Circular dichroism and infrared spectroscopic characterization of secondary structure components of protein Z during mashing and boiling processes

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ABSTRACT

In beer brewing, protein Z is hypothesized to stabilize beer foam. However, few investigations have revealed the relationship between conformational alterations to protein Z during the brewing process and beer foam. In this report, protein Z from sweet wort was isolated during mashing and boiling processes. Circular dichroism (CD) and Fourier transform infrared spectroscopy (FTIR) were used to monitor the structural characteristics of protein Z. The results showed that the α -helix and β -sheet content decreased, whereas the content of β -turn and random coil increased. The complex environment rich in polysaccharides may facilitate conformational alterations and modifications to protein Z. Additionally, the formation of extended structural features to protein Z provides access to reactive amino acid side chains that can undergo modifications and the exposure of hydrophobic core regions of the protein. Analyzing structural transformations should provide a deeper understanding of the mechanism of protein Z on maintaining beer foam.

Keywords: Protein Z, Secondary structure, Mashing and boiling processes, CD, FTIR

1. Introduction

Beer is one of the most consumed alcoholic beverages worldwide and its raw materials include malted cereals, hops and beer yeast (Colgrave, Goswami, Howitt, & Tanner, 2013). The balance and stability of constituents of chemicals in beer, such as proteinaceous components, polysaccharides and hop acids determine the quality of beer foam (Bamforth, 1985). In several previous investigations, proteins were demonstrated to be the main factor responsible for both positive and negative effects on beer foam (Bamforth, 2004). On average, beer contains 2–6 g/L peptide, which mainly originates from the cereal grains (Osman, Coverdale, Cole, Hamilton, Jersey, & Inkerman, 2002). During beer production, the germinated malt undergoes a series of controlled procedures and several changes occur to proteins, including degradation, glycation, acylation and structural unfolding (Perrocheau, Bakan, Boivin, & Marion, 2006). The residual polypeptide fractions are mainly responsible for the quality of the finished products (Gupta, Abu - Ghannam, & Gallagher, 2010). The protein fragments cover a broad range of molecular weight sizes and are involved in haze formation and foam stability (Kanerva, Sontag - Strohm, & Lehtonen, 2005). Among these proteins, protein Z and lipid transfer protein (LTP) are the main proteins found in beer. Both protein Z and LTP are heat-stable and resist enzyme degradation during the brewing process (Yokoi, Yamashita, & Kunitake, 1994). Protein Z was the first protein to be found and characterized as being associated with the stability of beer foam, and structurally belongs to the serine proteinase inhibitor family (Hejgaard, Rasmussen, Brandt, & Svendsen, 1985). Protein Z has a molecular mass of ~43 kDa and exists as three isoforms: Z4, Z7 and Zx. The Zx isoform has only been reported in a few reports (Fasoli, Aldini, Regazzoni, Kravchuk, Citterio, & Righetti, 2010; Konecná, Müller, Dosoudilová, Potesil, Bursíková, Sědo, et al., 2012). The genes encoding Z4 and Z7 are from chromosomes 4 and 7, respectively (Hejgaard, 1984). Protein Z4 is the predominant type found in malt, accounting for ~80% of the total protein Z (Evans, Sheehan, & Stewart, 1999). Protein Z has the highest surface viscosity and elasticity in beer proteins, as described by Maeda *et al.* (1991). Therefore, it has been suggested that protein Z

stabilizes beer foam (Maeda, Yokoi, Kamada, & Kamimura, 1991). In recent investigations, proteomic techniques have been used to study protein Z. The results show that protein Z is glycated by the Maillard reaction on the second day of the malting process (Bobálová, Petry-Podgórska, Laštovičková, & Chmelík, 2010). Approximately 16% of the lysine residues of protein Z are hypothesized to be modified during the brewing process (Hejgaard & Kaersgaard, 1983) and the modified protein Z promotes the stability of the foam (Janette Bobalova & Chmelik, 2007). A fraction of protein Z precipitates upon binding small-sized fragments from sweet wort during the boiling process (Iimure, Nankaku, Kihara, Yamada, & Sato, 2012). In contrast to the above research, other studies have indicated that protein Z plays no major role in beer foam stability. In this study, normal malt, Z4-deficient malt, Z7-deficient malt and double-deficient malt were used in beer brewing, and no significant beer trait differences were detected among the four types of beer produced, indicating that protein Z is not significantly correlated with the foam stability of beer (Iimure, Kimura, Araki, Kihara, Sato, Yamada, et al., 2012).

