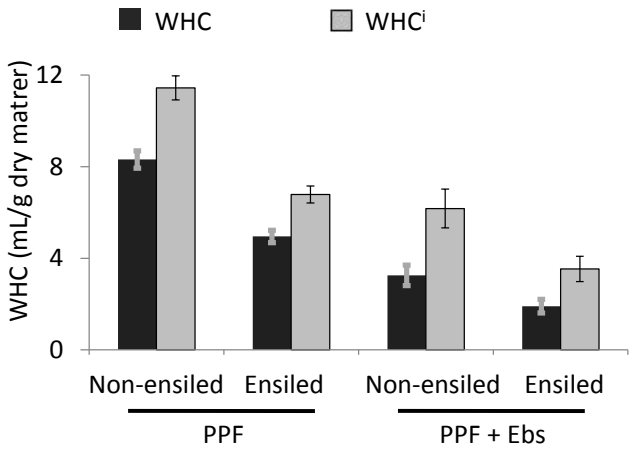

Figure 4: Water holding capacities of PPF and ensiled PPF and after treatment with the Ebs enzyme

Furthermore, WHC^{i} representing the amount of water held only by the insoluble residue, also reduced significantly (41%) upon ensiling (Figure 4). After treatment of ensiled PPF with Ebs, the WHC^{i} was also lower (3.5±0.6) than that of Ebs treated non-ensiled PPF. Ensilage of potato pulp has been shown to degrade non-starch carbohydrates and starch (Okine et al., 2007). The degradation of CWPs was supported by the HPSEC profiles (Figure 5) of the soluble matter from PPF and ensiled PPF, where in the latter mainly soluble matter with lower molecular weight (Mw) was observed.

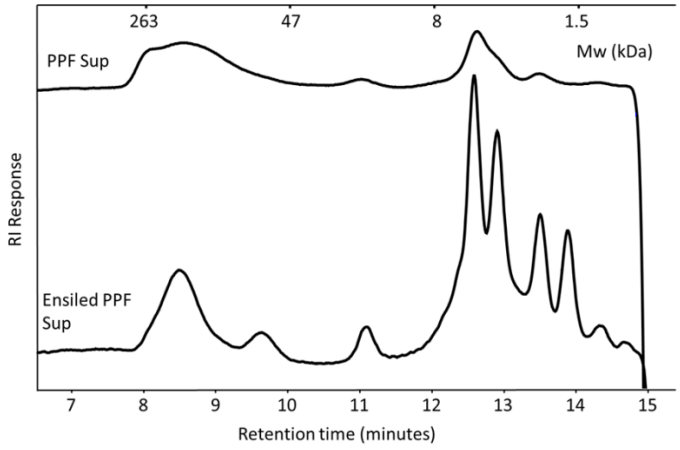

Figure 5: HPSEC profiles of supernatants (Sup): PPF Sup and ensiled PPF Sup (Take note that the PPF Sup is diluted twice more than Ensiled PPF Sup).

The carbohydrate compositions of PPF, ensiled PPF and their residues are shown in Table 4. Main differences were observed in Gal content, which was 21 mol% for PPF compared to 15 mol% for ensiled PPF (Table 3). Since data on the total amount of ensiled PPF recovered were not gathered, the yield of carbohydrates after ensiling from PPF (non-ensiled) could not be compared. Nevertheless, trends in the recovery of carbohydrates of PPF and ensiled PPF, before and after Ebs treatment, were obtained. The recovery of Gal and Ara in the residue from ensiled PPF (17% of Gal and 47% of Ara originally present in ensiled PPF) was lower than in the residue from PPF (39% of Gal and 59% of Ara originally present in PPF). This might point at the removal of arabinogalactan (AG) side chains from the residue upon ensiling. Also, the results might show negligible removal of XG or glucan

carbohydrates (cellulose, starch) compared to PPF. Therefore, from these results it seemed that ensiling of PPF caused degradation and solubilization of AG and HG and/or RG-I. A similar degradation of CWPs upon ensiling of chicory pulp has been reported to lower the WHC by 43% (Ramasamy et al., 2013) upon degradation of pectins (and side chains) and possibly of XGs, glucans and mannans. Clearly, ensiling was found to reduce the WHC of PPF, resulting from the degradation of AG and galacturonan rich carbohydrates (HG and RG-I).

