High-throughput microarray mapping of cell wall polymers in roots and tubers during the viscosity-reducing process

Yuhong Huang1,2,3
William G. Willats4
Lene Lange3
Yanling Jin1
Yang Fang1
Armando A. Salmeán4
Henriette L. Pedersen4
Peter Kamp Busk4
Hai Zhao1

1Environmental Microbiology Key Laboratory of Sichuan Province, Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, Sichuan, People’s Republic of China
2University of the Chinese Academy of Sciences, Beijing, People’s Republic of China
3Section for Sustainable Biotechnology, Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Copenhagen SV, Denmark
4Department of Plant Biology and Environmental Sciences, Faculty of Life Sciences, University of Copenhagen, Frederiksberg C, Denmark

Abstract

Viscosity reduction has a great impact on the efficiency of ethanol production when using roots and tubers as feedstock. Plant cell wall–degrading enzymes have been successfully applied to overcome the challenges posed by high viscosity. However, the changes in cell wall polymers during the viscosity-reducing process are poorly characterized. Comprehensive microarray polymer profiling, which is a high-throughput microarray, was used for the first time to map changes in the cell wall polymers of sweet potato (Ipomoea batatas), cassava (Manihot esculenta), and Canna edulis Ker. over the entire viscosity-reducing process. The results indicated that the composition of cell wall polymers among these three roots and tubers was markedly different. The gel-like matrix and glycoprotein network in the C. edulis Ker. cell wall caused difficulty in viscosity reduction. The obvious viscosity reduction of the sweet potato and the cassava was attributed to the degradation of homogalacturonan and the released 1,4-β-D-galactan and 1,5-α-L-arabinan. © 2015 International Union of Biochemistry and Molecular Biology, Inc. Volume 63, Number 2, Pages 178–189, 2016

Keywords: viscosity, roots and tubers, plant cell wall–degrading enzyme, comprehensive microarray polymer profiling, cell wall polymer

Abbreviations: CBMs, carbohydrate-binding modules; CDTA, diaminocyclohexane-tetraacetic acid; CoMPP, comprehensive microarray polymer profiling; mAbs, monoclonal antibodies; PCWDE, plant cell wall–degrading enzyme; VRR, viscosity-reducing rate.

*Address for correspondence: Professor Hai Zhao, Environmental Microbiology Key Laboratory of Sichuan Province, Chengdu Institute of Biology, Chinese Academy of Sciences, No. 9 Section 4, Renmin Nan Road, Chengdu, 610041 Sichuan, People’s Republic of China. Tel.: +86 28 82890725; Fax: +86 28 82890733; e-mail: zhaohai@cib.ac.cn.

Yuhong Huang and William G. Willats have contributed equally to this work.

Received 23 July 2014; accepted 5 March 2015
DOI: 10.1002/bab.1367
Published online 18 April 2016 in Wiley Online Library (wileyonlinelibrary.com)

1. Introduction

Roots and tubers, such as sweet potato (Ipomoea batatas), cassava (Manihot esculenta), and Canna edulis Ker., are preferentially used as nongrain-based feedstock for bioethanol production. Such roots and tubers are characterized by their high concentration of fermentable sugars, rich total energy resources, and wide availability worldwide, especially in China. China produces 117 million tons of sweet potatoes annually, which accounts for approximately 90% of the global production [1–4]. C. edulis Ker. is abundantly available in southwest China, such as the Guizhou province can harvest approximately 1 million tons of variable C. edulis Ker. tubers annually [5]. Cassava tubers are mainly harvested in southern China with approximately 6 million tons produced each year [6]. The use of these roots and tubers as food and feed has decreased over
time. Instead, their use as industrial materials is increasing significantly as the economy in China becomes more developed. Recently, the China National Petroleum Corporation planned to sign an agreement with the government to develop facilities to produce 600,000 tons of ethanol from sweet potatoes every year [2]. Therefore, roots and tubers have already attracted extensive research attention and have been used for ethanol fermentation [7–9]. However, the mash of these roots and tubers is a typical non-Newtonian fluid with high viscosity, which is difficult to transport through pipes. In addition, the high viscosity could also affect yeast growth and metabolism, ultimately decreasing the ethanol yield and fermentation efficiency and prolonging the fermentation time.

