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Structural changes in polysaccharides isolated from chestnut (*Castanea mollissima* Bl.) fruit at different degrees of hardening

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ABSTRACT

Hardening is an important physiological disorder of chestnut fruit during storage. In this study, polysaccharides of chestnut (PCs) at 0%, 50% and 100% degrees of hardening (DH) were extracted and then sequentially fractionated using 40% and 80% ethanol in order to obtain PCs I and II. For fresh chestnut, arabinose, fructose, glucose and galactose were the main monosaccharides of PCs I and II. Significantly positive correlations ($P < 0.01$) existed between xylose, galactose, arabinose contents and DH, while significantly negative correlations ($P < 0.01$) were present between glucose, fructose contents and DH for both PCs I and II. The appearance in hardening of chestnut fruit was accompanied with decreases in the contents of 1,3-fructose, 1,3- and 1,6-glucose, and increases of 1,4-arabinose, 1,6-galactose and 1,3-xylose. The results confirmed that cell wall degradation of chestnut induced the structural changes in PCs I and II, which further led to the occurrence of hardening.

1. Introduction

Chestnut is an important edible fruit in northern hemisphere, which has been consumed as extensively as potato in the past (Ferreira-Cardoso, Rodrigues, Gomes, Sequeira, & Torres-Perreira, 1999). It has a long growing history of over 2000 years in China with an important role in the economy. Literatures suggest that chestnut is a good source of bioactive substances including lectin, cysteine, proteinase inhibitor and quercetin (Wang & Ng, 2003). It also contains considerable vitamins, fibres, essential fatty acids and minerals (Borges, de Carvalho, Correia, & Silva, 2007; Borges, Gonçalves, de Carvalho, Correia, & Silva, 2008). Increasing evidences show that the consumption of chestnut become more important in human nutrition due to the health protection provided by the antioxidants (Blomhoff, Carlsen, Andersen, & Jacobs, 2006).

Chestnut hardening is a commonly physiological disorder during storage and deteriorates seriously edible quality. It can be observed by the colour of chestnut. The formation of lime-white region indicates the occurrence of hardening. Loss of moisture and disruption of cell walls during storage are two possible processes responsible for the hardening of chestnut (Yang, Jiang, Prasad, Gu, & Jiang, in press). However, understanding of this disorder is still unclear and requires to be investigated. It is well known that polysaccharides are the most important component of cell wall (Yang, Jiang, Wang, Zhao, & Sun, 2009a; Zhao, Yang, Yang, Jiang, & Zhang, 2007). The disruption of cell wall during the storage of chestnut might affect the compositions and structural characteristics of polysaccharides. Elucidation of the structural changes in polysaccharides of chestnut (PC) during storage will be helpful to understand the occurrence of hardening. Therefore, the objective of this work was to extract polysaccharides from chestnut at various degrees of hardening (DH) and then to identify their structures. The changes in monosaccharide composition and glycosidic linkage of PC were also determined to further evaluate the hardening disorder of chestnut during storage.

2. Materials and methods

2.1. Plant materials

Chinese chestnut (*Castanea mollissima* Bl.) fruits at different degrees of hardening were donated by Guangzhou University (Guangzhou, China). These fruits were peeled manually and cut into half. The colour of cross-section was recorded for the evaluation of DH. A well-trained panel of six persons was employed for the evaluation. Three DH levels were set as 0%, 50% and 100%. DH of 0% was defined that no lime-white region was observed from the cross-section of chestnut, while DH of 50% was that half area of the cross-section appeared as lime-white, DH of 100% was that the

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whole cross-section appeared as lime-white. These chestnut fruits were pulverized by a miller and then screened through a 100-mesh iron sieve.

2.2. Chemicals

Ethanol, phenol and sulphuric acid were obtained from Guangzhou Reagent Co. (Guangzhou, China). Standards of xylose, arabinose, glucose, galactose, fructose and rhamnose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the other chemicals used were of analytical grade.