Brewing, mashing and boiling processes create a complex environment in which parts of protein Z are modified. The environment and these modifications may induce structural changes to protein Z that enable the protein to maintain a better quality beer foam (Bamforth, 1985; Wüstneck, Wüstneck, Perez-Gil, & Pison, 2003). However, there is a paucity of data describing the secondary structure of protein Z during the mashing and boiling processes. In addition, modifications to protein Z have not been fully investigated. Thus, there are no clear explanations for the reason why protein Z can maintain beer foam. It is also unknown how structural changes to protein Z affect beer foam during the brewing process. Therefore, analyzing the structural evolution of protein Z should potentially reveal the mechanism of beer foam stabilization. Currently, circular dichroism (CD) and Fourier transform infrared spectroscopy (FTIR) are widely used techniques in predicting the structure of polypeptides and revealing the secondary structures of proteins (Pelton & McLean, 2000). CD in the far UV region provides information on the structural composition of the polypeptide backbone of proteins (Whitmore & Wallace, 2008). FTIR reflects changes

to function groups and structural transitions by detecting the amide regions of proteins. The conformational amide I region in the range of 1700–1600 cm⁻¹ of the FTIR spectrum can be used to reveal the secondary structure of proteins (Barth, 2007). Combined with deconvolution and curve-fitting of the amide I region, the secondary structural compositions of proteins can be predicted (Singh, 2000).

In this report, CD and FTIR were used to determine the effects of the environment and modifications on the structural transformations of protein Z during mashing and boiling processes. Structural alterations to protein Z with respect to stabilizing beer foam are also presented.

2. Materials and methods

2.1. Materials

Sodium dihydrogen phosphate, disodium hydrogen phosphate, ethanol, ammonium sulfate and sodium chloride were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). The protein markers sample was purchased from TAKARA Biotechnology Co., Ltd. (Dalian, China). Ultrafiltration centrifuge tubes were purchased from Millipore Co., Ltd (Boston, MA, USA). HiPrep 16/10 DEAE FF and Superdex 75 10/300 GL for trapping protein material and separation were purchased from GE Healthcare (Pittsburgh, PA, USA). Canada barley of Metcalfe was malted and purchased from COFCO (Beijing, China).

2.2. Sweet wort and boiled wort preparations

Sweet wort was prepared according to the mashing procedure. The crushed Metcalfe malt and deionized water were mixed at the proportion of 1:4.5 and kept at 48 °C for 30 min. The temperature was then raised to 63 °C for 40 min and then to 72 °C for 20 min. Before the mashing procedure finished, the iodine test was conducted and mashing was conducted at 78 °C. The rate of temperature increase during each step was 1 °C/min. Samples were taken at the onset and end of each temperature procedure.

After the mashing process, the sweet wort was filtered and adjusted to 12 °P and then boiled for 60 min according to the boiling procedure. 0.3% (w/v) of hops was added. Samples were obtained after boiling for 30 and 60 min.