Table 4: Carbohydrate composition of fractions of PPF and ensiled PPF

	Yield (w/w %) on dry matter		Carbohydrate composition (mol %); yield % carbohydrate residues [a,b]							
Sample	Dry matter	Carbohydrate	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
PPF	100	100	1	<1	5	2	1	21	55	13
			(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)
PPF Res	72	80	1	<1	4	3	2	10	73	7
			(47)	(84)	(59)	(102)	(120)	(39)	(106)	(43)
Ebs PPF Res	61	57	1	<1	4	2	1	3	77	12
			(29)	(93)	(36)	(34)	(37)	(9)	(79)	(53)
Ensiled PPF	100	100	1	<1	6	3	2	15	60	13
			(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)
Ensiled PPF Res	73	85	1	<1	3	3	2	3	82	6
			(42)	(113)	(47)	(94)	(89)	(17)	(117)	(38)
Ebs Ensiled PPF Res	50	54	1	<1	4	1	1	3	81	9
			(29)	(103)	(35)	(28)	(37)	(10)	(73)	(36)

[a] Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; UA, uronic acid

[b] Values in brackets indicates yields of carbohydrate residues

6.4.2 Effect of CWP modifications on the network

Non-enzyme treated insoluble residues, in general, showed to hold more water than soluble solids, due to the entrapment of water in pores as well as interactions of CWPs with water (chapters 2 and 3). Modification of CWPs is known to affect pore volumes and the packing of the particles. The reduced pore volumes and condensed packing of cavities leads to reduced WHC (Kneifel et al., 1991). Therefore, CWP content and composition directly or indirectly influenced the ability of the CWP network to hold water. Hitherto, the importance of xyloglucan (XG) and arabinogalactan (AG)(in the insoluble CWP network) on the WHC of PPF (chapter 2) was described in chapters 3 and 4. The enzymatic degradation of AG, hemicelluloses (XG), and possibly HG, decreased the WHC. However, it was not clear whether XG or AG contributed the most to the WHC.

Higher recoveries of AG and XG in USP3 “fibre” compared to Ebs Res (Table 5), caused the WHC also to drop to 16% for USP3 “fibre” versus 26% for Ebs Res. From these results, a reduction in the WHC by the substantial removal of AG, as well as that of XGs seems to be important.

Table 5: The recovery (%) of arabinogalactan, homogalacturonan and xyloglucan in residue (Res) and “fibre” after enzyme treatment, and their corresponding decrease in WHC (%) compared to PPF and non-enzyme treated “fibre”, respectively.

Sample treated	Treatment	Insoluble sample after treatment	% Recovery			WHC decrease (%)	WHC^R (%)
			AG[a]	**XG**[a]	**HG**[a]		
PPF (chapter 3)	Treatment with Ebs	Ebs Res[b]	9%	44%	56%	26%	48
Potato pulp (chapter 4)	Treatment with USP 1	"Fibre"[c]	71%	87%	78%	0%	88
Potato pulp (chapter 4)	Treatment with USP 2	"Fibre"[c]	41%	81%	59%	13%	60
Potato pulp (chapter 4)	Treatment with USP 3	"Fibre"[c]	17%	63%	38%	16%	40

[a] AG is based on Gal, XG is based on Xyl, and HG is based on UA

[b] is compared to PPF (chapter 3)

[c] is compared to "fibres" of the non-enzyme treated trial (chapter 4)

[R] is retained WHC (see chapter 4)

The degradation of XG is known to disrupt the framework that holds cellulose microfibrils, thereby shrinking the CWP network. Studies point at the role of AG in filling spaces and maintaining "pores" in the CWP network (Renard and Jarvis, 1999). This was suggested to be due to their mobility and interaction with water compared to the rigid xyloglucans and cellulose in hydrated cell walls (Ha et al., 2005; Fenwick et al., 1999; Larsen et al., 2011). This could explain that clearing the "pores" by degrading AG might be necessary to access XG (see 6.3.1).

