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Purification and characterisation of arabinoxylan arabinofuranohydrolase I responsible for the filterability of barley malt

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ABSTRACT

Dan'er is a widely grown malt barley cultivar in China, but its filterability defects have severely impeded its application in beer brewing. Previous investigations have suggested that we should identify the malt filterability correlated proteins, one of which was postulated to be arabinoxylan arabinofuranohydrolase I (AXAH-I). To verify this hypothesis, we purified AXAH-I from Dan'er malt, characterised its enzyme performance, and investigated its influence on filterability by adding different amounts of purified enzyme to the mash. With 6 mU g^{-1} malt AXAH-I supplemented, the wort separation rate increased by 31.8%, viscosity decreased by 3.6%, and the endosperm reserve contents declined concomitantly. Unexpectedly, the wort turbidity increased with increasing AXAH-I. We also tried to optimise the use of currently available commercial enzyme products for filterability improvement in beer brewing, by supplementing them with purified AXAH-I and β-amylase. AXAH-I could be a functional component for novel commercial enzyme products in the beer industry.

1. Introduction

Malt, made from barley (*Hordeum vulgare* ssp. *vulgare*), is the most important raw material for beer production (Jamar, du Jardin, & Fauconnier, 2011). In China, malt produced from the widely grown Dan'er cultivar, has encountered severe filterability problems, such as high viscosity and turbidity, and a low wort separation rate. The deficiency in Dan'er malt filterability not only delays the production process, but also increases the risk of haze formation in the final beer product, and impedes Dan'er malt's application in the beer industry (Sungurtas, Swanston, Davies, & McDougall, 2004; Zhang, Wang, & Chen, 2002).

Filtration and viscosity problems can largely be attributed to incomplete degradation of endosperm reserves during barley germination (Schneider, Krottenthaler, Back, & Weisser, 2005). Arabinoxylans (AXs) are the major components of the endosperm, making up 20–25% of the cell wall (Fincher, 1989). Barley grain AXs consist of a (1 → 4)-β-linked backbone of D-xylopyranosyl residues, with α-L-arabinofuranosyl residues linked at C3 and C2. AXs may also be partially cross-linked by diferulic acid bridges (Vinkx & Delcour, 1996) and other polysaccharides such as β-glucan, which is another major constituent of the cell wall (Izydorczyk & Biliaderis, 1995). These adjuncts in AXs have been found to decrease filterability and increase wort viscosity (Lu & Li, 2006; Sadosky, Schwarz, & Horsley, 2002), and thus contribute to filtration problems and haze formation during brewing (Vietor, Voragen, & Angelino, 1993).

In our previous work, we have applied comparative proteomic analysis to malts produced from domestic and imported Australian and Canadian barley cultivars for breeding improvement. The differentially expressed proteins are mainly hydrolases and pathogen-related proteins (PRs). Several of these, including the positive effector β-amylase and the negative effector serpin Z7, have been purified and verified to be associated with filterability (Jin et al., 2013a, 2013b; Jin et al., 2014). Additionally, the AXAH-I (a family 51 arabinoxylan arabinofuranohydrolase), which removes α-L-arabinofuranosyl side chains from the backbone of AXs (Ferre, Broberg, Duus, & Thomsen, 2000), was found to be less abundant in Dan'er malt than in the imported Canadian malt Metcalfe (Jin et al., 2013a). The near full-length cDNA encoding barley AXAH-I obtained by Lee, Burton, Hrmova, and Fincher (2001), indicated

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that the mature enzyme consists of 626 amino acid residues and has a calculated *pI* of 4.8, and a MW of 65 kDa. AXAH-I has been proposed to participate in the modification of the fine structure of AXs and to play roles in the hydrolysis of AXs in germinated grain. The aim of this research was to purify AXAH-I from the Dan'er malt and further verify AXAH-I's key role in malt filterability by supplementation analysis, with a view to optimising malting conditions and improving the filterability of domestic barley for the beer industry.