2.3. Extraction and quantification of PC

PC was extracted with hot water by the method of Yang et al. (2009b). The fine chestnut seed powder (5 g) was extracted for 120 min with 100 ml of distilled water in a 150-ml conical flask submerged in a water bath at 55 °C. The extract was filtered through a Whatman No. 1 paper (Whatman Plc., Shanghai, China) and then concentrated to 25 ml using a vacuum rotary evaporator (BC-R203, Shanghai Biochemical Equipment Co., Shanghai, China) at 65 °C. The proteins in the extract were removed by Sevag reagent (Navarini et al., 1999). Then, anhydrous ethanol was added into the extract to obtain a final concentration of 40% and maintained overnight at 4 °C to precipitate large molecular-weight polysaccharides (PC I), which was then obtained after centrifugation at 4000*g* for 15 min. Ethanol was then added into the supernatant to obtain a final concentration of 80%. The above programme was repeated to obtain small molecular-weight polysaccharides (PC II).

The content of polysaccharides was determined by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and expressed as glucose equivalent. Protein content was calculated from nitrogen content ($N \times 6.25$), which was determined by a PE-2400 series II automatic elemental analyser (Perkin-Elmer, Waltham, Massachusett, USA). The moisture and ash contents of PCs I and II were also determined according to the method of Singthong, Ningsanond, and Cui (2009).

2.4. Analysis of monosaccharide composition

PCs I or II (10 mg) was hydrolysed for 4 h with 10 ml of 2 M trifluoroacetic acid at 100 °C (Erbing, Jansson, Widmalm, & Nimmich, 1995). Derivatisation of the released monosaccharides was then carried out using trimethylsilylation reagent according to the method of Guentas et al. (2001). The trimethylsilylated derivatives were loaded onto a HP-5 capillary column and then determined by a flame ionization detector. The following programme was used for gas chromatography analysis: injection temperature, 230 °C; detector temperature, 230 °C; column temperature programmed from 130 to 180 °C at 2 °C/min, holding for 3 min at 180 °C, then increasing to 220 °C at 10 °C/min and finally holding for 3 min at 220 °C. N_2 was used as the carrier gas and maintained at 40.0 ml/min. The speeds of air and H_2 were 400 and 40 ml/min, respectively. The split ratio was set as 10:1. Inositol was used as the internal standard.

2.5. Methylation analysis

Methylation of PCs I or II was carried out by the method of Needs and Sevendran (1993) with minor modification. Five milligrams of sample was weighted precisely and then dissolved in 5.0 ml of dimethyl sulfoxide before 200 mg of NaOH was added. The mixture was treated for 10 min at 120 W by ultrasonic wave using an ultrasonic cleaner (KQ-300DE, 40 kHz, Kunshan Ultrasonic Equipment Co., Kunshan, China). After 1 h of incubation at room temperature (25 °C), 1.5 ml of methyl iodide were added for methylation. The sample solution was kept for 1 h in dark before 4 ml of distilled water was used to decompose the remained methyl iodide. The methylated polysaccharides were extracted with 3×2 ml of chloroform and then dried at a low pressure by a rotary evaporator (RE52AA, Yarong Instrument Co., Shanghai, China). After hydrolysis with 10 ml of 2 M trifluoroacetic acid, the PC hydrolysates were dissolved in 4 ml of 1% (w/w) NaOH. Twenty milligrams of $NaBH_4$ was added to reduce the uronic acid and hemiacetal bond. After 30 min of incubation at 40 °C, one hundred microlitres of glacial acetic acid were used to terminate the reduction. The sample was dried under low pressure, and then acetylated with 2 ml of acetic anhydride and 2 ml of pyridine. The reaction was kept at 100 °C for 1 h. Two millilitres of distilled water was used to hydrolyse the remained acetic anhydride. The acetylated derivatives were extracted with 4 ml of methylene chloride. A gas chromatography/mass spectrometer (GCMS-QP 2010, Shimadzu, Kyoto, Japan) was used to analyse the glycosidic linkage. The acetylated derivatives were loaded into a HP-1 capillary column. The temperature programme was set as follows: the initial temperature of column was 150 °C, increased to 180 °C at 10 °C/min, then from 180 to 260 °C at 15 °C/min, holding for 5 min at 260 °C, with an injection temperature of 220 °C. The ion source of mass spectrometer was set at 200 °C. One microlitre of sample was injected, with a split ratio of 50:1.

