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Short communication

POD promoted oxidative gelation of water-extractable arabinoxylan through ferulic acid dimers. Evidence for its negative effect on malt filterability

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ABSTRACT

As a major component of non-starch polysaccharide in barley, arabinoxylan (AX) plays an important role in quality traits of malt and the final beer product. The Chinese barley malt has encountered filterability problems for a long time. The main reason caused by barley cultivar has been accepted in the malting and brewing industries. In our previous proteomic study, the peroxidase (POD) BP1 was found to be in quite high abundant in the filterability defect Chinese barley malt. Therefore, the present study tried to verify its negative effect on filterability, by surveying its activity in different malt samples and detecting effects of POD on AX gelation and filterability. The results showed that the activity of POD, as well as the content of AX bounded ferulic acid, were both negatively correlated with filterability, while the feruloyl esterase activity was positively correlated with it. In addition, AX gelation catalyzed by POD caused worse filterability, and the natural inhibitor of POD, vitamin C, could blocked the cross linking catalyzed by POD and thus improve the filterability. These results all suggested the great negative effect of POD on malt filterability.

1. Introduction

Arabinoxylan (AX) is the major component of non-starch polysaccharides (NSP) in barley (Dervilly-Pinel, Rimsten, Saulnier, Andersson, & Aman, 2001), which plays important role in the malting of barley and in subsequent steps of brewing process. AX contains a linear backbone of D-xylopyranose residues linked by β-(1 → 4)-glycosidic bonds with units such as L-arabinofuranose attached as branches by β-(1 → 2)- or β-(1 → 3)-linkages (Vietor, Voragen, Angelino, & Pilnik, 1991). Typically, L-arabinofuranose or other side chains are carried on the main chain as non-reducing end groups, which could be attached by other polysaccharides such as β-glucan, another major constituent of the cell wall (Lee, Burton, Hrmova, & Fincher, 2001), or esterified with ferulic acid (3-methoxy, 4-hydroxy cinnamic acid, FA), the most abundant phenolic acid in barley (Wang, Cao, Sun, & Wang, 2011). Because of the high degree of substitution and linkage with other compounds, AX alone or synergistically with other compounds, could result in lautering and/or filtering problems during brewing (Han, 2000). In view of the importance of AX in the quality traits of barley malt and even the final beer product, it is desirable to have a more detailed knowledge of AX in barley, malt and wort. According to the solubility, AX could be classified into two major groups, the water-extractable arabinoxylans (WEAX) and the water-unextractable arabinoxylans (WUAX) (Jaekel, da Silva, Steel, & Chang, 2012). One of the most important properties of WEAX is capable to form highly viscous solutions and gel by covalent cross-linking of AX chains through dimerization of FA by chemical or enzymatic oxidation (Dervilly-Pinel et al., 2001).

In our previous work, the behavior of AX during malting and brewing has been extensively investigated, the effects of mashing parameters on solubilization and hydrolysis of AX have also been examined, a mathematical model predicting AX concentration during the mashing process was also developed (Li, Lu, & Gu, 2005; Li, Lu, Gu, Shi, & Mao, 2005). Besides of that, arabinoxylan arabinofuranohydrolase I (AXAH-I), the key enzymes involved in the modification of the fine structure of AX and the hydrolysis of AX in germinated grain, was discovered to be different between malts with distinct filterability, which produced from the Chinese barley cultivar Dan'er and the Canadian barley cultivar Metcalfe (Jin et al.,

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2013). The role of AXAH-I in filterability has also been characterized (Li et al., 2015). By proteomic comparison, the peroxidase (POD) BP1 was also found to be with more abundance in domestic Dan'er malt facing filterability problems than Metcalfe malt with superior filterability (Jin et al., 2013). POD catalyze the conversion of a large number of substrates, notably phenolic compounds for biosynthetic and catabolic functions (Dicko, Gruppen, Hilhorst, Voragen, & van Berkel, 2006). In the current study, we surveyed the filterability properties of different barley malt samples, compared the enzyme activities of peroxidase and feruloyl esterase, as well as the AX bounded FA (AX-FA) content in each malt, and identified the effects of WEAX gel catalyzed by POD on filtration rate of wort, in order to find out the relationship of POD activity, WEAX cross linking, and the malt filterability for their quality improvement.