2. Materials and methods

2.1. AXAH-I activity assay in different malt samples

Dan'er and Metcalfe malts were collected from a commercial maltster in China. Dan'er malt was produced from barley which was harvested in 2011 in the Jiangsu Province of China, and the Metcalfe malt was made from imported barley in 2011. Congress wort was prepared with Dan'er and Metcalfe malt (Jin et al., 2013a). AXAH-I activity was assayed by determining the 4-nitriphenol content at 410 nm (Ferre et al., 2000). The standard reaction mixture contained 0.5 mL 0.5 mM 4-nitrophenyl-α-L-arabinofuranoside (4NPA) and an appropriate amount of mash. Reactions were initiated by incubating the mixture at 40 °C for 30 min, and terminated by adding 1 mL 0.5 M Na_2CO_3. One unit of enzyme was defined as the amount of AXAH-I that produced 1 μmol 4-nitriphenol per minute.

2.2. AXAH-I purification

AXAH-I was purified from the Dan'er malt according to previously described methods, with several modifications (Ferre et al., 2000; Lee et al., 2001). Briefly, 1 kg of Dan'er malt was ground using a grinder, and then suspended in 40 mL buffer A (0.1 M acetic acid–sodium acetate, pH 5.0) and incubated at 4 °C with 50 rpm shaking overnight. The lysate containing the total protein extract was clarified by centrifugation at 10,000*g* for 15 min at 4 °C, and then fractionally precipitated by stepwise addition of ammonium sulphate to saturation levels of 20% and 40%. The final precipitant was then suspended in buffer B (0.05 M acetic acid–sodium acetate, pH 5.0) and dialyzed with the same buffer.

The following purification was performed with GE AKTA™ avant 25 (GE Healthcare, Sweden). The crude extract was loaded onto a cation exchange column (HiTrap 16/10 SP FF, GE Healthcare, Sweden). The unbound flow through peak was then collected, concentrated, dissolved in buffer C (0.05 M acetic acid–sodium acetate, pH 5.2), and loaded onto an anion exchange column (HiPrep 16/10 Q FF, GE Healthcare, Sweden). Protein elution was carried out with buffer C, supplemented with 1 M NaCl at a flow rate of 4 mL min^{-1}. The collected fractions were analysed using AXAH-I activity assay described above (Ferre et al., 2000). The fractions containing AXAH-I were pooled, concentrated, and submitted to the size exclusion column (HiPrep 16/60 Sephacryl S-100 HR, GE Healthcare, Sweden), with a flow rate of 0.5 mL min^{-1}. Fractions with AXAH-I activity were further identified by SDS–PAGE and MALDI-TOF/TOF as previously described (Jin et al., 2013a), and finally were lyophilised for future analysis.

2.3. Enzymatic characterisation of AXAH-I

To determine the optimum pH, AXAH-I activity was measured at 40 °C in the pH range 2.0–8.0 using 0.1 M Na_2HPO_4-citrate acid buffer. To determine the optimum temperature, AXAH-I activity was measured in the standard reaction mixture at pH 4.5 with the temperature range 20–80 °C. To determine the thermal stability of the AXAH-I, the enzyme was incubated in a 0.1 M Na_2HPO_4-citrate acid buffer (pH 4.5) at various temperatures (20–80 °C) for 10 min before measuring activity. The effect of metal ions, including K^+, Na^+, Ca^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+} and Cu^{2+} on AXAH-I activity was examined by carrying out the standard reaction in the presence of selected ions (2 mM). The effects of EDTA and SDS were determined in the presence of 1 mM EDTA and 0.05% SDS respectively. Kinetic parameters were determined for 4NPA at concentrations in the range of 0.1–20-fold K_m. Assays were performed in triplicate in the standard reaction mixture containing a fixed amount of AXAH-I and varied amounts of 4NPA. The apparent values of K_m and *V*max were calculated from Lineweaver–Burk plots.

2.4. Measurement of wort filterability with extra AXAH-I

A series amount of purified AXAH-I (0, 2, 4, 6 mU g^{-1} malt) was added at the start of EBC Congress mashing process using Dan'er malt. At the end of mashing, the filterability of the product, including its separation rate, turbidity and viscosity, was determined according to the method described by Jin et al. (2013a).

2.5. Determination of macromolecule content in wort with supplemented AXAH-I

After mashing, the concentrations of major macromolecules that correlated with wort filterability were determined. The AXs content was determined using the Douglas method (Douglas, 1981). The wort was precipitated with 80% ethanol to collect high molecular weight AXs for polymeric arabinoxylan (PAX) determination (Li, Lu, & Gu, 2005). The concentration of β-glucan in the Congress wort was determined with the Congo-red method (Naoyuki, Masaru, Masaharu, & Noriko, 1998).