In chapter 3 it was shown that RG-I linked AG adsorbed to cellulose and increased the WHC. So, the degradation of the linkage between RG-I and AG or truncation of the side chains is important to lower the WHC (chapter 3). Since truncating the side chains has been shown to reduce the hydrophilic nature of RG-I (Larsen et al., 2011), the removal of AG side chains might be sufficient to prevent adsorption to cellulose and to lower the WHC.

Although the recovery of HG in the residue and "fibre" fractions decreased upon enzyme treatment (Table 5), the contribution of polymeric HG to the WHC of PPF is unknown. Nevertheless, the contribution of HG is expected to be lower than that of AG since the drop in WHC was accompanied by a greater decrease in AG than HG (Table 5). The state of ionization of HG was indicated to have an effect on the WHC. Lowering the pH, probably due to de-esterification, lowered the WHC (chapter 3) due to the presence of HG in their non-dissociated forms. Also, ensiled PPF showed a lower pH (less than 3.5) compared to fresh PPF (pH 5.2), and consequently, partly due to this lower pH, a lower WHC (figure 4). The effect of enzymatic de-esterification of HG containing pectin and the degradation of HG on the WHC of the CWP network should be confirmed.

These findings, along with the results in chapters 3 and 4, indicate that the enzyme activities needed to lower the WHC of PPF, can be narrowed down. Based on the results in this thesis, only enzymes hydrolyzing AG and XG are essential. The assistance of cellulases and alpha-amylases (Olsen, 2000) can be eliminated.

6.5 Fate of CWP degradation by enzyme inhibitors in potato juice

The enzymatic degradation of soluble arabinan in potato juice (PJ) was inhibited by components that are water soluble, small molecules (<3 kDa), excluding glucose, saccharose, peptides and polyphenols (chapter 5). The degradation of high molecular weight arabinan was inhibited more for the Ebs than the USP enzyme in PJ (chapters 4 and 5). This observed inhibition was, most likely, also the reason why in chapter 4, a higher amount of Ara was recovered from potato in Ebs treated "fibre" (31%) compared to USP treated "fibre" (15%). In buffer both enzymes were equally active on arabinan at the doses applied (chapters 4 and 5).

Hydrolysis of other CWPs, like AG, XG and PGA, by Ebs and USP were not inhibited by PJ (Figure 3). But, at the same enzyme doses, the higher recovery of Gal residues from Ebs treated "fibre" (36%) compared to USP treated fibre (10%) at the pilot scale (chapter 4), pointed at the inhibition of AG hydrolysis. This inhibition can be the result of inhibited hydrolysis of the different side chains coupled to rhamnogalacturonan-I (RG-I; see chapter 1; Figure 6).

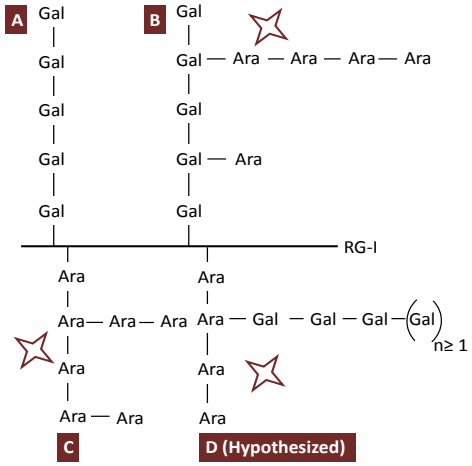

Figure 6: Simplified depiction of potato RG-I and its side chains: The points of inhibition of arabinan degradation (marked with a star) according to possibilities of side chain structures, A-D, attached to RG-I (adapted from Obro et al., 2004).