2.6. Statistical analysis

Data were expressed as mean ± standard deviation of three replicated determinations. One way of variance analysis was applied for determining the significant difference at $P < 0.05$. Statistical analysis software SPSS Version 10.0 (SPSS Inc., Chicago, Illinois, USA) was used to analyse the correlation coefficient between relative molar percentage of monosaccharide, glycosidic linkage and DH.

3. Results and discussion

3.1. Chemical compositions of PCs I and II

Ethanol precipitation is a useful method for fractionating water-soluble polysaccharides in terms of molecular weight (Yang, Zhao, Shi, Yang, & Jiang, 2008). The water-soluble polysaccharides with high molecular weight are precipitated in ethanol with low concentration, while those having low molecular weight can only be precipitated by high-concentration ethanol. In this work, hot water extraction was used to prepare water-soluble polysaccharides from chestnut with different DHs (0%, 50% and 100%). Fractionation by ethanol solution led to the precipitation of PCs I and II. Table 1 shows the chemical compositions of PCs I and II. The protein contents of all the PC fractions were close to 2.0%. Peña-Méndez, Hernández-Suárez, Díaz-Romero, and Rodríguez-Rodríguez (2008) have determined the chemical compositions of various chestnut cultivars and have found the total proteins account for approximately 3% of the fresh weight of chestnut. During the extraction, proteins are usually co-precipitated with the PC fraction in ethanol solution. The Sevag reagent effectively removed most of the proteins from the PC fraction. This explains that only few proteins were detected in PCs I and II. Small amount of ash was also detected in the PC fraction. The ash content of PC I at 0% DH was 2.2%, while that of PC II at 0% DH was 3.1%. The mineral content of chestnut ranged from 1% to 3% on dry weight basis, mainly including K, Mg, P and Ca (Miguelez, Bernárdez, & Queijeiro, 2004). Furthermore, the carbohydrate contents of PC I (or PC II) at DHs of 0% and 50% were not significantly different ($P > 0.05$).

Table 1
Chemical compositions of PCs I and II (%, w/w).

Chemical composition	PC I			PC II		
	0%	50%	100%	0%	50%	100%
Protein	2.1 ± 0.2	2.1 ± 0.1	1.9 ± 0.2	2.4 ± 0.2	2.2 ± 0.2	2.6 ± 0.1
Ash	2.2 ± 0.3	2.5 ± 0.5	2.3 ± 0.5	3.1 ± 0.3	3.4 ± 0.3	3.6 ± 0.4
Moisture	10.2 ± 0.8	9.8 ± 0.7	9.4 ± 0.9	9.8 ± 0.7	10.3 ± 0.9	10.2 ± 0.5
Carbohydrate[a]	73.1 ± 2.9	73.4 ± 3.2	80.2 ± 2.7	79.3 ± 3.5	79.8 ± 3.1	82.5 ± 2.9

[a] The carbohydrate content is expressed as glucose equivalent.

3.2. Monosaccharide compositions of PCs I and II

PCs I or II was hydrolysed by trifluoroacetic acid into individual monosaccharides that were further trimethylsilylated for gas chromatography analysis. In comparison with the retention time of standards, the monosaccharide composition was identified in Table 2. Four monosaccharides, including arabinose, fructose, glucose and galactose, were identified for PC I at 0% DH, while arabinose, fructose, glucose, galactose and xylose were detected for PC Is at 50% and 100% DH, as well as PC IIs at 0% and 50% DH, respectively. Besides the above-mentioned five monosaccharides, rhamnose was uniquely detected in PC II at 100% DH. Except for low quantity of xylose, the relative mole percentages of arabinose (3.0%), fructose (20.2%), glucose (71.9%) and galactose (4.1%) in PC I at 50% DH were comparatively similar to those in PC I at 0% DH. Moreover, the similar mole percentages of monosaccharides in PC IIs at 0% and 50% DH were observed, which indicated that when the DH reached 50%, the microenvironmental change in the cell wall could not apparently affect the chemical compositions of PC. PC I at 100% DH exhibited different relative mole percentages of monosaccharides, compared with those at 0% and 50% DHs. The relative mole percentage of xylose increased sharply up to 24.0% while that of glucose decreased obviously. Furthermore, the occurrence of rhamnose was found in PC II at 100% DH. A significant difference in the relative mole percentage of monosaccharides between PC IIs at 100% and 50% or 0% DH was obtained.