2.6. Determination of arabinose/xylose (A/X) ratio in wort

The monosaccharide composition of the Congress wort, supplemented with different amounts of AXAH-I, was analysed using a set of Dionex ICS-5000 HPAEC/PAD (Thermo Fisher scientific, Waltham, MA, USA) as previously described, with several modifications (Krahl, Muller, Zarnkow, Back, & Becker, 2009). Operating conditions were as follows: column, Dionex CarboPac PA20 (0.4 × 150 mm, 6.5 μm, Thermo Fisher scientific, Waltham, MA, USA), mobile phase A, H_2O, mobile phase B, 0.25 M NaOH, mobile phase C, 1 M NaAc, flow rate, 0.5 mL min^{-1}. Elution was carried out in gradient mode as shown in Supplementary Table S1. Pre-column derivatization was performed by mixing 2.0 mL sample with 3.0 mL 4 M HCl, heating in a water bath for 60 min for hydrolysis, and then neutralizing with 3.0 mL of 4 M NaOH. The A/X ratio was then calculated by comparing the peak area of each monosaccharide.

2.7. Preparation of commercial enzyme

In beer manufacture, supplementation with commercial enzyme products at 0.4 mg enzyme g^{-1} malt is common for filterability improvement. To find the optimum commercial enzyme product for Dan'er malt, purified AXAH-I (6 mU g^{-1} malt) and purified β-amylase (40 U g^{-1} malt) were added to the malt, as in our previous work (Jin et al., 2013a). The mixed enzyme preparations were then added to the mash to determine their effects on filterability as described above. Due to commercial sensitivity, the provider of commercial enzyme product will not be named.

3. Results and discussion

3.1. AXAH-I activity in Dan'er and Metcalfe worts

The activity of AXAH-I was monitored during the EBC mashing process, using Dan'er and Metcalfe malts (Fig. 1). With both malts, AXAH-I displayed increased activity at the beginning of the mashing process, got maximum activity as the mashing temperature was raised to 55 °C, lost activity rapidly when the temperature was higher than 65 °C, and then showed no activity when the temperature was kept at 70 °C for 20 min. However, throughout the mashing process, the AXAH-I activity of Dan'er malt was always lower than the AXAH-I activity of Metcalfe. The highest AXAH-I activity shown by Dan'er wort was 9 mU g^{-1} malt, which was only 60% of the activity of Metcalfe (13 mU g^{-1} malt), a disparity of 4 mU g^{-1} malt. In the same action period, AXAH-I in Metcalfe could degrade more AXs and other macromolecules with synergistic reactions, thus leading to better filterability.

3.2. Purification of AXAH-I from Dan'er malt

AXAH-I was extracted from milled Dan'er barley malt and purified approximately 99-fold using a combination of cation exchange, anion exchange, and size exclusion chromatography (Supplementary Fig. 1 and Table S2). The initial purification step was cation exchange chromatography on HiTrap 16/10 SP FF at pH 5.0. The unbound fraction displayed the highest specific AXAH-I activity (Supplementary Fig. 1), and thus was collected for further purification and characterisation. The AXAH-I preparation contained two polypeptides in almost equivalent amounts with apparent molecular weights of 65 and 60 kDa (Fig. 2), both of which were identified as AXAH-I by MALDI-TOF/TOF (Supplementary Table S3). The SDS–PAGE gel presented two separated bands corresponding to AXAH-I, even after treatment with PNGase F, indicating that they were not caused by N-glycosylation of AXAH-I. This result was inconsistent with the previous germinated barley isolated AXAH-I (Ferre et al., 2000; Lee et al., 2001). The 60 kDa polypeptide was therefore proposed to be partially digested, or an isoenzyme with different modifications (Ferre et al., 2000).

3.3. The enzymatic characteristics of AXAH-I

AXAH-I was active in the pH range 2.0–8.0, and the maximum activity was detected at pH 4.5 (Fig. 3a). AXAH-I activity was detected at temperatures between 20 and 80 °C, with the