Short oligosaccharides of Ara (≤DP4) on AG side chains (structure B) will hinder the activity of endo-galactanase (Christgau et al., 1995). Likewise, in structure D (Figure 6), the substitution of galactan on arabinan could prevent endo-galactanases from hydrolyzing galactan near the sites of substitution. Therefore, inhibition of arabinan hydrolyzing enzymes leaves arabinan and AG side chains still attached to RG-I. Due to this, the hydrolysis of RG-I can be hindered, especially if the side chains wrap around the backbone (Willats et al., 2000).

It seems likely that the removal of UA residues by Ebs in potato pulp (containing PJ) was also inhibited (chapter 4), since the Ebs "fibres" showed less removal of UA residues than USP "fibres" (chapter 4) despite the fact that Ebs degraded PGA equally well as USP, both in buffer and in PJ (Figure 3). If the RG-I backbone is wrapped with side chains such that it also includes adjacent segments of homogalacturonan (HG), it is possible that the degradation of HG was also inhibited. Therefore, the inhibition of enzyme hydrolysis of other CWPs present, might be explained on the basis of inhibition of arabinan hydrolyzing enzymes.

6.6 Future perspectives

The effect of CWP degrading enzymes in improving starch recovery and lowering the WHC of the "fibres" in the pilot scale set-up (chapter 4) is also expected to occur in the industrial isolation of starch. The USP enzyme performed better than the Ebs enzyme in degrading CWPs, releasing starch and in maintaining the purity of starch (chapter 4). Further studies are needed to investigate the nature of inhibition of the enzymatic hydrolysis of arabinan (chapter 5), and the options to overcome this inhibition. Technically, Ebs seems to be a promising enzyme to lower the WHC of PPF. It showed the activities needed to degrade arabinogalactan and xyloglucan in helping to lower the WHC (chapters 3 and 4). The extent to which pectin de-esterification is needed to lower the pH needs to be investigated further to define the esterases (acetyl, methyl) needed to lower the WHC.

However, for application at the industrial scale, enzyme costs need to be reasonable in view of the benefit obtained. Even at lower protein content, USP lowers the WHC more than Ebs (chapter 4). Nevertheless, the liquefaction of PPF with Ebs was evaluated to be economically feasible from a sensitivity analysis (FBR, unpublished, 2011). In this study, the price of Ebs was assumed to be 5€/Kg, based on a range of costs determined for producing enzymes for second generation bioethanol (Hong et al., 2013). Treating 50,000 tonnes of PPF (as is) per year using 45 tonnes of Ebs (liquid weight) in a batch operation was found to cost 225,000€ per year. In that study, an internal rate of return of 16.2% showed that the enzyme costs were reasonable.

The liquefied PPF can either be directly applied as feed or can also be de-watered easily due to a quick phase separation after which the cellulose rich residue (chapter 3) can be used in several applications. The low WHC of the residue facilitates its drying so that it can be stored until it is transported to the site of application.

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Summary

SUMMARY Cell wall polysaccharides (CWPs) contribute to the water holding capacity (WHC) of fibres. However, the effect of individual CWPs and the CWP network in holding water in dicotyledonous fibres, such as pressed potato fibres (PPF), was unidentified so far. Next to this, it was not known if the solubilization of individual CWPs in potato pulp assists starch recovery from potatoes.