The components of these roots and tubers are complex; they contain storage polysaccharides (starch) and structural polysaccharides, such as dynamic and complex plant cell wall polymers. The plant cell wall polymers include cellulose, pectin, and hemicelluloses, which, together with the phenolic polymer lignin and proteins, form a complex and rigid structure [10]. Cellulose is a β-(1,4)-d-linked glucose molecule that is a highly crystalline and linear insoluble branched polymer [11]. All hemicelluloses have β-(1,4)-linked backbones with an equatorial configuration at C1 and C4, which are mainly composed of xylglucans, xylans, mannans, glucomannans, galactoglucomannans, arabinoxylans, and other heteropolysaccharides [12]. The variety of branched sugar polymers with acetyl groups results in a highly heterogeneous population of polysaccharides in hemicelluloses. Pectin is mainly formed by homagalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II polymers. Homagalacturonan is the polygalacturonic acid background, and rhamnogalacturonan II is the most complex structure with different sugars and linkages [13]. Among these polymers, hemicelluloses bind to the surface of crystalline cellulose and pectin embeds in the cellulose–hemicelluloses network, which forms a hydrated gel phase [11]. These complex and diverse cell wall polymers in roots and tubers were found to affect the rheological properties of the mash. The high viscosity problem has been overcome by using plant cell wall-degrading enzymes (PCWDEs), which provide great advantages for ethanol production using roots and tubers as feedstock. Recently, ethanol production from sweet potato feedstock has been scaled up to industrial levels with xylanase and cellulase treatment [14, 15]. Cassava [16] and C. edulis Ker. mash [5] also exhibited a significantly decreased viscosity and increased ethanol fermentation efficiency after the mash was treated with PCWDEs. Huang et al. [17] evaluated the physical and chemical changes of C. edulis Ker. mash by using high-performance liquid chromatography (HPLC), scanning electron microscopy, atomic force microscopy, and confocal laser scanning microscopy when the mash treated with acid xylanase and β-glucanase. Despite the fact that PCWDEs have the ability to reduce mash viscosity, and even though the physical and chemical properties of the mash have been investigated, analytical methods such as HPLC, liquid chromatography–mass spectroscopy, and nuclear magnetic resonance have only been used to measure the soluble sugars [18]. However, the soluble sugar of the roots and tubers does not indicate actual information about the structures of plant cell wall polymers. For example, the measured glucose by HPLC or ion chromatography cannot be assumed to only come from the cellulose because it may also come from hemicellulose. Therefore, we still do not understand the extent to which plant cell wall polysaccharides affect the viscosity of the mash. In addition, changes in the structural features of the cell walls that contribute to viscosity reduction are not fully clarified.

A high-throughput comprehensive microarray polymer profiling (CoMPP) technique was recently established [19–22]. This method includes a sequential extraction of the major classes of cell wall polysaccharides (diaminocyclohexane-tetraacetic acid [CDTA], NaOH, and cadoxen are used to solubilize pectin, noncellulosic polysaccharides, and cellulose, respectively) [21]. This approach can provide a systematic, semiquantitative, and high-throughput mapping of cell wall polysaccharides. A large repertoire of monoclonal antibodies (mAbs) and carbohydrate-binding modules (CBMs) are currently available to specifically identify defined glycan structures (epitopes) with high specificities in the plant cell wall mAb database (http://glycomics.crcr.uga.edu/wall2/antibodies/antibodyHome.html). This technique is designed to provide more information about cell wall polymer composition [23, 24], characterize cell wall specificities (e.g., when plants grow under different conditions), assess the occurrence of cell wall polymers after different pretreatments [18, 25], and track enzymatic decomposition of harvested leaves in fungus gardens grown by leaf-cutting ants [22].

In the present study, the CoMPP technique was applied to track the detailed changes of cell wall polymers in roots and tubers mash over the entire viscosity-reducing process. This is the first use of the CoMPP technique to investigate the viscosity reduction mechanism.

2. Materials and Methods
2.1. Materials
Sweet potato (I. batatas in the Convolvulaceae family) (varieties Shangshu19 and Nanshu007) and cassava (M. esculenta in the Euphorbiaceae family) harvested in November 2010 were kindly provided by the Sichuan Academy of Agriculture Sciences of China. They were stored at room temperature (at approximately 25 •C for 30 days).

C. edulis Ker. (in the Cannaceae family) was harvested in December 2010 and purchased in the Jinyan of Sichuan province, People’s Republic of China. The tubers were stored under ventilated conditions at room temperature (at approximately 25 •C for 30 days).

2.2. Enzymes
The selected liquefaction enzyme (Liquozyme Supra, Novozymes, People’s Republic of China) was a thermostable
α-amylase (EC 3.2.1.1) from *Bacillus licheniformis* with a declared activity of 90 KNU/g. The glucoamylase (EC 3.2.1.3) (Suhong GAI, Novozymes, People’s Republic of China) from *Aspergillus niger* had a declared activity of 500 AGU/g (an Amyloglucosidase Novo Unit as defined by Novo Industry). One KNU (a Novo α-amylase Unit as defined by Novo Industry) is defined as the amount of enzyme that hydrolyzes 5.26 g of soluble starch per hour at pH 5.6 and 37 °C. One AGU (an Amyloglucosidase Novo Unit as defined by Novo Industry) is the amount of enzyme that cleaves 1 μmol of maltose per minute at pH 4.3 and 25 °C. The xylanase (Shearzyme 500 L) was purchased from Genencor Danisco (Rochester, NY, USA), and it had a declared activity of 4,200 U/g. The GC220 was purchased from Genencor Danisco (Rochester, NY, USA), and it had a declared activity of 552.46 U/g. The cellulase was purchased from Sichuan Habio Bioengineering Co., and it had a declared activity of 524 U/g. The glucanase came from Imperial Jade Bio-technology Co., and it had a declared activity of 1,000,000 U/g.