2. Materials and methods

2.1. Barley malts and filterability measurement

Eighteen malts made from various barley cultivars were provided by different Chinese commercial malting company in 2013 (as shown in Table 1). Malt samples were stored at 4 °C following receipt. Wort was produced by the European Brewery Convention (EBC) congress mash using each malt. And the filterability that represented by filtration rate, viscosity and clarity of the Congress wort, were measured by EBC official analytical methods (2006). Briefly, the filtration rate was calculated by the volume of filtered wort for 30 min after recirculation of the initial turbid wort. Viscosity was determined at 20 °C by a falling ball viscometer (Hoppler, Germany) and turbidity was measured according to the 90° scattering method on an EBC turbimeter.

2.2. Determination of FA

Free FA in the wort was extracted as previously described and analyzed by Agilent 1260 HPLC-UV system (Agilent CA, USA) (Dervilly-Pinel et al., 2001). Operating conditions were as follows: column, Zorbax Eclipse XDB-C_{18} (250 mm × 4.6 mm i.d., 5 μm, Agilent CA, USA), mobile phase A, 0.1% (v/v) acetic acid in deionized water, mobile phase B, 0.1% (v/v) acetic acid solution in HPLC-grade methanol, flow rate, 1 mL/min, column temperature, 25 °C. Detection was followed by UV absorbance at 320 nm. Elution was carried out in gradient mode as showed in Table 2. For determination of AX bounded FA (AX-FA), the wort was firstly treated with alkali to hydrolyze AX-FA into free FA. The total FA was then detected as mentioned above. The concentration of AX-FA was calculated by subtracting free FA from total FA.

2.3. Activity measurement of POD and feruloyl esterase

For crude enzyme preparation, the malt was homogenously milled in liquid nitrogen. Five grams of the powder was mixed with 50 mL phosphate buffer (50 mM, pH 6.0), and vortex for 1 h at 4 °C to extract cellular protein. After centrifugation at 10,000*g*, 4 °C for 20 min, the supernatant was collected and stored at 4 °C for enzyme activity determination. POD activity was measured spectrophotometrically by monitoring the H_2O_2-dependent oxidation of guaiacol, at 37 °C. Incubations were performed in 3 mL of 50 mM phosphate buffer containing 5 mM guaiacol and 20 μL of crude enzyme extract. For feruloyl esterase activity determination, 250 μL of methyl ferulate (1 mM in phosphate buffer pH 6.0) (Sigma, St. Louis, USA) was mixed with equal volume of diluted crude enzyme and incubated at 50 °C for 10 min. The reaction was stopped by adding of 0.5 mL glacial acetic acid. And the amount of released ferulic acid was determined by Agilent 1200 series HPLC-VWD system using a C18 column (250 mm, 4.6 mm, i.d., 5 μm, Agilent, CA, USA) as described (Szwajgier, 2009). One unit (U) of feruloyl esterase activity was defined as the amount of enzyme that used to liberate 1 μmol ferulic acid per min.