2.3. Protein extraction and mass spectrometry

All samples were salted out with ammonium sulfate using a saturation range of 40–60%. The precipitates were collected by centrifugation at $6000 \times g$ for 10 min and resuspended in deionized water for dialyzing. Subsequently, the crude protein Z solution was loaded onto an anion exchange column HiPrep 16/10 DEAE FF connected to an AKTA avant protein purification system (GE Healthcare, Pittsburgh, PA, USA). Elution of the proteins was achieved with a linear gradient of 0–150 mM sodium chloride/200 mM phosphate buffer (pH 6.5). Eluted fractions were concentrated by centrifugal concentrators at $4000 \times g$ for 20 min. The concentrated target fraction was loaded onto a size-exclusion column Superdex 75 10/300 GL and the protein eluted using a 150 mM sodium chloride/200 mM phosphate buffer (pH 7.0). The eluted fractions were collected and the protein detected by SDS-PAGE (Leiper, Stewart, & McKeown, 2003). The stained protein band from SDS-PAGE was in-gel digested by trypsin and analyzed via an Ultraflex MALDI-TOF/TOF mass spectrometer. The positive ion reflect mode was used to analyze the peptides and the Bruker peptide calibration standard II method was used to calibrate the spectra. Flex Analysis software and BioTools software were used to analyze the peptide fragments. The proteins were identified with an in-house Mascot server (<http://www.matrixscience.com>) on the Green Plant taxonomy database of the National Center for Biotechnology Information (Bruker-Daltonics, Germany) (Jin, Mu, Sun, Li, Gao, & Lu, 2012). The samples of sweet wort at the end of the 48 °C (48 °C End), 63 °C (63 °C End) and 72 °C (72 °C End) steps were purified. The samples of sweet wort at the beginning of the 63 °C (63 °C Start) and 72 °C (72 °C Start) steps were purified. The samples of mashing (Mashing), boiling wort for 30 min (Boiling) and wort (Wort) were also purified.

2.4. Circular dichroism (CD) spectrophotometry

CD spectra were collected on a MOS-450/AF-CD spectropolarimeter (BioLogic, Grenoble, France). The purified proteins from all sweet wort samples were freeze-dried and dissolved in deionized water to 0.1 mg/mL. A 1-mm path-length quartz cuvette was used and data were recorded from 190 to 250 nm with deionized water as the background at 25 °C. Each CD spectrum was scanned at 30 nm/min, data points were recorded at 1-nm intervals and the response time was 2 s (Kelly, Jess, & Price, 2005).

2.5. Fourier transform infrared spectroscopy (FTIR) spectra

The FTIR spectra were obtained using a Thermo Nicolet Nexus 470 FTIR spectrometer (Thermo Nicolet Analytical Instruments, MA, USA). The purified and freeze-dried proteins from all sweet wort samples were mixed with grinding potassium bromide and then scanned in the range of 4000 to 400 cm⁻¹. The scanning number was 64 times with a resolution of 4 cm⁻¹ (Barth, 2007; M Jackson & Mantsch, 1993). To further reveal information on protein Z, the amide I region in the original spectra was analyzed via Omnic software combined with deconvolution and curve fitting (Kauppinen, Moffatt, Mantsch, & Cameron, 1981; Kong & Yu, 2007).

3. Results

3.1. Identification of purified proteins

Protein Z was isolated by HiPrep 16/10 DEAE FF and Superdex 75 10/300 GL column chromatography after salting out with ammonium sulfate. All of the collected fractions were initially confirmed by SDS-PAGE (Supplementary Figure 1). SDS-PAGE of purified protein Z from all samples is shown in Figure 1. The result shows a purified band between 29 and 44.3 kDa in all lanes. The purified protein was identified by MALDI-TOF MS/MS and the result showed the purified band corresponded to protein Z (Supplementary Table 1).

3.2. Structural analysis of protein Z during the mashing process

Changes to the secondary structure of protein Z during the mashing process was monitored by CD. As shown in

Figure 2A, the broad negative peaks at ~220 nm correspond to the absorption of α -helices in the $n-\pi^*$ region, which is consistent with a previous study (Sreerama & Woody, 2003). However, the other negative peak typical for the presence of α -helix elements in a protein at ~208 nm was not obvious in the spectra. Therefore, protein Z does not display the typical CD spectral features of a protein that contains α -helix, as described previously (Levitt & Chothia, 1976). A typical feature of a CD spectrum for a protein with α -helices is the presence of a positive peak at ~192 nm; however, a strong positive peak was presented at ~195 nm, which demonstrates the existence of β -sheets. Therefore, protein Z appears to contain β -sheet (Whitmore & Wallace, 2008). As the temperature increased during the mashing process, the peaks at ~220 nm shifted towards negative wavelength, whereas the intensity of the peak at ~195 nm gradually decreased in intensity. These changes suggested an increase in disorder of the protein, i.e., random coil due to thermal unfolding. The destabilization of α -helices and an increase in random coil content both contribute to an increase in the intensities of peaks in the range of 200 to 220 nm. The negative peak at ~212 nm increased in intensity (i.e., greater negative values) in the CD spectrum of the mashing sample and the moderate change in the CD spectrum also showed that the β -sheet content decreased. As the temperature increased, the spectra showed that a structural transformation from standard secondary structure elements to turns and random coil had occurred. However, β -turns were still the main structure. The conformational transformations indicate that the secondary structure of protein Z loosened or decreased because of denaturation during mashing process. Such a transformation leads to the exposure of amino acid residues from the normally buried hydrophobic core of protein Z. During the mashing process, malt starch was degraded into polysaccharides and was rich in sweet wort, which would provide more opportunities to modify the less well-structured protein Z (Perrocheau, Bakan, Boivin, & Marion, 2006).