### 2.3. Viscosity-reducing procedure

Fresh roots and tubers were washed, cut into cubes of approximately 3 cm³, and milled in a Philips Juicer HR2826 (Royal Philips Electronics Co., Amsterdam, Netherlands). Water was added according to the ratio of mash to water (3:1). The mash was initially treated by adding thermostable α-amylase (0.12 KNU/g of root and tuber mash), and then incubating at 85 °C for approximately 10–20 Min until a sorrel color response was obtained with iodine solution. The liquefied mash was then cooled to room temperature, and water was added to replace the evaporated water during the liquefaction. The mash was homogenized well, and aliquots of 100 g per 250-mL Erlenmeyer flask were sterilized at 110 °C for 20 Min. For viscosity reduction, the mash was cooled to room temperature, and then PCWDEs were added and incubated at 50 °C, 0.58 g for 2 h. The doses of xylanase, cellulase, GC220, and glucanase were 100 μL/100 g, 0.1 g/100 g, 100 μL/100 g, and 100 μL/100 g, respectively. Samples were collected at every step (milled, liquefied, 110 °C treatment for 20 Min, and viscosity reduction) for the rheological property assay and the CoMPP assay.

### 2.4. Analytical methods

**Rheological property assay**

The viscosity of the mash was evaluated by DIN spindles with a digital viscometer (DV-II+ PRO; Brookfield, Middleboro, MA, USA), which was equipped with a recirculating water bath (TC 200; Brookfield) and ULA-DIN-85 spindles. Viscosity changes were determined at 30 °C at a paddle speed of 0.15 g.

**CoMPP**

An overview of the key stages from the CoMPP collection summarized in Figs. 1A and 1C–H). After sample collection and homogenization (A, C). 1.5 g (fresh weight) of cell wall components from each stage was sequentially extracted by using two solvents, namely 50 mM CDTA and 4 M NaOH with 1% v/v NaBH₄ (D). First, 9 mL 50 mM CDTA was added to each sample and incubated with shaking for 2 h at room temperature. After centrifuging for 10 Min at 4,000g, the supernatants (CDTA extracts) were collected. The remaining pellets were resuspended in 9 mL 4 M NaOH with 1% v/v NaBH₄ and also incubated with shaking for 2 h at room temperature. After centrifuging for 10 Min at 4,000g, the supernatants (NaOH extracts) were collected. The collected supernatants (supts.) were stored at −20 °C until printing. Extracts were then printed in triplets onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) (Fig. 1F). The extracts were diluted to three concentrations (undiluted, diluted fivefold and 25-fold) in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.7 mM NaH₂PO₄, pH 7.5) and three printing replicates. Then, every sample was represented by nine spots. Printing was carried out using split pins (MicroSpot 2500, Genomic Solutions, Ann Arbor, MI, USA) equipped in a microarray robot (Microgrid II; Genomic Solutions). Pins were washed twice in dH₂O after deposition of each sample. All samples were printed on the same sheet of nitrocellulose membrane as arrays (Fig. 1F).

Arrays were blocked by incubation for 1 h in PBS containing 5% (w/v) fat-free milk powder. After blocking, arrays were incubated in primary mAbs or CBMs for 1 h. The mAbs and CBMs used in this study were listed in Table 1. Then, the arrays were washed twice with PBS and once with PBS for 10 Min. After washing, arrays were incubated with secondary antibodies conjugated to alkaline phosphatase for 1 h followed by washing twice with PBS, once with PBS for 10 Min and then with PBS and dH₂O for 1 Min. Then, the arrays were developed in 5-bromo-4-chloro-3-indolyphosphate/nitro-blue tetrazolium chloride substrate. The arrays were then washed with dH₂O to stop the development (Fig. 1G). The dried arrays were scanned and the spot signals were quantified and analyzed by Imagen 6.0 microarray analysis software (BioDiscovery, Hawthorne, CA, USA; http://www.biodiscovery.com) as described by Moller et al. [20] (Fig. 1H). For each data set, the maximal mean spot signal was set to 100% and all other values within that data set were adjusted accordingly; the cutoff value was set to 5%. Then, the sets were generated into heatmap using online heatmapper software (http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools/heatmapper_plus.cgi).

### 2.5. Calculation

Viscosity-reducing rate (VRR) = (the viscosity of the control mash – the viscosity of the mash treated with cell wall polysaccharide-degrading enzymes)/the viscosity of control mash × 100%.