2.4. Isolation of WEAX from Dan'er malt

Malts were milled in a DLFU W23050 mill (Buhler, Braunschweig, Germany) to pass a 0.5 mm screen and then heated at 130 °C for 90 min to inactivate enzymes. The flour was extracted by suspension in distilled water (500 mL) and held in a water bath at 30 °C for 60 min with constant stirring. After centrifugation at 3000*g* for 15 min, the supernatant was heated in a boiling water bath for 30 min to precipitate soluble proteins, which were removed by centrifugation at 3000*g* for 15 min. The supernatant was then incubated at 90 °C for 30 min with thermal stable α-amylase (EC 3.2.1.1, Megazyme, 400 U). To further degrade the residual proteins, neutral protease was added to the supernatant and held in a water bath at 50 °C for 60 min, followed by heating in a boiling water bath for 15 min for enzyme inactivation. After centrifugation, the aliquot was precipitated overnight at 4 °C with 65% ethanol for two cycles. The precipitants were collected by centrifugation at 10,000*g* for 30 min and freeze dried to give purified WEAX.

Table 1
Filterability of collected barely malt samples in China produced in recent 3 years.

Malt samples	Filtration rate (mL/30 min)	Turbidity (EBC)	Viscosity (mPa s)	Production area	Production year
Dan'er-1[a]	140 ± 6	3.53 ± 0.09	1.52 ± 0.05	Jiangsu, China	2011
Dan'e-2[a]	145 ± 4	3.53 ± 0.05	1.60 ± 0.05	Jiangsu, China	2012
Dan'er-3[a]	240 ± 3	2.45 ± 0.07	1.55 ± 0.05	Jiangsu, China	2013
Dan'er-4[a]	245 ± 6	8.70 ± 0.09	1.60 ± 0.05	Jiangsu, China	2013
KA4B-1[a]	75 ± 4	1.95 ± 0.03	1.63 ± 0.03	Jiangsu, China	2012
KA4B-2[a]	160 ± 2	2.42 ± 0.04	1.59 ± 0.04	Jiangsu, China	2013
Supi 6–1[a]	130 ± 4	6.21 ± 0.14	1.65 ± 0.03	Jiangsu, China	2012
Supi 6–2[a]	215 ± 3	14.44 ± 0.23	1.59 ± 0.04	Jiangsu, China	2013
Kenpi 1	150 ± 4	1.78 ± 0.048	1.64 ± 0.04	Jiangsu, China	2012
Ganpi 4–1[a]	195 ± 7	2.66 ± 0.12	1.53 ± 0.04	Gansu, China	2012
Ganpi 4–2[a]	285 ± 4	1.43 ± 0.12	1.47 ± 0.05	Inner Mongolia, China	2012
Kenpi 7	240 ± 6	1.91 ± 0.08	1.47 ± 0.04	Inner Mongolia, China	2012
Mexican 500	60 ± 2	2.65 ± 0.10	1.57 ± 0.03	Yunnan, China	2012
Metcalfe-1[a]	320 ± 3	0.99 ± 0.07	1.44 ± 0.04	Canada	2012
Metcalfe-2[a]	300 ± 4	1.00 ± 0.08	1.47 ± 0.02	Canada	2013
Baudin-1[a]	80 ± 5	7.03 ± 0.11	1.51 ± 0.05	Australia	2011
Baudin-2[a]	255 ± 4	0.92 ± 0.09	1.46 ± 0.05	Australia	2012
Vlamingh	270 ± 4	1.42 ± 0.06	1.48 ± 0.03	Australia	2013

[a] The malt produced with same cultivar but in different year or area were indicated with numbers.

Table 2
Effect of vitamin C on the concentration and MW of PAX in wort.

	Added vitamin C (mg/g malt)			
	0	3	4	5
PAX concentration (mg/L)	934.4 ± 2.4	893.5 ± 3.1	726.9 ± 5.6	689.2 ± 4.4
PAX MW (kDa)	140.1 ± 0.9	116.7 ± 0.8	95.1 ± 0.8	93.8 ± 0.7