FTIR experiments were also conducted to monitor conformational modifications and structural alterations due to the mashing procedure. The FTIR absorptions of protein Z during the mashing process after baseline correction and vectorial normalization are shown in Figure 2B. All FTIR spectra showed two strong absorption bands at 3295

and 2961 cm^{-1} . The two absorption bands were associated with the amide A region and the amide B region, which are contributions from N–H stretching vibrations and C–N stretching vibration, respectively. As the temperature increased, the absorption peaks of both regions moved to high wavenumbers and the relative intensities at ~ 3300 cm^{-1} were observed to broaden gradually. These changes indicated that the N–H stretching vibration strengthened during the mashing process. It has been reported that D-glucose reacts with free amine groups to be a Schiff base in the Maillard reaction and then the Schiff base is arranged to form Amadori compounds (Schalkwijk, Ligtoet, Twaalfhoven, Jager, Blaauwgeers, Schlingemann, et al., 1999). After structural arrangement as the Maillard reaction, the N–H groups in the protein increased. Glycated lysine residues in the Maillard reaction were detected in the protein. Protein Z has a high content of lysine residues (Hejgaard & Kaersgaard, 1983), therefore, multiple modifications of lysine residues may occur to protein Z during the mashing process. The absorption peaks in the range of 1100–1000 cm^{-1} resulted from C–OH and C–C stretching vibration of the saccharides, and the peaks vibrated repeatedly in all FTIR spectra, which indicated that the protein was modified by saccharides. In the FTIR spectra there are three sensitive regions for analyzing structural transformations of proteins. The amide I region caused by the C=O stretching vibration of the peptide backbone ranges between 1700 and 1600 cm^{-1} . The amide II region caused by the C–N stretching vibration and N–H bending vibration ranges between 1600 and 1500 cm^{-1} , and the amide III region arises from the C–N stretching vibration and N–H bending vibration and ranges between 1360 and 1200 cm^{-1} (Michael Jackson, Choo, Watson, Halliday, & Mantsch, 1995). The absorption peaks of the amide I, II and III regions in the FTIR spectra were found at ~ 1655 , 1536 and 1238 cm^{-1} , respectively (Figure 2B). Among all the regions, the frequencies of the amide I region components closely correlated to each secondary structure element in the protein (Barth, 2007). In Figure 2C, the amide I region of protein Z was analyzed to investigate the structural alterations that occur during the mashing process. The absorption peaks at ~ 1635 cm^{-1} gradually decreased and the absorption of the amide I region moved towards high wavenumbers. To reveal the changes in internal structure of