### 3. Results and Discussion

#### 3.1. The rheological properties of roots and tubers during the entire viscosity-reducing process

Many nongrain-based feedstocks such as sweet potato and cassava were identified as biofuel crops by the Chinese...
FIG. 1

Overall strategy for analysis of the viscosity reduction. Rheological properties assay (A→B) and comprehensive microarray polymer profiling (CoMPP) (A, C→H). (A) Samples were collected at various stages of the processing. The rheological properties assay was followed by a viscosity evaluation using a digital viscometer (B). The CoMPP technique was followed by homogenizing the collected samples (C). Cell wall components were sequentially extracted with CDTA and NaOH (D). The supernatants (supts.) were pooling, diluted, and printed from the extraction onto the microarray. (E and F) The microarray was probed with monoclonal antibodies (mAbs) or with carbohydrate-binding modules (CBMs) (G). Finally, spot signals were qualified and analyzed (H).

The traditional ethanol production process that employs roots and tubers as feedstock typically involves milling, application of α-amylase to convert starch into glucose and maltose (liquefaction), autoclaving, and simultaneous saccharification and fermentation. Recently, viscosity reduction by PCWDE treatment, which is performed between autoclaving and ethanol fermentation or together with ethanol fermentation, has shown a great advantage for improving the fermentation efficiency and ethanol yield. Therefore, we mainly focused on the viscosity-reducing process, including milling, liquefaction, 110 °C for 20 Min, and viscosity reduction by adding different PCWDEs for further research.

The rheological properties during the entire process for each tuber are shown in Table 2. The viscosities of the sweet potato, cassava, and C. edulis Ker. mashes clearly increased during the liquefaction process. The hydration and radial swelling of starch during the liquefaction process might cause this high viscosity. After the following high-temperature treatment (110 °C for 20 Min), the viscosities of the sweet potato and C. edulis Ker. mashes decreased; however, it was still difficult to transport the mashes by pumping through pipes (Fig. 2). This difficulty in turn affects the mass and heat transfer and influences the growth and metabolism of yeast, which ultimately results in decreasing ethanol production. PCWDEs were added after the high-temperature process and incubated at 50 °C, 0.58 g for 2 H. Interestingly, the changes in viscosity of these three roots and tubers mashes varied. Here, xylanase, cellulase, GC220, and glucanase were chosen because government [52]. C. edulis Ker. is also a potential feedstock for ethanol production [5]. All of these roots and tubers can be cultivated on marginal lands, have low nutrient demand, produce high content of fermentable carbohydrate, and provide high ethanol yield. Therefore, in this study, we chose these three roots and tubers to further investigate the relationship of cell wall polysaccharides and viscosity reduction. Two varieties of sweet potato, Shangshu 19 and Nanshu 007, were included because the viscosity of Shangshu 19 mash was much easier to be reduced when compared with the viscosity of Nanshu 007 mash. Therefore, it was very interesting to elucidate the reason why the same PCWDE had different effects on the two sweet potato varieties.
of their differing abilities to reduce mash viscosity according to previous work [14]. The results indicated that cellulase, GC220, and glucanase have great viscosity-reducing capabilities (the VRR ranges from 86% to 95%) for Shangshu 19 and cassava (Table 2). Although Zhang et al. [9] reported that xylanase has a substantial viscosity-reducing capability, no significant viscosity reduction for xylanase was observed in the current investigation; by contrast, the viscosity of the Nanshu 007 mash increased. This may be due to the fact that the xylanases from different companies are composed of different enzyme composition blends. In addition, the structures of plant cell wall polysaccharides are highly complex, varying from one species to another, between varieties, from tissue to tissue, and from cell to cell within each plant [53]. The two varieties of sweet potatoes that were studied had different polysaccharides and starch characteristics. Furthermore, pasting properties of native starches also contributed to the viscosity differences. Sweet potato and cassava are dicotyledons, which have predominant xyloglucan polysaccharides, whereas glucuronarabinoxylans are abundant in monocotyledons such as *C. edulis* Ker. [54]. All of these variations may result in the different rheological properties for the processed root and tuber mashes.

### 3.2. A detailed mapping of cell wall polymers in roots and tubers during the viscosity-reducing process

According to the rheological properties described in Section 3.1, we found three interesting phenomena: (1) the viscosity was different even though the same enzyme was used to treat one type of tuber (cellulase/GC220/glucanase-treated sweet potato Shangshu 19 and Nanshu 007), (2) different types of roots and tubers (sweet potato, cassava, and *C. edulis* Ker.) exhibited various viscosities during the entire process, and (3) Shangshu19 and cassava mashes presented clear changes when treated with enzymes. Thus, additional studies should be undertaken that focus on these three interesting phenomena. The CoMPP technique can be used to characterize cell wall polymer changes in a high-throughput setup. Here, CoMPP was used to analyze the samples collected over the entire viscosity-reducing process as described in Fig. 1. Twenty-six types of mAbs and CBMs (Table 1), which have specificity on extensin, pectin, hemicellulose, cellulose, and others, were used in this study. The heatmap of Shangshu 19, Nanshu 007, cassava, and *C. edulis* Ker., exhibiting the mean CoMPP signals obtained from 15 types of mAbs and CBMs, is shown in Fig. 3.