Chapter 1 provides information on the CWPs present in potato and PPF, highlighting the composition of CWPs and their abundance known so far. The information was mainly pertaining to potato CWPs, since detailed information on the CWPs in PPF is not known. Secondly, the concept of WHC is introduced and factors affecting the WHC of plant fibre-rich materials are discussed. Thirdly, potential enzymes capable of modifying CWPs in potato are reviewed. In PPF, the CWP network is abundant in cellulose and pectic polysaccharides, particularly arabinogalactan (AG). Therefore, lastly, the effect of pectinases (including galactanase) and glucanase rich enzymes in solubilizing PPF is reviewed. The effect of enzymatic degradation of CWPs on the WHC and recovery of starch for other materials is discussed as well.

Chapter 2 reveals the composition of CWPs and their structural features in PPF, next to obtaining a first impression of the WHC of PPF and its insoluble fractions. Via sequential extractions, it was found that half of all CWPs was recovered in the hot buffer soluble solids extract (HBSS). This suggested that the CWP network originally present in potatoes was loosened during the starch extraction process. The WHC of PPF was 7.4 mL/g of dry matter (mL/g). The WHC of the fractions obtained via sequential extractions were studied. Reconstituting HBSS (pectin-rich) with buffer insoluble solids (hemicellulose and cellulose rich) in water gave a WHC comparable to that of PPF. In addition, the removal of alkali soluble solids, which mainly comprised xyloglucans (XGs), lowered the WHC of the final residue. The findings in this chapter indicated that interactions between CWPs involving pectic CWPs and XGs could affect the WHC of PPF.

In **chapter 3**, the WHC of CWPs was probed further using an enzyme as a tool and recombining PPF fractions with soluble CWPs. It was found that the WHC of PPF was caused by a network of mainly insoluble, non-cellulosic CWPs. This was observed from the fact that although one-fourth of the CWPs were solubilized from PPF as pectic CWPs, the

insoluble residues still showed similar WHCs (mL/g dry matter) as PPF. The WHC of PPF decreased substantially (by 61%) only after the enzymatic hydrolysis and solubilization of non-cellulosic CWPs. Among these CWPs, the content of XG (40 - 70% w/w of the amount originally present) decreased, along with pectic CWPs (homogalacturonan-rhamnogalacturonan-I-arabinogalactan (HG-RG-I-AG)). Therefore, the results confirmed the importance of these non-cellulosic CWPs in immobilizing water in the CWP network in PPF. After enzyme addition, combining the cellulose-rich residue with a polymeric HG-RG-I-AG extract, increased the WHC, which was found to be caused by the adsorption of HG-RG-I-AG to the residue.

The effect of CWP degrading enzymes in improving starch recovery from potatoes and in simultaneously lowering the WHC of the constituent “fibres” at the pilot scale was studied in **chapter 4**. The use of a pectinase-rich preparation removed 58% of the starch present in the “fibre” fraction and removed 60% of the weight of water present in the “fibre” before treatment. The decrease in WHC was caused by the degradation of mainly pectic CWPs, in particular AG side chains. The effect of HG on the WHC needs to be proved.

The performance of CWP degrading enzymes in the presence of potato juice (PJ) was tested in **chapter 5** since PJ was expected to contain components that could inhibit CWP degrading enzymes. Opposed to the enzymatic hydrolysis of other CWPs, the hydrolysis of arabinan was inhibited by PJ. This was substantiated from the decreased release of the most abundant oligosaccharide, arabinotriose, from arabinan in the presence of 40 (v/v) % of PJ. Only about 11% of the arabinotriose was released compared to that in the absence of PJ. After 24 hours of hydrolysis of arabinan, a similar amount of arabinotriose released (≥ 85%) compared to that in the absence of PJ indicated that the hydrolysis was delayed. This inhibition was caused by low molecular mass components, smaller than 3 kDa, without affinity for a reversed phase (C18) column. Results indicated that these components did not include glucose or sucrose, peptides, chlorogenic acid, caffeic acid, coumaric acid, sulphonated ChA or oxidized phenolic acids. The components causing this inhibition could not be identified.