Small molecular-weight sugars (monosaccharide and disaccharide) are important parameters for the assessment of the commercial quality of chestnut, since the special sweet taste is preferred by consumers (Senter, Payne, Miller, & Anagnostakis, 1994). Bernárdez, Miguélez, and Queijeiro (2004) pointed out that sucrose in the chestnut is the major free sugar which contributes to the sweet taste. The analysis of monosaccharide contents in PCs I and II showed that glucose and fructose were the major monosaccharides of PCs I and II for fresh chestnut (0% DH). Hardening disorder of chestnut is accompanied with the content decreases of glucose and fructose, and the content increases of arabinose and galactose. It was interesting that no xylose was detected in the PC I of fresh chestnut but it was found in PC I at 50% or 100% DH. Moreover, a positive relationship between xylose content and DH was observed.

Water-soluble polysaccharides are important component of plant cell wall, which play a critical role in controlling the size and shape of cell, and physiological functions like intercellular communication and interaction with environment (Cosgrove, 1997). The disruption of cell wall is related to the hardening disorder of chestnut, which might also affect the polysaccharide structure to a certain extent. From the results obtained in this work, at 50% DH, no significant difference was observed for monosaccharide composition of PC, as compared with fresh chestnut. However, an apparent change in monosaccharide composition of PC was detected at 100% DH. These changes proved that the degradation of cell wall polysaccharides was occurred. The degradation of cell wall polysaccharides is dependent on the action of numerous enzymes, including exopolysaccharidases, endopolysaccharidases and other hydrolases (Minic & Jouanin, 2006). Exopolysaccharidases attach polysaccharides from the non-reducing terminus, releasing monosaccharides and sometimes disaccharides. Attachment by endopolysaccharidases into the polysaccharide backbone at any position leads to a big impact on the molecular weight of polysaccharides (Fry, 2004). Many types of glucosidases, galactosidases, fructosidases, xylosidases and arabinosidases have been identified in plant cell walls (Iglesias et al., 2006; Monroe et al., 1999). With increasing DH of chestnut, xylose was introduced into PC I and its content increased. The degradation of other hemicelluloses in chestnut cell wall, especially water-insoluble polysaccharides, into water-soluble polysaccharides should be responsible for the hardening disorder. The occurrence of rhamnose in PC II at 100% DH was also hypothesized to be attributed to the degradation of water-insoluble hemicelluloses. The content decreases of glucose and fructose might be due to the degradation of PC into small molecules by glucosidases and fructosidases.

3.3. Glycosidic linkages of PCs I and II

Methylation and acetylation were taken to derivatize PCs I and II. The glycosidic linkage of each monosaccharide was identified by gas chromatography/mass spectrometry (GC/MS). As shown in Table 3, both arabinose and xylose have 1,3- and 1,4-linkages. The existence of 1,4-arabinose and 1,4-xylose indicated that they had a pyran ring structure. The 1,4-glycosidic linkage of xylopyranose is common in hemicellulose, such as the arabinoxylan of *Plantago*

Table 2
Monosaccharide composition of PCs I and II at different DHs[a].