#### Changes in the cell wall polymers of one type tuber

Raw (milled) sweet potato (Shangshu 19 and Nanshu 007) had high homogalacturonan signals for both LM18 and LM19 and low methyl-esterified homogalacturonan, 1,4-β-D-galactan, and 1,5-α-L-arabinan signals for JIM5, LM5, and LM6, respectively (Fig. 3). These results indicate that the epitopes of the cell wall polymers of the two original sweet potatoes are very similar. However, the extracted samples from Shangshu 19 and Nanshu 007 behaved differently after liquefaction. Shangshu 19 was characterized by much higher JIM5, JIM7, LM18,
TABLE 2

| Treatment       | Viscosity (mPa·s) | VRR (%) | Viscosity (mPa·s) | VRR (%) | Viscosity (mPa·s) | VRR (%) | Viscosity (mPa·s) | VRR (%) |
|-----------------|-------------------|---------|-------------------|---------|-------------------|---------|-------------------|---------|
| Milled          | 4,138             | ——      | 19,103            | ——      | 9,566             | ——      | 12,016            | ——      |
| Liquefied       | 23,938            | ——      | 27,936            | ——      | 11,654            | ——      | 25,497            | ——      |
| 110 °C, 20 Min  | 17,992            | ——      | 16,354            | ——      | 17,263            | ——      | 13,032            | ——      |
| Xylanaseb       | 10,245 ± 24       | —4 ± 0  | 22,976 ± 16       | —46 ± 0 | 15,565 ± 115      | 17 ± 1  | 15,516 ± 365      | 7 ± 2   |
| Cellulaseb      | 1,409 ± 141       | 86 ± 1  | 7,600 ± 159       | 52 ± 1  | 859 ± 62          | 95 ± 0  | 6,744 ± 220       | 60 ± 1  |
| GC220b          | 917 ± 102         | 91 ± 1  | 10,585 ± 564      | 33 ± 4  | 876 ± 62          | 95 ± 0  | 7,353 ± 260       | 56 ± 2  |
| Glucanaseb      | 892 ± 49          | 91 ± 0  | 3,234 ± 112       | 79 ± 1  | 863 ± 5           | 95 ± 0  | 10,590 ± 487      | 37 ± 3  |

The viscosities of roots and tubers over the entire viscosity-reducing process as follows: milling, liquefaction, 110 °C, 20 Min treatment, and viscosity reduction through adding enzymes (xylanase, cellulase, GC220, and glucanase) and incubating at 50 °C for 2 H at a paddle speed of 0.58 g. Sweet potatoes (Shangshu 19 and Nanshu 007), cassava, and Canna edulis Ker. were used as feedstock.

JIM7, and LM20 reportedly recognized the homogalacturonan backbone domains with differing degrees and patterns of methyl esterification. LM18 and LM19 preferentially bind to deesterified homogalacturonan, and LM18 also shows some binding to a trigalacturonide. Thus, these results indicate that more methyl-esterified homogalacturonans in Shangshu 19 mash were exposed or released when compared with Nanshu 007 after the mash was treated with thermostable α-amylase at 85 °C. It was reported that homogalacturonan can influence cell wall properties, including cell expansion, cell development, intercellular adhesion, and defense mechanisms in the primary cell wall matrix [37]. The deesterified homogalacturonan can be cross-linked by calcium, resulting in gel formation and intercellular adhesion maintenance [55, 56]. A highly
Comprehensive microarray polymer profiling (CoMPP) of Shangshu 19, cassava, and Canna edulis Ker. during the entire viscosity-reducing process. The heatmap represents the total intensity for CDTA and NaOH extractions. The numbers in the heatmap matrix indicate the signal intensity (0 → 150, signal from low (black color) to high (green color)). Twenty-six types of mAbs and CBMs (Table 1) were applied to map the cell wall polymers of roots and tubers, and 15 types of mAbs and CBMs have signals according to this heatmap. ME: methyl-esterified.