2.5. WEAX gelation by POD/H_2O_2 oxidation system

A WEAX solution (2% w/v in AX) was prepared in 0.1 mM phosphate buffer (pH 6.0) filtered through 0.22 μm screen. Since peroxidase BP1 from barley has been shown to share structure similarities and catalytic properties with horseradish peroxidase (HRPC) (Dicko et al., 2006), the HRPC (Sino-American Biotech., China) was used instead of purifying POD from barley malt for WEAX gelation. Cross-linking agent containing certain amount of HRPC (0.14 U/mg AX, the average activity of POD in the 18 malt samples) and different amount H_2O_2 (0, 0.125, 0.5 and 2.0 mg/g AX) were added to WEAX solutions for gel development at 25 °C for 2 h (Carvajal-Millan et al., 2005). The molecular weight (MW) of WEAX gel was determined by HPGFC-RID (Waters, USA) with tandem analytical Ultrahydrogel 1000 column (300 mm × 7.8 mm i.d., Millipore Co, USA). A total of 20 μL of WEAX solution was injected and isocratic elution was carried out with 0.1 M $NaNO_3$ at a flow rate of 0.9 mL/min.

2.6. Adding of POD, WEAX gels and vitamin C to mashing process

At the beginning of EBC congress mashing of Supi 6 malt I (50 g), a series amount of HRPC (0, 13×10^3, 19.5×10^3, 26×10^3 U/g malt), or WEAX gel originated from 150 mg of extracted WEAX catalyzed by 0.14 U/g AX of POD with the existence of 0, 0.125, 0.5 and 2 mg/g AX of H_2O_2, or vitamin C (3, 4, 5 mg/g malt) was added, respectively. At the same time, EBC congress mashing of Supi 6 malt without extra POD or WEAX gel or vitamin C was performed as control. At the end of mashing, filterability (wort filtration rate, viscosity, and turbidity) was measured. In addition, in vitamin C added wort, PAX (the high MW arabinoxylan) was precipitated with 80% (V/V) ethanol for concentration determination using the Douglas method (Douglas, 1981; Li, Lu, Gu, Shi et al., 2005). The precipitant was further treated with amyloglucosidase and lichenase (endo-1,3(4)-β-D-glucanase) (Megazyme, Ireland) to remove dextrin and β-glucan in PAX, the average MW of PAX were then detected with HPGFC-RID (high-performance gel filtration chromatography system with differential refraction detector) system with a tandem analytical Ultrahydrogel™ Linear Column (300 × 7.8 mm i.d.) as described (Shang, Li, Cai, & Lu, 2015).

2.7. Statistical analysis

All data were analyzed with SPSS 19.0. The routine statistical calculations were applied (mean values calculation with standard deviation). A value of $p \leqslant 0.05$ was taken as the measure statistically significance results.

3. Results and discussion

3.1. Monitoring 18 malt samples with different filterability

Filterability is one of the key quality traits of barley malt in beer brewing. In the present study, 18 malt samples produced from Chinese, Australian and Canadian barley cultivars in recent three years were collected for filterability survey (Table 1). The results showed that Chinese barley malts, especially malt produced with Dan'er, a widely grown barley cultivar in Jiangsu Province of China, has encountered severe filterability problems, including high viscosity and turbidity of wort, and a low wort filtration rate. Use of the filterability defect malt will delay the beer production process, and increase the risk of haze formation in the final beer product.

The activity of POD during mashing process using malt samples Dan'er, Supi 6, KA-4B and Metcalfe were monitored. POD in Metcalfe kept low activity during the whole mashing process, while the activity of POD in the other three samples was almost 10 times higher than that in Metcalfe in the first 30 min of mashing, and then declined as mashing going on (Fig. 1), suggesting that POD may catalyze formation of filterability negative factors during mashing. POD was able to oxidize di-FA or tri-FA bridges formation causing the coupling of AX chains (Schooneveld-Bergmans, Dignum, Grabber, Beldman, & Voragen, 1999), while the feruloyl esterase could release FA from them. Therefore, the activities of POD and feruloyl esterase as well as the concentrations of AX-FA in the wort made from each malt sample were measured at the same time. There showed negative correlations of wort filtration rate between POD activity and AX-FA concentration, with correlation coefficient of −6.028 and −34.194, respectively (Fig. 2a and b), and positive correlation of wort filtration rate between feruloyl esterase activity with correlation coefficient of 0.620 (Fig. 2c). These results further suggested that the formation of FA bridges among AX chains significantly contribute to the filterability defect.