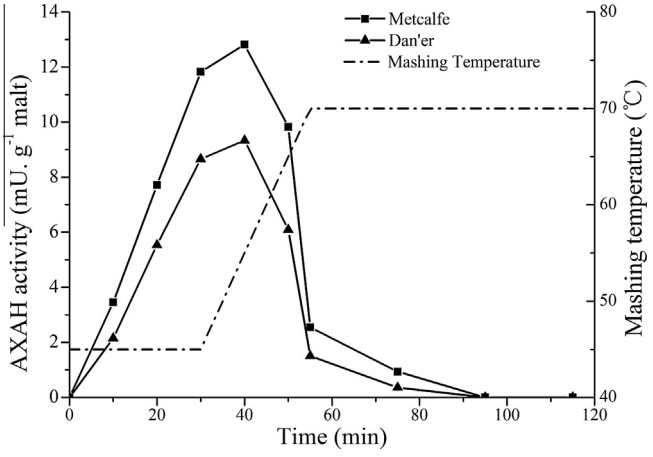

Fig. 1. AXAH-I activity during the mashing process with Dan'er and Metcalfe malts.

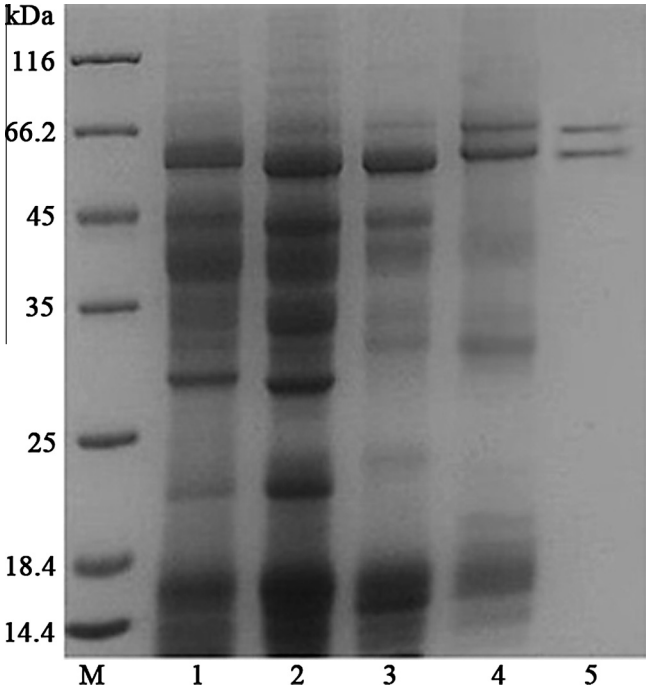

Fig. 2. SDS–PAGE of AXAH-I during purification process of crude extract (lane 1), fractional precipitate (lane 2), cation exchange column (lane 3), anion exchange column (lane 4) and size exclusion column (lane 5). Lane M, molecular mass markers.

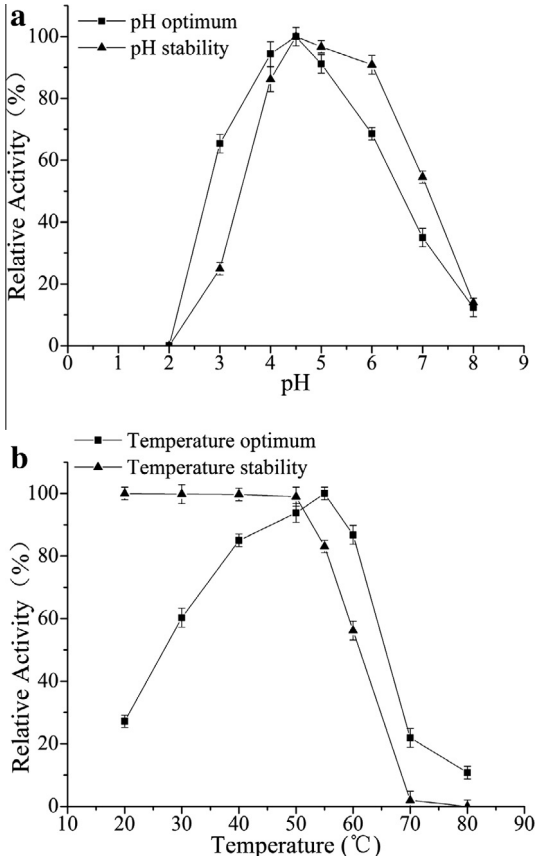

Fig. 3. The pH optimum and stability (a) and temperature optimum and stability (b) of AXAH-I.

Table 1
Effect of metal ions and chelators on AXAH-I activity.