protein Z, deconvolution combined with curve fitting of the amide I region were carried out, and the original spectra were separated into 10 peaks based on the Gauss formula, as depicted in Figure 3 (Byler & Susi, 1986). In each figure, the top spectrum is the original data of the amide I region and the fitting peaks are presented in the bottom panels of the figure. Since the area and amplitude of the amide I region showed significant differences, the quantities of various peak areas were studied. From Table 1, differences in secondary structure content based on the amide I region were observed during the mashing and boiling processes. Along with mashing process, an increased content of aggregated strands at around 1615 cm^{-1} was observed. The total content of β -sheet decreased during the mashing process with a change from 35.03 to 29.55%, indicating parts of the cohesion inside the molecules was destroyed. The same change of intermolecular β -sheets at 1625 cm^{-1} and intramolecular β -sheets at 1635 cm^{-1} was observed, and the value of intermolecular β -sheets and intramolecular β -sheets fell from 14.90 to 10.03% and 18.33 to 15.08%, respectively. The results showed that the inter-hydrogen and intra-hydrogen bonds weakened. The ratio of intermolecular β -sheets to intramolecular β -sheets fell from 0.81 to 0.67, indicating the relative contents of intramolecular β -sheets had increased, and the intramolecular β -sheets had a dominant effect on the β -sheet structure. The ration was found to increase to 0.82 at the end of the $63\text{ }^{\circ}\text{C}$ procedure, indicating a large number of intra-hydrogen bonds were disrupted. Moreover, a high relative disruption of inter-hydrogen bonds during the $72\text{ }^{\circ}\text{C}$ procedure was observed because the ratio dropped to 0.67. These changes indicated that a structural transition from intermolecular β -sheets to intramolecular β -sheets occurred (Dong, Kendrick, Kreilgård, Matsuura, Manning, & Carpenter, 1997). Although the total number of hydrogen bonds in β -sheets decreased, changes from intermolecular to intramolecular hydrogen bonds were observed and the content of random coil, as measured by the value at 1645 cm^{-1} , increased. The content of helix structure, as measured by the intensity of the signal at $\sim 1653\text{ cm}^{-1}$, had clearly decreased during the mashing process and reached a minimum value by the end of the mashing process. While the total content of β -turns increased significantly, as determined from the values in the range of $1660\text{--}1693$

cm⁻¹. The areas of peaks at 1660, 1667, 1673, 1682 and 1682 cm⁻¹ varied significantly during the mashing process. This phenomenon showed that the variability of β -turns of protein Z was reversible and the internal β -turns of protein Z did not alter readily during these processes. The ratio of random coil to helices increased from 1.10 to 1.73, as monitored by the C=O stretching vibration of protein Z. Moreover, regions of helices transformed into random coil elements. The structural investigations on protein Z from FTIR spectra showed the same alterations as observed in the CD data (Singh, 2000).

3.3. Structural analysis of protein Z during the boiling process

The CD spectra of protein Z during the boiling process are shown in Figure 4A. All spectra moved towards high wavelengths during the different steps of the process. Compared with the CD spectra of the mashing process, the negative peak present at ~223 nm and the profile over the range of 215 to 225 nm were more apparent in the wort sample. The α -helix and β -sheet content continued to decrease. In contrast, the amount of β -turns and random coil was observed to increase. The FTIR spectrum after baseline correction and vectorial normalization presented the same regularity as the CD spectra and was shown in Figure 4B. In the amide A region, one new peak appeared at 3411 cm⁻¹ in the spectrum of the wort sample, which arised from the N-H stretching vibration. During the boiling process the high temperature destoryed the disulfide bonds of protein Z and a moderate intensity absorption peak caused by S-H stretching vibration appeared at around 2431 cm⁻¹. All spectra during the boiling process indicated that the secondary structure of protein Z continued to be an extended conforamtion. The amide I region spectra of protein Z during the boiling process are shown in Figure 4C and the absorption peaks were observed to move to higher wavenumbers as the process proceeded through the steps. Compared with the spectra of the mashing process, the absorption peaks at around 1635 cm⁻¹ gradually decreased in intensity and finally disappeared in the spectrum of the wort sample. The signal of the C=O stretching vibration became weak in all spectra, indicating the random coil content had increased and reached a maximum at the end of the boiling process. Deconvolution and curve fitting

were also used to analyze structural components of protein Z during the boiling process. The results are shown in Figure 5 and Table 1. As shown in Figure 5 and Table 1, aggregated strands at around 1615 cm^{-1} decreased from 4.44 to 1.36%. The extreme high temperature in the boiling procedure may denature the protein. Both intermolecular β -sheets and intramolecular β -sheets dropped from 10.03 to 8.91% and 15.08 to 14.78%, respectively. The variations may be caused by the destruction of the intra- and inter-hydrogen bonds of protein Z. In addition, the ratio of intermolecular β -sheets to intramolecular β -sheets decreased from 0.67 to 0.6, which also indicated that the intra- and inter-hydrogen bonds were damaged in the high temperature environment. The total α -helix content and β -sheet content increased continuously during the whole process, whereas opposite changes in the total content of β -turns and random coil were observed. As a result, the secondary structure of protein Z further unfolded during the boiling process.