3.2. The negative effect of extra POD on filterability

POD was resistant to extreme temperatures associated with ale malt kilning and mashing (Clarkson, Large, & Bamforth, 1992). The mean activity of POD in Dan'er and Metcalfe malt was 18.8×10^3 U/g malt and 5.9×10^3 U/g malt, respectively. To further confirm the negative effect of POD on wort filterability, a series amount of POD (0, 1, 1.5 and 2 times of the difference-value of enzymatic activity between Dan'er and Metcalfe cultivars) was added at the beginning of mashing process using Supi 6 malt, which has the best filterability among domestic malt samples. It was found that with more extra POD added, the filterability of wort became worse, the filtration rate declined by 20%, the turbidity and viscosity increased by 10.3% and 4.4%, respectively (Fig. 3a), suggested that the high concentration or activity of POD was one of the key factors of malt filterability.

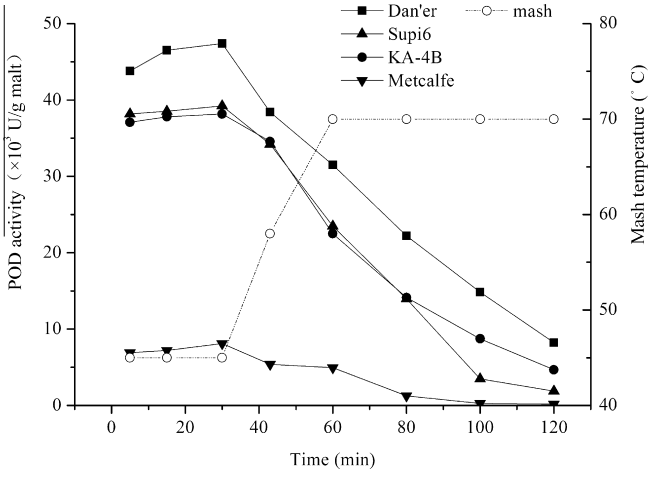

Fig. 1. POD activities tracking during mashing using malts with distinct filterability.