Metal ion/chelator	Relative activity[a]
None	100
Na^+	78.72
K^+	84.04
Ca^{2+}	81.38
Mg^{2+}	83.16
Fe^{2+}	38.65
Cu^{2+}	2.48
Zn^{2+}	48.05
Mn^{2+}	104.08
EDTA	51.24
SDS	53.01

[a] The relative activity of 100% corresponds to 2.31 mU mg^{-1} for AXAH-I with no metal ions nor chelators added in the standard reaction mixture.

maximum activity at 55 °C. Incubation at 20–50 °C for 30 min did not affect the activity of AXAH-I. When incubated at 50–65 °C, the enzyme retained more than half of the initial activity. However, when the temperature was higher than 65 °C, AXAH-I was inactivated rapidly (Fig. 3b). The optimal pH and temperature of AXAH-I from Dan'er malt was a little different from the AXAH-I previously isolated from germinated barley, which showed maximum activity at pH 4.2 and 60 °C (Ferre et al., 2000). The temperature sequence used in the industrial mashing process is 40 °C for 20 min, 40–45 °C for 5 min, 45 °C for 30 min, 45–52 °C for 5 min, 52 °C for 30 min, 52–62 °C for 5 min, 62 °C for 30 min, 62–72 °C for 5 min, 72 °C for 20 min, 72–78 °C for 2 min, and then 78 °C for 10 min. Therefore, AXAH-I could actively catalyse AXs degradation when the mashing temperature was increased to 62 °C, but could not work when the temperature reached 72 °C.

The effects of metal ions, EDTA and SDS on enzyme activity were also examined (Table 1). The activity of AXAH-I was strongly inhibited by Cu^{2+} (>95% inhibition). Chelators and metal ions, apart from Mn^{2+}, also inhibited the AXAH-I activity to different extents (17–62%). In contrast to most of the metal ions we tested, Mn^{2+} enhanced the activity of AXAH-I by 4%.

The K_m for AXAH-I on 4NPA was 0.46 ± 0.007 mg mL^{-1}, and V_{max} was 0.22 ± 0.005 U mg^{-1}.

3.4. Effects of added AXAH-I on wort filterability and macromolecules contents

During the mashing process, the highest activity of AXAH-I in Dan'er was 4 mU g^{-1} malt lower than that of AXAH-I from Metcalfe malt. Therefore, a series amount of purified AXAH-I (0, 2, 4 and 6 mU g^{-1} malt) was added at the beginning of EBC mashing using Dan'er malt, to test the effect of AXAH-I on wort filterability. As shown in Fig. 4, extra AXAH-I added to the mash enhanced the separation rate, decreased the viscosity, and also, unexpectedly, increased the turbidity. When 6 mU g^{-1} malt of AXAH-I was added, the separation rate and turbidity were increased by 34.2% and 32.5% and the viscosity was reduced by 5.5%.

In the brewing industry, soluble fibre is often believed to increase wort viscosity and thus to result in prolonged lautering and filtration time. Therefore, the extraction ratio, and the concentrations of the major macromolecules that correlate with malt filterability, were determined (Table 2). The extraction ratio and AXs concentration increased continually with increasing AXAH-I, while PAX and β-glucan were decreased. The arabinose/xylose (A/X) ratio, which was found to be positively related with the AXs branching degrees, decreased with increasing AXAH-I.

Low membrane filtration efficiency causes low extraction ratios of wort. The extraction ratio increased when AXAH-I was added, suggesting that filterability was enhanced. The increased AXs content in the wort suggested that extra AXAH-I improved the solubility of AXs. PAX, defined as high molecular weight AXs, is a negative indicator of the degradation rate of AXs (Li, Lu, Gu, & Mao, 2005). The decrease of PAX indicated that added AXAH-I promoted the degradation of AXs. However, AXAH-I has been reported to have no hydrolysing activity on β-glucan, another major constituent of the barley endosperm cell wall (Lee et al., 2001). It has been postulated that intermolecular alignment between polymer chains or non-covalent interactions of AXs and other non-starch polysaccharides like β-glucan cause the formation of multicomponent gels in the cell wall complex. Therefore, we might assume that AXAH-I changed the fine structure of AXs which caused marked effects on its physiochemical properties, such as solubility, and its interactions with other wall polysaccharides like β-glucan. These effects lead to increased degradation of AXs and β-glucan, both of which are associated with filterability problems, especially viscosity. However, no study has explored the relationship between wort turbidity and AXs, and the mechanism by which AXAH-I could increase wort turbidity is unclear.