4. Discussion

Previous studies have shown that protein Z maintains the stability of beer foam after the brewing process (Janette Bobalova & Chmelik, 2007). Protein Z is not readily degraded by proteases during the mashing and boiling processes, and modifications by glycation to protein Z could be one reason for protease resistance. As reported, the presence of adducts of protein Z with a molecular mass of 162 Da during malting process have been monitored, and the detected glucose suggests the Maillard reaction occurs to protein Z (Janette Bobalova & Chmelik, 2007; J. Bobalova, Petry-Podgorska, Lastovickova, & Chmelik, 2010). The Maillard reaction is a non-enzymatic reaction. Covalent linkages can form between protein-free amino groups and reducing sugar. During mashing and boiling processes, the high temperature treatments and high contents of reducing sugar in the sweet wort are favorable factors for the Maillard reaction to occur. α -NH₂-terminal amino acids and ϵ -NH₂ of the side chain of lysine residues are the most reactive groups that combine with reducing sugar and condense to stable Amadori compounds (J.

Bobalova, Petry-Podgorska, Lastovickova, & Chmelik, 2010). Protein Z has a high content of lysine residues that become potential sites for glycations. The components of sweet wort are complex and the low molecular weight carbohydrates from the degradation of malt starch are multitudinous; therefore, protein Z could be heterogeneously modified by the presence of the various carbohydrates, such as glucose and maltose in malt. In this work, we showed that the transformations to protein Z occurred during mashing and boiling processes. Some of the disulfide bonds were disrupted, as shown in the FTIR results, which suggests that the structural transformations could occur when the disulfide bonds are reduced. The alterations to the polypeptide chain and destruction of disulfide bonds could cause structural loosening or partial denaturation. Moreover, the complex environment and modifications may disrupt hydrogen bonds and the hydrophobic core fold of protein Z, which further promotes structural unfolding into an extended state. The variability of the internal structure could contribute to a rapid increase in the number of possible amino acid modification sites and this leads to exposure of the hydrophobic regions. The less compact structure provides a larger number of reactive sites for reactions to occur with the surrounding carbohydrate rich environment, and the carbohydrates that combine with the protein can protect protein Z from precipitating during the brewing process (Pierce, 1978). More modifications and the relative content of the hydrophobic regions confer protein Z with greater innate ability to maintain the stability of beer foam. Since glycations can reinforce the surface properties of protein Z by enhancing the amphiphilicity, the modified hydrophilic carbohydrates may increase the viscosity of protein Z to prevent liquid drainage from beer foam and localize in the liquid between bubbles (Roberts, 1975). Hydrophobic groups and amphipathic domains of extended protein Z may easily attach to the air-water interfaces of beer foam. Partial disulfide bond disruptions can be beneficial to air-water interfaces (Kella, Yang, & Kinsella, 1989). The loose structure combined with modifications to protein Z enables this protein to better maintain beer foam. Therefore, glycations, disulfide bond reduction and extension of the secondary structure during mashing and boiling processes may provide an explanation for the better efficiency of protein Z in stabilizing beer foam.

In summary, the structural characterization of protein Z showed distinct differences during the mashing and boiling processes. Protein Z is tolerant to high temperature and resistant to enzyme degradation. The destruction of hydrogen bonds during the processes leads to the formation of a more extended protein structure. The structural alterations exposes normally buried hydrophobic groups and provides a larger number of potential reactive modification sites. Meanwhile, the transformations provide evidence for the importance of surface-exposed amino acids and modifications to these amino acids can affect protein function. These changes provide a basis for understanding the function of protein Z in maintaining foam stability. Investigations on the functional groups and structural transformations triggered by environmental factors of protein Z should be further studied.

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Figure legends

Fig. 1. SDS-PAGE of protein Z samples after purification from the mashing and boiling processes. Lane M:

Protein molecular weight markers (Mr: kDa); Lanes 1–9: 48 °C, End; 63 °C, Start; 63 °C, End; 72 °C, Start; 72 °C, End; Mashing; Boiling 30 min; Wort.

Fig. 2. Analysis of protein Z during the mashing process. Arrows indicate the samples of purified protein Z

from Canada Metcalfe malt. (A): Circular dichroism spectra of the purified protein Z during the mashing process.