Monosaccharides	PC I			PC II		
	0%	50%	100%	0%	50%	100%
Arabinose	3.0 ± 0.2	3.0 ± 0.1	22.5 ± 0.4	16.2 ± 0.2	16.3 ± 0.3	36.6 ± 0.6
Fructose	20.2 ± 0.5	16.8 ± 0.4	13.7 ± 0.4	25.8 ± 0.3	26.0 ± 0.4	4.1 ± 0.2
Glucose	72.7 ± 0.6	72.2 ± 0.4	23.8 ± 0.6	34.3 ± 0.3	34.1 ± 0.5	5.2 ± 0.2
Galactose	4.1 ± 0.2	4.9 ± 0.2	16.0 ± 0.3	20.8 ± 0.4	20.7 ± 0.4	44.3 ± 0.5
Xylose	–	3.1 ± 0.1	24.0 ± 0.5	2.9 ± 0.1	2.9 ± 0.2	5.9 ± 0.1
Rhamnose	–	–	–	–	–	3.9 ± 0.1

–, undetected.
[a] The monosaccharide content is expressed as relative mole percentage.

Table 3
Relative mole percentages of the glycosidic linkages of PCs I and II at different DHs*.

Glycosidic linkages	PC I			PC II		
	0%	50%	100%	0%	50%	100%
1,3-Arabinopyranose	2.4 ± 0.2	2.3 ± 0.1	2.2 ± 0.1	3.0 ± 0.1	3.0 ± 0.3	3.5 ± 0.2
1,4-Arabinopyranose	0.6 ± 0.1	0.7 ± 0.1	20.3 ± 0.5	13.2 ± 0.4	13.3 ± 0.2	33.1 ± 0.6
1,3-Fructopyranose	20.2 ± 0.6	16.8 ± 0.4	13.7 ± 0.3	25.8 ± 0.7	26.0 ± 0.8	4.1 ± 0.3
1,2,4-Glucopyranose	2.9 ± 0.1	3.0 ± 0.2	1.9 ± 0.1	–	–	–
1,3-Glucopyranose	33.6 ± 0.7	33.5 ± 0.9	5.4 ± 0.2	7.1 ± 0.3	7.1 ± 0.4	0.5 ± 0.2
1,4-Glucopyranose	–	–	–	0.9 ± 0.2	0.9 ± 0.1	–
1,6-Glucopyranose	35.4 ± 0.5	36.6 ± 0.7	16.5 ± 0.3	26.3 ± 0.9	26.1 ± 0.8	4.7 ± 0.4
1,4-Galactopyranose	4.5 ± 0.2	3.8 ± 0.1	3.4 ± 0.1	18.8 ± 0.4	18.6 ± 0.6	4.1 ± 0.1
1,6-Galactopyranose	0.4 ± 0.1	0.3 ± 0.1	12.6 ± 0.4	2.0 ± 0.1	2.0 ± 0.2	40.2 ± 0.6
1,3-Xylopyranose	–	0.2 ± 0.1	20.1 ± 0.5	0.3 ± 0.1	0.3 ± 0.1	5.5 ± 0.2
1,4-Xylopyranose	–	2.9 ± 0.1	3.9 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	0.4 ± 0.1
1,5-Rhamnofuranose	–	–	–	–	–	3.9 ± 0.2

* –, undetected.

ovata Forsk (Craeyveld, Delcour, & Courtin, 2009). Galactose was detected to have 1,4- and 1,6-linkages in this work, which was also reported in the galactan structure of New Zealand red algae (Falshaw & Furneaux, 2009). Fructose and rhamnose were found to appear as 1,4- and 1,5-linkages, respectively. GC/MS analysis suggested that rhamnose existed as a furan ring structure. Glucose was measured to have four linkages, 1,2,4-, 1,3-, 1,4- and 1,6-linkages. Along with the increase of DH from 0% to 100%, the relative mole percentage of 1,4-arabinose increased from 0.6% to 20.3% for PC I and from 13.2% to 33.1% for PC II. Furthermore, 1,2,4-glucose was only found in PC I while 1,4-glucose was only detected in PC II at 0% or 50% DH with a low relative mole percentage but it was not detectable at 100% DH. The dominant linkages of glucose were identified as 1,3- and 1,6-linkages which decreased sharply with increasing DH. 1,6-Galactose and 1,3-xylose increased as the DH increased and a similar tendency to 1,4-arabinose was shown.