In **chapter 6**, the effect of the enzymatic degradation of the CWP network in PPF on the WHC was addressed. In addition, the inhibition of the degradation of AG side chains and RG-I via the inhibition of the enzymatic hydrolysis of arabinan observed by PJ (in chapter 4) was hypothesized. This hypothesis was used to explain the limited degradation of CWPs by Ebs compared to USP in potato pulp. The findings in the thesis pointed that the AGs were degraded to a larger extent than XGs. This increased degradation is proposed to result either due to increased accessibility of AGs or due to the insolubility of XG. Since XGs were found to be equally important in contributing to the WHC as AGs, the substantial removal of AGs and XGs could be conducive to lower the WHC. Therefore, enzymes hydrolysing AGs and XGs are hypothesized to be necessary to lower the WHC of PPF.

SAMENVATTING Celwand polysachariden (CWPs) dragen bij aan het water houdend vermogen (WHC) van vezels. Het effect van individuele CWPs en het CWP netwerk op het vasthouden van water in dicotyledon vezels, zoals geperste aardappelvezels (PPF), is nog niet bekend. Daarnaast is het niet bekend of een verhoogde oplosbaarheid van individuele CWPs in aardappel bijdraagt aan het winnen van zetmeel.

Hoofdstuk 1 geeft informatie over welke CWPs aanwezig zijn in aardappel en in PPF, met name over de tot nu toe bekende samenstelling van deze CWPs in aardappel. Als tweede wordt het concept WHC geïntroduceerd en de factoren die op het WHC van plantaardig vezel rijk materiaal van invloed zijn besproken. Ten derde worden potentiële enzymen die in staat zijn aardappel CWPs te modificeren behandeld. Het CWP netwerk in aardappel bestaat voornamelijk uit cellulose en pectine-achtige polysachariden, zoals arabinogalactaan (AG) -rijke rhamnogalacturonaan. Daarom wordt het effect van pectinases (inclusief galactanase) en glucanase-rijke enzymen op de oplosbaarheid van aardappelvezel besproken. Ook het effect van enzymatische afbraak van CWPs op het WHC en de zetmeel opbrengst uit andere gewassen dan aardappel wordt kort beschreven.

Hoofdstuk 2 beschrijft de samenstelling van CWPs en hun moleculaire structuren zoals aanwezig in PPF. Daarnaast wordt een eerste indruk van het WHC van PPF gegeven en van een aantal onoplosbare fracties verkregen uit PPF. Na zetmeelverwijdering werd de helft van alle CWPs in het warm water extract (HBSS) van PPF teruggevonden. Dit suggereert dat het rigide CWP netwerk dat van nature in aardappelen aanwezig is, minder rigide was geworden in het commerciële proces voor extractie van zetmeel. Het WHC van PPF was 7,4 mL/g droge stof. Wanneer de HBSS (pectine-rijke fractie) met de buffer-onoplosbare fractie (hemicellulose- en cellulose-rijke fractie) werd samengevoegd in water, gaf dit een WHC die vergelijkbaar is met die van PPF. Het verwijderen van de loog-oplosbare fractie, die vooral uit xyloglucanen (XG) bestaat, verlaagde het WHC van het uiteindelijke residu. De bevindingen in dit hoofdstuk wijzen erop dat interacties tussen CWPs, waarbij pectine-achtige CWPs en XGs betrokken zijn, een belangrijk effect hebben op het WHC van PPF.