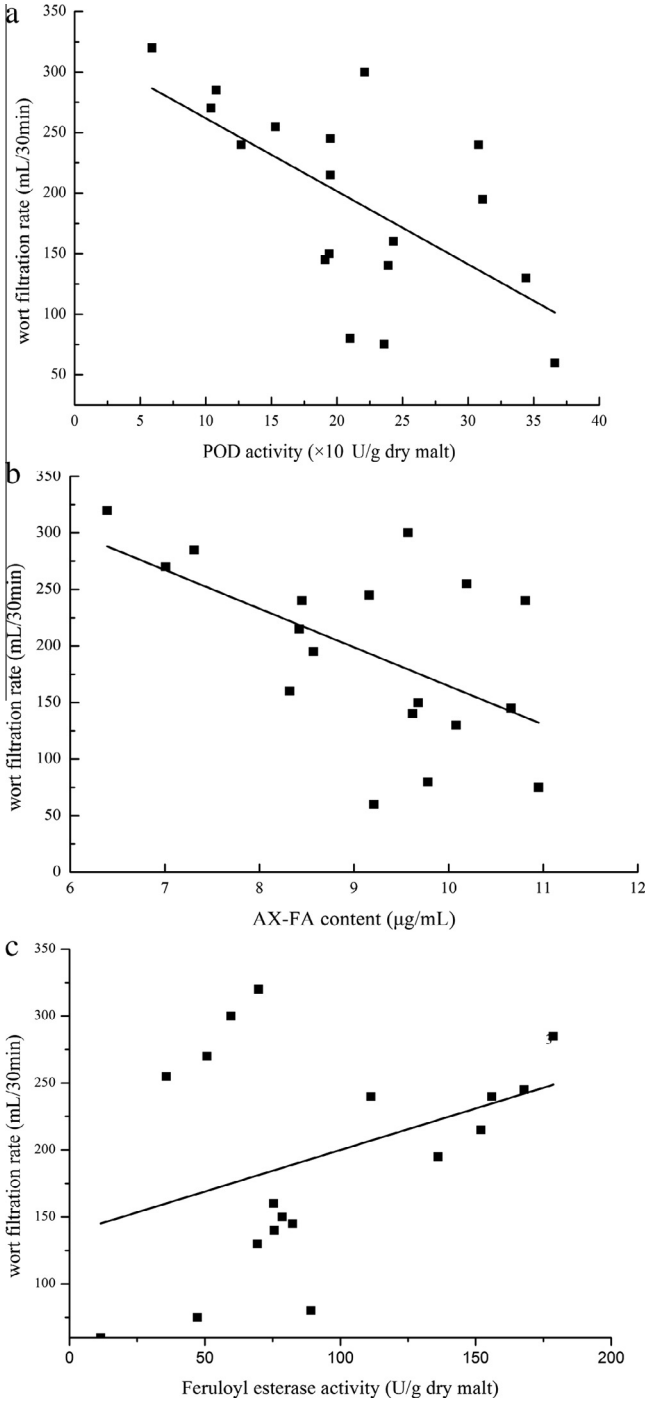

Fig. 2. The correlation of malt filterability between POD activity (a), AX-FA concentration (b) and feruloyl esterase activity (c).