| Sample          | LM 1: extensin | LM 3: extensin | JIM 5: Homogalacturonan (partially ME) | JIM 7: Homogalacturonan (partially ME) | LM 18: Homogalacturonan | LM 19: Homogalacturonan | LM 20: Homogalacturonan (ME) | LM 5: 1,5-β-D-galactan | LM 6: 1,5-α-L-arabinan | LM 11: Xylan/Arabinosylan | LM 12: Furfurylated arabinan | LM 13: 1,5-α-L-arabinan | LM 15: Xyloglucan | MAC 207: Arabinogalactan Protein | CBM 3a: Crystalline cellulose |
|-----------------|----------------|---------------|---------------------------------------|----------------------------------------|-------------------------|-------------------------|-------------------------------|---------------------------|--------------------------|-------------------------------|--------------------------------|------------------------|------------------------|--------------------------------|---------------------------------|
| Shangshu19      |                | 0             | 13                                    | 0                                      | 65                      | 60                      | 0                             | 10                        | 18                       | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | liquefied     | 0             | 84                                     | 89                                     | 69                      | 49                      | 99                            | 19                        | 24                       | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | 110 °C, 20 Min| 5             | 67                                     | 69                                     | 55                      | 46                      | 71                            | 68                        | 68                       | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | xylanase      | 0             | 68                                     | 59                                     | 52                      | 42                      | 64                            | 49                        | 55                       | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | cellulase     | 7             | 77                                     | 76                                     | 62                      | 46                      | 85                            | 101                       | 86                       | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | GC220         | 6             | 66                                     | 66                                     | 56                      | 53                      | 68                            | 147                       | 120                      | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | glucanase     | 9             | 70                                     | 65                                     | 53                      | 51                      | 59                            | 100                       | 89                       | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | milled        | 10            | 24                                     | 75                                     | 63                      | 27                      | 26                            | 0                         | 0                        | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | liquefied     | 20            | 67                                     | 51                                     | 38                      | 27                      | 50                            | 25                        | 21                       | 5                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | 110 °C, 20 Min| 26            | 80                                     | 10                                     | 0                       | 0                       | 38                            | 27                        | 6                        | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | xylanase      | 6             | 8                                       | 13                                     | 0                       | 0                       | 18                            | 19                        | 0                        | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | cellulase     | 7             | 30                                     | 35                                     | 25                      | 20                      | 52                            | 52                        | 36                       | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | GC220         | 9             | 77                                     | 65                                     | 78                      | 69                      | 80                            | 142                       | 118                      | 9                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | glucanase     | 10            | 7                                       | 33                                     | 6                       | 19                      | 75                            | 48                        | 0                        | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | milled        | 0             | 7                                       | 0                                      | 0                       | 14                      | 64                            | 45                        | 0                        | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | liquefied     | 24            | 6                                       | 0                                      | 13                      | 22                      | 94                            | 70                        | 0                        | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | 110 °C, 20 Min| 21            | 67                                      | 47                                     | 72                      | 74                      | 33                            | 96                        | 102                      | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | xylanase      | 17            | 70                                      | 50                                     | 77                      | 69                      | 45                            | 81                        | 87                       | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | cellulase     | 27            | 50                                      | 35                                     | 38                      | 39                      | 27                            | 107                       | 91                       | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | GC220         | 22            | 68                                      | 49                                     | 66                      | 66                      | 41                            | 108                       | 103                      | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | glucanase     | 25            | 30                                      | 21                                     | 43                      | 43                      | 70                            | 63                        | 0                        | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
| Cassava         | milled        | 0             | 0                                       | 0                                      | 0                       | 0                       | 22                            | 18                        | 0                        | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | liquefied     | 5             | 0                                       | 0                                      | 0                       | 7                       | 31                            | 33                        | 0                        | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | 110 °C, 20 Min| 5             | 28                                      | 16                                     | 23                      | 30                      | 15                            | 39                        | 42                       | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | xylanase      | 2             | 40                                      | 25                                     | 36                      | 30                      | 23                            | 12                        | 23                       | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | cellulase     | 0             | 39                                      | 25                                     | 36                      | 29                      | 22                            | 9                         | 19                       | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | GC220         | 0             | 21                                      | 11                                     | 17                      | 12                      | 11                            | 33                        | 24                       | 7                         | 0                           | 0                      | 0                       | 0                             | 0                               |
|                 | glucanase     | 0             | 0                                       | 0                                      | 0                       | 0                       | 20                            | 16                        | 0                        | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
| Canna edulis Ker| milled        | 0             | 0                                       | 0                                      | 0                       | 0                       | 22                            | 18                        | 0                        | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | liquefied     | 5             | 0                                       | 0                                      | 0                       | 7                       | 31                            | 33                        | 0                        | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | 110 °C, 20 Min| 5             | 28                                      | 16                                     | 23                      | 30                      | 15                            | 39                        | 42                       | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | xylanase      | 0             | 40                                      | 25                                     | 36                      | 30                      | 23                            | 12                        | 23                       | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | cellulase     | 0             | 39                                      | 25                                     | 36                      | 29                      | 22                            | 9                         | 19                       | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
|                 | GC220         | 0             | 21                                      | 11                                     | 17                      | 12                      | 11                            | 33                        | 24                       | 7                         | 0                           | 0                      | 0                       | 0                             | 0                               |
|                 | glucanase     | 0             | 0                                       | 0                                      | 0                       | 0                       | 20                            | 16                        | 0                        | 0                             | 0                         | 0                      | 0                       | 0                             | 0                               |
Changes in cell wall polymer structure of Shangshu 19, cassava, and Canna edulis Ker. before and after viscosity reduction. Shangshu 19 has abundant homogalacturonan; cassava has abundant 1,4-β-D-galactan and 1,5-α-L-arabinan, and Canna edulis Ker. is covered by gel-like matrices and a cellulose cross-linking protein network, resulting in enzyme difficulty in accessing the surface of the substrate. After viscosity reduction, Shangshu 19 released a high quantity of 1,4-β-D-galactan and 1,5-α-L-arabinan; cassava mash was characterized by the degradation of homogalacturonan and 1,5-α-L-arabinan and by the release of 1,4-β-D-galactan. PCWDEs, plant cell wall–degrading enzymes; ME, methyl-esterified.