3.5. Compound enzymes for Dan'er malt filterability improvement

In beer manufacture, commercial enzyme product mainly consisting of β-glucanase, xylanase and protease, has frequently been used to overcome filterability problems of malts. However, commercial enzyme product has not always been effective on Dan'er

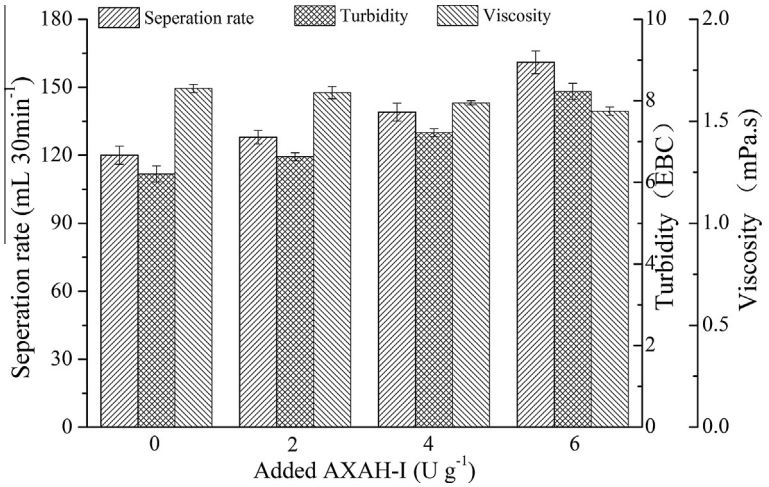

Fig. 4. Effects of AXAH-I adding to the mash using Dan'er malt on the filterability.

Table 2
The contents of macromolecules in EBC wort of Dan'er with added AXAH-I.

Macromolecules	Added AXAH-I (mU/g malt)			
	0	2	4	6
Extraction ratio (%)	72.74 ± 0.02	73.32 ± 0.01	74.49 ± 0.03	75.66 ± 0.01
AX (mg L^{-1})	934.37 ± 4.24[a]	946.94 ± 5.05[a]	962.66 ± 3.85[a]	987.80 ± 2.35
PAX (mg L^{-1})	849.51 ± 4.13	836.94 ± 3.47	821.23 ± 4.96[a]	782.94 ± 3.59[b]
β-Glucan (mg L^{-1})	220.78 ± 2.13	212.94 ± 2.64	193.33 ± 2.95[b]	189.17 ± 3.01[b]
Ratio of A/X	0.87 ± 0.06	0.83 ± 0.03	0.72 ± 0.04[a]	0.6 ± 0.05[b]

[a] $p < 0.05$.
[b] $p < 0.01$.

malt. It was proposed that Dan'er malt lacked other key enzymes. Therefore, we determined the activities of potentially important enzymes (Supplementary Table S4). The commercial enzyme product was found to contain trace activities of AXAH-I and β-amylase. In our previous work (Jin et al., 2013a), we proved that β-amylase could improve filterability. Therefore, we combined AXAH-I and β-amylase from Dan'er malt with commercial enzyme product, and added them to the mash process using Dan'er malt. It was found that, supplemented with 6 mU g^{-1} malt AXAH-I and 40 U g^{-1} malt β-amylase, the new enzyme compound could raise the separation rate from 177 ± 4 mL 30 min^{-1} to 214 ± 5 mL 30 min^{-1}, and reduce the turbidity from 8.53 ± 0.03 EBC to 3.44 EBC and the viscosity from 1.56 ± 0.02 mPa s to 1.52 mPa s, respectively. Unlike supplementation with AXAH-I alone, the combination of enzymes improved all three parameters of Dan'er malt filterability, which suggested that filterability was a complex issue affected by several factors. AXAH-I and β-glucan are potential new components for an innovative and effective commercial enzyme product to improve filterability of Dan'er malt in the malting and beer industries.

In conclusion, the present study has purified AXAH-I from Dan'er barley malt, which has severe filtration problems. By enzymatic characterisation and supplementation experiments, we found that AXAH-I was active during mashing and thus changed the structure of AXs. This structure change promoted the degradation of AXs and β-glucan, which have negative effects on filtration and lautering, and thus enhanced the separation rate, viscosity and wort extraction ratio. Improved understanding of AXAH-I and its role in filterability could allow the creation of an innovative and effective combined enzyme agent for overcoming Dan'er malt's filterability deficiency.

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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.2014.11.024.

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