In **hoofdstuk 3** werd het WHC van CWPs verder onderzocht. Het WHC van PPF bleek te worden veroorzaakt door een netwerk van met name onoplosbare, niet-cellulose CWPs. Ondanks dat een kwart van de CWPs uit PPF oplosbaar was, hadden de onoplosbare residuen namelijk vergelijkbare WHCs als PPF. Het WHC van PPF werd substantieel verlaagd (met 61%) na enzymatische hydrolyse door het in oplossing brengen van vooral andere CWPs dan cellulose. De hoeveelheid XG (40-70% w/w van de originele aanwezige hoeveelheid) die in oplossing kwam was gelijk aan de hoeveelheid pectine-achtige CWPs (homogalacturonaan-rhamnogalacturonaan-I-arabinogalactaan (HG-RG-I-AG)). De resultaten bevestigden dus het belang van deze CWPs voor het immobilizeren van water in het PPF-netwerk. Wanneer, na toevoeging van enzymen, het overgebleven cellulose-rijke residu werd gecombineerd met een HG-RG-I-AG-extract nam het WHC toe. Dit werd veroorzaakt door de adsorptie van HG-RG-I-AG aan het residu.

Het effect van CWP-afbrekende enzymen op de opbrengst van zetmeel uit aardappelen en tegelijkertijd op het verlagen van het WHC van de vezels werd bestudeerd in **hoofdstuk 4**. Door toevoeging van een pectinase-rijk enzymmengsel werd 58% van het zetmeel aanwezig in de vezel-fractie verwijderd en tegelijkertijd ook 60% van het water aanwezig in de vezelfractie, vergeleken met het proces zonder pectinase toevoeging. De verlaging van het WHC werd veroorzaakt door afbraak van voornamelijk pectine-achtige CWPs, met name AG zijketens. Het effect van HG op het WHC moet nog worden bevestigd.

De activiteit van CWP afbrekende enzymen in de aanwezigheid van aardappelsap werd onderzocht in **hoofdstuk 5**, omdat aardappelsap mogelijk componenten bevat die CWP-afbrekende enzymen hinderen. Tegengesteld aan de enzymatische hydrolyse van andere CWPs, werd de hydrolyse van arabinaan geremd door aardappelsap. In de aanwezigheid van 40% (v/v) aardappelsap werd minder arabinotriose de meest voorkomende oligosacharide, vrijgemaakt uit arabinaan vergeleken met in afwezigheid van aardappelsap. Slechts 11% van de arabinotriose werd vrijgemaakt in 4 uur, ten opzichte van de hoeveelheid die vrijgemaakt werd in afwezigheid van aardappelsap. Na 24 uur hydrolyse van arabinaan werd een vergelijkbare hoeveelheid arabinotriose vrijgemaakt (≥ 85%) als in afwezigheid van aardappelsap. De remming werd veroorzaakt door moleculen met een moleculaire massa kleiner dan 3 kDa, welke geen binding gaven

aan een reversed phase (C18) kolom. Deze componenten waren niet: glucose, sucrose, peptiden, chlorogeenzuur, caffeïnezuur, coumaarzuur, gesulfoneerd chlorogeen zuur of geoxideerde fenolische zuren. De remmende componenten konden niet worden geïdentificeerd.

Hoofdstuk 6 beschrijft het effect van enzymatische hydrolyse van het CWP-netwerk op het WHC. Daarnaast wordt beschreven dat mogelijk ook remming, in de aanwezigheid van aardappelsap, van enzymatische hydrolyse van AG zijketens en RG-I optreedt door remming van arabinaan hydrolyse (hoofdstuk 4). Deze hypothese werd gebruikt om de beperkte afbraak van CWPs door Ebs vergeleken met USP in aardappelpulp te verklaren. De bevindingen in deze thesis wijzen erop dat AGs verder werden afgebroken dan XGs. Dit verschil in afbraak wordt ofwel door de toegenomen toegankelijkheid van AGs veroorzaakt of door de onoplosbaarheid van de XGs. Aangezien de XGs even belangrijk gevonden werden in het bijdragen aan het WHC als AGs, zou het substantiëel verwijderen van AGs en XGs kunnen bijdragen aan het verlagen van het WHC. Daarom worden enzymen die AGs en XGs hydrolyseren van belang geacht om het WHC van PPF te verlagen.