Changes in cell wall polysaccharide of the three types of roots and tubers (sweet potato, cassava, and C. edulis Ker.) during liquefaction and high-temperature treatments significantly affect the viscosity reduction. The degradation of the homogalacturonan and release of 1,4-β-D-galactan and 1,5-α-L-arabinan were essential for reducing viscosity of sweet potato mash.

Changes in cell wall polymers of different roots and tubers
Changes in cell wall polysaccharide of the three types of roots and tubers (sweet potato, cassava, and C. edulis Ker.) during
Overall changes in homogalacturonan (HG), 1,4-β-D-galactan, and 1,5-α-L-arabinan occurrence during the viscosity-reducing process of Shangshu 19. The data are the sums of the mean signals from CDTA and NaOH extractions.

The milling, liquefaction, and 110 °C for 20 Min processes varied (Fig. 4). As described above, the original Shangshu 19 mash has strong homogalacturonan (LM18 and LM19) and weak methyl-esterified homogalacturonan (JIM5), 1,4-β-D-galactan (LM5), and 1,5-α-L-arabinan (LM6) epitopes. However, the original cassava mash showed very weak LM18 and JIM5 and much higher LM5 and LM6 binding capability. It also had weak 1,5-α-L-arabinan (LM13) and xyl glucan (LM15) epitopes (Figs. 3 and 4). LM13 binds to longer stretches of 1,5-linked arabinosyl residues that are likely to be linear arabinoxylans. Its epitope is conformational and generally specific to epidermal cell walls [45]. Xyloglucan is one of the most abundant hemicelluloses, which can cross-link glycan to the primary cell walls and tether the cellulose microfibrils by hydrogen bonds. Marcus et al. [47] found that abundant sets of xyloglucan epitopes are masked by the presence of homogalacturonan in primary cell walls of certain organs, indicating that xyloglucan is also associated with pectin. Thus, the plant cell wall polymers of sweet potato and cassava tubers were different. Although both of these are dicotyledons, they belong to different families (Sweet potato and cassava belong to the Convolvulaceae and Euphorbiaceae families, respectively). The original mash of *C. edulis* Ker. had weak LM5- and LM6-binding capabilities; interestingly, it had a MAC207 binding signal for arabinogalactan protein, which was not present in the sweet potato or the cassava (Figs. 3 and 4). After liquefaction, both the 1,4-β-D-galactan (LM5) and 1,5-α-L-arabinan (LM6) epitopes of cassava and Shangshu 19 significantly increased when compared with *C. edulis* Ker. However, the feruloylated arabinan (LM12) and arabinogalactan protein (MAC207) epitopes were only observed in *C. edulis* Ker. mash. Cross-linking feruloylated arabinan reportedly results in gel formation [60]. Furthermore, as Sehlbach et al. [61] discovered, the polypeptide portion of *J. curcas* arabinogalactan proteins have O-glycosidic and N-glycosidic linkages to the carbohydrate moieties and primarily include fasciclin-like arabinogalactan, xylan-like proteins, and LysM domain-containing proteins. These gel-like matrices at cell wall interfaces [54] and the cellulose cross-linking protein network [47] extensively masked hemicelluloses and cellulose, making PCWDEs inaccessible to the substrate. The viscosity of *C. edulis* Ker. mash was therefore very difficult to reduce even though the LM12 and MAC207 signals were relatively weak.
Changes in the cell wall polymers of Shangshu 19 and cassava with obvious viscosity reduction
The viscosities of Shangshu 19 and cassava were clearly decreased when the mash was treated with cellulase, GC220, and glucanase (Table 2, Fig. 2). The CoMPP data provided detailed insight into the cell wall polysaccharide changes after the enzyme treatment (Fig. 3). There were small changes among the JIM5, JIM7, LM18, LM19, and LM20 epitopes in the Shangshu 19 extracts after adding enzymes (cellulase, GC220, and glucanase). However, when compared with the levels of epitopes between the 110 °C, 20 Min and enzyme treatments, the LM5 and LM6 epitopes clearly increased (from 0.47- to 1.16-fold and 0.26- to 0.76-fold, respectively) after adding enzymes (Fig. 5). The relative levels of LM5 and LM6 epitopes decreased when the mash was treated by xylanase with a low viscosity reduction capability. These results indicate that the release of a high quantity of 1,4-β-D-galactan and 1,5-α-L-arabinan resulted in changes in the Shangshu 19 cell wall polymer structure and reducing the mash viscosity.