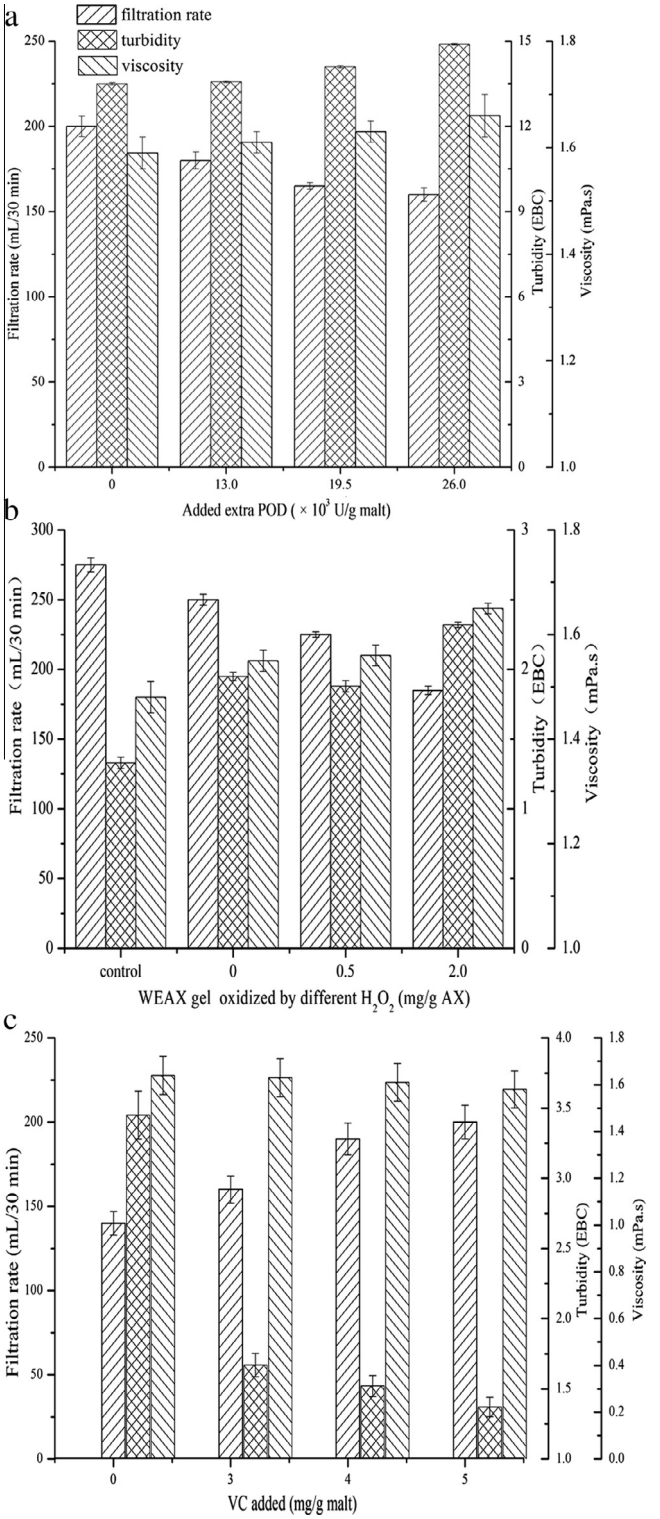

Fig. 3. The effects of extra POD (a), WEAX gel with different cross linking degrees (b), and vitamin C (c) on wort filterability of Supi 6.

3.3. Cross linked WEAX gel catalyzed by POD decreased the filtration rate

The WEAX was extracted from Dan'er malt with average MW of 94 kDa. Partially gelation of WEAX was obtained by the POD/H_2O_2 oxidation system containing different amount of H_2O_2 according to the studies by Schooneveld-Bergmans et al. (1999). The concentrations of FA, as well as the MW of AX in each WEAX gel were determined. With more H_2O_2 existed, the contents of AX-FA declined from 16.3 μg/mL to 0.7 μg/mL, and the MW of AX increased from 93.6 to 1797.7 kDa (Supplementary Table S1), suggested more FA bridges formed in WEAX gels. These WEAX gel with different cross linking degrees originated from 0.5 time of the WEAX content in 50 g malt was added at the beginning of mashing to investigate its effect on filterability. It was found that with cross linked WEAX gel, the filtration rate decreased significantly, while the turbidity and viscosity increased (Fig. 3b). With extensively cross linked WEAX gel catalyzed with POD and 2 mg/g AX of H_2O_2, the filtration

rated decreased by 32.7%, the turbidity and viscosity increased by 74.4% and 11.5%, respectively. These results indicated that during mashing process, the cross linking of FA among AX chains catalyzed by POD in the wort was an important factor that negatively impacted wort filterability.

3.4. Vitamin C eliminate ill effects of POD on wort filterability

Vitamin C was known to be a strong antioxidant, thus could be used as the POD inhibitor to further confirm the negative role of POD on wort filterability. With the different amount of vitamin C added to the start of mashing process, the wort filterability has greatly improved, especially the wort turbidity (Fig. 3c). With 5 mg/g malt of vitamin C added, the turbidity of wort decreased by 60.3%, and the filtration rate increased by 30%, the viscosity lightly decreased by 3.7%. Meanwhile, the POD activity in wort was almost inhibited, with reduction rate of 96.1%. The PAX concentration and MW in all the wort samples were determined (Table 2). It showed that with more vitamin C added, the cross linking of AX and FA was much greater blocked, leading to less content and lower MW of PAX formed.

In conclusion, the present study verified the negative effect of POD on wort filterability. By survey a large amount of barley malt in Chinese market, it was found that malt with defect filterability contained high amount of POD and AX-FA, and exhibited lower feruloyl esterase activity. POD was active during mashing process and thus promoted the gelation of AX chains by di-FA and tri-FA cross linking bridges, forming highly viscous solutions, therefore caused low filtration rate, high turbidity and viscosity of the wort. By addition of the POD inhibitor could improve filterability by some degree.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.10.130.

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