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Urmila

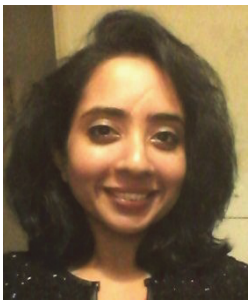
About the author

Curriculum vitae

List of publications

Completed training activities

Curriculum vitae



Urmila Ramasamy was born on December 2nd, 1984 in Bangalore, India. After graduating with a Bachelor's degree in fermentation technology from Garware College, University of Pune, India, she chose to pursue a Master's programme in food technology at Wageningen University, The Netherlands in September 2007. In February 2009, she completed her MSc. thesis titled "kinetic modelling of enzymatic starch hydrolysis" in the Food Process Engineering group. In September 2009, she graduated with an MSc degree in Food Technology after completing an internship as junior product developer in Friesland Campina, Wageningen, The Netherlands. After graduating, in November 2009 she began her PhD studies in "Pre-treatment of agricultural by-products" which was within the framework of the Carbohydrate Competence Centre and performed in the Laboratory of Food Chemistry, Wageningen University. The results of her studies are presented in this thesis.

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List of publications

Ramaswamy, U.R.; Kabel, M. A.; Schols, H.A; Gruppen, H., 2013. Structural features and water holding capacities of pressed potato fibre polysaccharides. Carbohydrate Polymers, 93(2), 589-596.

Ramasamy, U.R.; Gruppen, H; Kabel, M.A; 2014. Water holding capacity of soluble and insoluble polysaccharides in pressed potato fibres. *To be submitted*.

Ramasamy, U.R.; Steef Lips; Robert Bakker; Kabel, M.A; Gruppen, H., 2014. Imrpoved recovery of starch assisted by enzymes and reduced water holding of residual fibre on a pilot scale. *Submitted* for publication to Carbohydrate Polymers.

Ramasamy, U.R.; Gruppen, H; Kabel, M.A; 2014. Inhibition features of enzymatic hydrolysis of arabinan in potato juice. *To be submitted*.

Overview of completed training activities

Discipline specific activities

Courses

- Summerschool glycosciences (VLAG), Wageningen, The Netherlands, 2010 ✓
- Advanced food analysis (VLAG), Wageningen, The Netherlands, 2010 ✓
- Chromeleon user meeting DIONEX, Wageningen, The Netherlands, 2011
- Food and biorefinery enzymology (VLAG), Wageningen, The Netherlands, 2011 ✓

Conferences and meetings

- CCC scientific days, Groningen, The Netherlands, 2010 – 2013 ✓, +
- Plant cell wall meeting, Porto, Portugal, 2010 ✓
- Workshop Cosun, Rosendaal, The Netherlands, 2010 +
- Plant and seaweed polysaccharides symposium, Nantes, France, 2012 ✓
- European polysaccharide network of excellence (EPNOE) conference, Wageningen, The Netherlands, 2011 ✓

General Courses

- PhD week (VLAG), 2010.
- Techniques for writing and presenting a scientific paper (WGS), 2010
- Information literacy and introduction to endnote (WU), 2009
- Interpersonal communication to PhD students (WGS), 2013
- Career Perspectives (VLAG), 2013
- Mobilizing your scientific network (WGS), 2013

Optionals

- Preparation of PhD research proposal
- PhD trip to Switzerland and Italy, WU (FCH), 2010 ✓, +
- PhD trip to Singapore and Malaysia, WU (FCH), 2012 ✓, +
- PhD presentations, WU (FCH), 2009 - 2013
- BSc/MSc thesis student presentations and colloquia, WU(FCH), 2009 - 2013
- Project meetings - consortium, 2009 - 2014

✓ *poster presentation,* + *oral presentation*

Abbreviations used:

WU: Wageningen university; **VLAG**: Graduate school for nutrition, food technology, agrobiotechnology and health science; **WGS**: Wageningen graduate school; **FCH**: Laboratory of food chemistry; **CCC**: Carbohydrate competence centre.

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