However, subtly different cell wall polymer profiles were observed in the cassava mash after the PCWDEs treatment (Fig. 6). All of the mAbs recognitions (JIM5, JIM7, LM18, LM19, LM20, and LM6) were decreased except for LM5 when the mash was treated with cellulase and GC220. The decreased homogalacturonan and 1,5-α-L-arabinan may attribute to the degradation of these polymers after adding cellulase and GC220, which resulted in reduction of the viscosity. Glucanase also has the distinct ability to reduce the cassava viscosity. The changes in the mAbs epitopes were similar to those of xylanase, of which the viscosity-reducing capability is not obvious. However, the binding of JIM5, JIM7, LM18, LM19, and LM20 of the glucanase-treated mash were reduced to very low levels. Thus, much more homogalacturonan was degraded, which further resulted in viscosity reduction when glucanase was added to the cassava mash.

Therefore, the viscosity reduction of Shangshu 19 and cassava was due to the degradation of homogalacturonan and the release of 1,4-β-D-galactan and 1,5-α-L-arabinan after cellulase, GC220, and glucanase treatments.
4. Conclusions

Nongrain-based roots and tubers are promising feedstock for ethanol production. The high viscosity bottleneck of the mash has been solved by PCWDEs. This study used the CoMPP technique to investigate changes in the cell wall polymers of different roots and tubers over the entire viscosity-reducing process. The results indicated that the composition of cell wall polymers among these three roots and tubers were markedly different, the gel-like matrix and glycoprotein network resulted in difficult viscosity reduction, and the viscosity reduction of mash was attributed to the degradation of homogalacturonan and the release of 1,4β-D-galactan and 1,5-α-L-arabinan.

5. Acknowledgements

This study was supported by the International Cooperation Key Project (No. 2014DFA30680), the China Agriculture Research System (No. CARS-11-B-17), the “Western Light” talent cultivation program of the Chinese Academy of Sciences (No.Y2C5021100), and the Sino-Danish Center (SDC) for Education and Research. The authors have no conflicts of interest to declare.

6. References

[1] Ziska, L. H., Runion, G. B., Tomecek, M., Prior, S. A., Torbet, H. A., and Sicher, R. (2009) Biomass Bioenerg. 33, 1503–1508.
[2] Jin, Y., Fang, Y., Zhang, G., Zhou, L., and Zhao, H. (2012) Acta Oecol. 39, 33–37.
[3] Srichuwong, S., Orikasa, T., Matsuki, J., Shiina, T., Kobayashi, T., and Tokuyasu, K. (2012) Biotechnol. Bioeng. 109, 120–127.

For the rest of the references, please refer to the text.
[47] Marcus, S., Verhertbruggen, Y., Herve, C., Ordez-Ortiz, J., Farkas, V., Pedersen, H., Willats, W., and Knox, J. P. (2008) BMC Plant Biol. 8, 60.

[48] Bradley, D. J., Wood, E. A., Larkins, A. P., Galfre, G., Butcher, G. W., and Brewin, N. J. (1988) Planta 173, 149–160.

[49] Pennell, R. I., Knox, J. P., Scofield, G. N., Selvendran, R. R., and Roberts, K. (1989) J. Cell Biol. 108, 1967–1977.

[50] Yates, E. A., and Knox, J. P. (1994) Carbohydr. Polym. 24, 281–286.

[51] Blake, A. W., McCartney, L., Flint, J. E., Bolam, D. N., Boraston, A. B., Gilbert, H. J., and Knox, J. P. (2006) J. Biol. Chem. 281, 29321–29329.

[52] Initiative, G. S., (2008) Biofuels–at what cost? Government support for ethanol and biodiesel in China, International Institute for Sustainable Development, Geneva.

[53] Bauer, S., Vasu, P., Persson, S., Mort, A. J., and Somerville, C. R. (2006) Proc. Natl. Acad. Sci. USA 103, 11417–11422.

[54] Willats, W. T., McCartney, L., Mackie, W., and Knox, J. P. (2001) Plant Mol. Biol. 47, 9–27.

[55] Jarvis, M. C. (1984) Plant Cell Environ. 7, 153–164.

[56] Knox, J. P. (1992) Plant J. 2, 137–141.

[57] Zhang, G. F., and Staehelin, L. A. (1992) Plant Physiol. 99, 1070–1083.

[58] Vignon, M. R., Heux, L., Malainine, M. E., and Mahrouz, M. (2004) Carbohydr. Res. 339, 123–131.

[59] Zykwinska, A., Thibault, J. F., and Rale, M. C. (2007) J. Exp. Bot. 58, 1795–1802.

[60] Oosterveld, A., Poi, I. E., Beldman, G., and Voragen, A. G. J. (2001) Carbohydr. Polym. 44, 9–17.

[61] Sehlbach, M., König, S., Mormann, M., Sendker, J., and Hensel, A. (2013) Carbohydr. Polym. 98, 522–531.