The protein concentration was 1 mg/mL in deionized water. (B): The FTIR spectra of the purified protein Z during

the mashing process after baseline correction and vectorial normalization. Numbers indicate the main peak positions.

(C): The FTIR spectra of the amide I region of protein Z during the mashing process.

Fig. 3. Original (top) and curve-fitting (bottom) spectra of the amide I region of protein Z during the mashing

process. a–f: amide I region in the FTIR spectra of protein Z for the samples of: 48 °C End; 63 °C Start; 63 °C End;

72 °C Start; 72 °C End; Mashing.

Fig. 4. Analysis of protein Z during the boiling process. Arrows indicate the samples of protein Z from Canada

Metcalfe malt. (A): Circular dichroism spectra of the purified protein Z during the boiling process. The protein

concentration was 1 mg/mL in deionized water. (B): The FTIR spectra of the purified protein Z during the boiling

process after baseline correction and vectorial normalization. Numbers indicate the main peak positions. (C): The

FTIR spectra of the amide I region of protein Z during the boiling process.

Fig. 5. Original (top) and curve-fitting (bottom) spectra of the amide I region of protein Z during the boiling

424 process. a–b: amide I region of the FTIR spectra of protein Z samples after boiling for 30 min and Wort,
425 respectively.
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Table1. Quantitative estimation of the secondary structure of protein Z

Secondary structure	48°C End		63°C Start		63°C End		72°C Start		72°C End		Mashing		Boiling 30min		Wort	
	Peak	Area	Peak	Area	Peak	Area	Peak	Area	Peak	Area	Peak	Area	Peak	Area	Peak	Area
	(cm ⁻¹)	(%)	(cm ⁻¹)	(%)	(cm ⁻¹)	(%)	(cm ⁻¹)	(%)	(cm ⁻¹)	(%)	(cm ⁻¹)	(%)	(cm ⁻¹)	(%)	(cm ⁻¹)	(%)
Aggregated strands	1613	1.80	1615	2.72	1614	2.71	1614	3.33	1615	4.03	1615	4.44	1616	4.07	1614	1.36
Intermolecular sheets	1625	14.90	1625	14.08	1625	13.24	1625	10.87	1625	10.32	1625	10.03	1625	10.08	1625	8.91
Intramolecular sheets	1635	18.33	1635	17.98	1635	16.21	1635	16.22	1635	15.93	1635	15.08	1634	15.16	1634	14.78
Random coils	1645	14.57	1645	14.45	1644	14.91	1645	16.70	1645	17.34	1645	18.38	1645	19.06	1645	19.24
Helices	1653	13.25	1653	11.97	1652	11.92	1653	11.79	1653	10.89	1653	10.61	1653	10.33	1653	10.09
Turns	1660	9.76	1660	9.85	1660	12.06	1660	11.22	1660	11.24	1660	12.90	1660	11.20	1660	14.03
Turns	1667	8.65	1667	8.81	1667	8.65	1667	7.81	1667	8.32	1667	8.51	1667	9.06	1667	8.74
Turns	1674	6.03	1674	10.08	1674	8.03	1673	9.87	1673	10.04	1674	7.05	1674	9.11	1674	8.34
Turns	1682	10.03	1684	7.86	1683	10.16	1683	9.55	1683	10.09	1683	10.46	1683	9.64	1683	12,20

Turns	1692	2.68	1693	2.20	1693	2.11	1692	2.64	1692	1.80	1692	2.54	1693	2.29	1692	2.31
Total helices	13.25		11.97		11.92		11.79		10.89		10.61		10.33		10.09	
Total sheets	35.03		34.78		32.16		30.42		30.28		29.55		29.31		25.05	
Total turns	37.15		38.80		41.01		41.09		41.49		41.46		41.30		45.62	
Total random coils	14.57		14.45		14.91		16.70		17.34		18.38		19.06		19.24	
Random coils/helices ratio	1.10		1.21		1.25		1.42		1.59		1.73		1.85		1.91	
Intermolecular sheets/Intramolecular sheets ratio	0.81		0.78		0.82		0.67		0.65		0.67		0.66		0.60	

Figure 1

Figure 2

(A)

(B)

(C)

Figure 3

Figure 4

(A)

(B)

(C)

Figure 5