Further analysis of glycosidic linkage by GC/MS indicated that PCs I and II had similar linkage types, but significant differences in the relative mole percentages existed. 1,3-Fructose, 1,3- and 1,6-glucose were the major linking types for PC I, while 1,4-arabinose, 1,3-fructose, 1,6-glucose and 1,4-galactose were the main linkages for PC II of fresh chestnut. 1,6-Glucose was also detected as an important component of longan fruit pericarp polysaccharides (Yang et al., 2009b). The degradation of cell wall is highly involved in the hardening disorder of chestnut. The results of this work indicated that the degradation leads to not only the changes of monosaccharide content but also the relative mole percentages of glycosidic linkages. The increases of 1,4-arabinose, 1,6-galactose and 1,3-xylose of both PCs I and II during hardening might be due to the degradation of water-insoluble polysaccharides by endo-polysaccharidases. The decreases in fructose and glucose were further confirmed by reduced 1,3-fructose, 1,3- and 1,6-glucose contents. All these results confirmed that the degradation of cell wall induced the changes in polysaccharide structure, which further facilitated the hardening of chestnut during storage.

3.4. Correlation analysis

Correlation test is important to confirm the correlation between two variables. To confirm the significant correlation between hardening and PC structure, the correlation coefficients between relative mole percentages of monosaccharides and DH were calculated. Moreover, the correlation coefficients between relative mole percentage of glycosidic linkages and DH were also determined. As shown in Table 4, the study indicated that the change in content of each monosaccharide was significantly correlated with DH. Significantly positive correlations ($P < 0.01$) were determined for arabinose, galactose and xylose while significantly negative correlations ($P < 0.01$) were obtained for fructose and glucose. Except for 1,3-arabinose, all the other glycosidic linkages showed significantly positive or negative correlations ($P < 0.01$) with DH. These results proved that the change in PC structure was one of the mechanisms involved in the hardening of stored chestnut.

Table 4
Correlation coefficients between the relative mole percentages of monosaccharides, glycosidic linkages and DH for PCs I and II.

	PC I	PC II
Arabinose	0.866**	0.868**
Fructose	−0.992**	−0.862**
Glucose	−0.870**	−0.869**
Galactose	0.894**	0.864**
Xylose	0.919**	0.863**
Rhamnose	–	0.866**
1,3-Arabinopyranose	−0.577*	0.693**
1,4-Arabinopyranose	0.868**	0.868**
1,3-Fructopyranose	−0.990**	−0.861**
1,2,4-Glucopyranose	−0.801**	–
1,3-Glucopyranose	−0.867**	−0.863**
1,4-Glucopyranose	–	0.840**
1,6-Glucopyranose	−0.837**	−0.869**
1,4-Galactopyranose	−0.957**	−0.871**
1,6-Galactopyranose	0.862**	0.866**
1,3-Xylopyranose	0.870**	0.865**
1,4-Xylopyranose	0.962**	−0.863**
1,5-Rhamnofuranose	–	0.865**

* and ** represent correlations were significant at $P < 0.05$ and $P < 0.01$, respectively; –, not computable.

4. Conclusions

PCs I and II were extracted from chestnut at 0%, 50% and 100% DH in this work. The analysis of chemical composition indicated that both PCs I and II had small amounts of proteins and ash. For fresh chestnut, arabinose, fructose, glucose and galactose were detected for PC I, while arabinose, fructose, glucose, galactose and xylose were found for PC II. Glucose and fructose were the dominant monosaccharides in both PC fractions. The occurrences of xylose and rhamnose in PCs I and II were detected when the DH increased. The main linkages of PC I from fresh chestnut were 1,3-fructose, 1,3- and 1,6-glucose, while 1,4-arabinose, 1,3-fructose, 1,6-glucose and 1,4-galactose were the major linkages of PC II from fresh chestnut. The hardening of chestnut was accompanied with the content decreases of 1,3-fructose, 1,3- and 1,6-glucose and the content increases of 1,4-arabinose, 1,6-galactose and 1,3-xylose. The correlation analysis proved the significant correlation between PC structure and DH. The study indicated that degradation of cell wall

induced the structural change in PCs I and II, which further induced the hardening of chestnut during storage. Further investigation on other carbohydrates and/or chemical compositions of chestnut during storage can help understanding well the mechanism of hardening.

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