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Role of non-covalent interactions in the production of visco-elastic material from zein ☆

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ABSTRACT

The role of non-covalent interactions in the formation of visco-elastic material from zein was investigated. Hydrophobic interactions were evaluated through the addition of various salts from the Hofmeister series. Urea, ethanol, and beta mercaptoethanol (β-ME) were used to evaluate the effects of protein denaturation and disulfide bonds on zein's ability to form a visco-elastic material. The addition of NaI and NaSCN altered the properties of visco-elastic materials made from zein, making them softer and more extensible, as did urea and ethanol. The addition of NaCl and Na_2SO_4 negatively impacted the ability of zein to from a visco-elastic material and at higher concentrations completely disrupted the formation of visco-elastic material. These results indicate that manipulating non-covalent interactions in zein can alter and in some cases, completely disrupt the formation of a visco-elastic material. Specifically this may be due to disruption of hydrophobic interactions within individual zein proteins or interactions between proteins. The reducing agent β-ME had little effect on zein's ability to form a visco-elastic material. Therefore, the visco-elastic properties of zein arise as a result of non-covalent interactions.

1. Introduction

Zein has played a vital role in many industrial applications throughout the last century (Lawton, 2002; Shukla & Cheryam, 2001). In more recent times, zein has been shown to be able to produce a wheat like dough for the production of gluten-free breads for people with coeliac disease (Mejia, Mauer, & Hamaker, 2007; Oom, Pettersson, Taylor, & Stading, 2008; Schober, Bean, Boyle, & Park, 2008; Schober, Bean, Tilley, Smith, & Ioerger, 2011; Schober, Moreau, Bean, & Boyle, 2010). While there has been substantial research conducted on the properties of zein–starch dough systems, the mechanism of how isolated zein forms a visco-elastic material is not well known. Commercial zein isolates are comprised almost entirely of α-zeins (Lawton 2002). This fraction of zein is known to contain low levels of cysteine, with the 19 kDa α-zein containing only one cysteine residue and the 22 kDa α-zein having two (Shewry & Tatham, 1990). It is well known that cysteine residues in wheat proteins play a key role in the unique visco-elastic properties of gluten, via the formation of very high molecular weight disulfide linked protein complexes. When the disulfide bonds in gluten are cleaved, wheat gluten loses its visco-elastic functionality (Shewry & Tatham, 1997). How zein forms wheat-like dough with little to no cysteine residues is not currently known.

Because commercial zein contains little to no cysteine residues, and typically does not contain appreciable amounts of very high molecular weight covalently linked disulfide bonded protein polymers, the presence of large gluten-like polymeric protein complexes is probably not the reason for zein being able to form a visco-elastic material. Commercial zein isolates alone are capable of forming a visco-elastic material (VEM) when mixed with water above the glass transition of the proteins in the absence of starch (Oom et al., 2008). Thus, functionality is due to protein–protein interactions, which give zein the ability to form a VEM. As large covalently linked protein complexes are not present in commercial zein isolates, non-covalent protein interactions probably play a key role in zein's visco-elastic functionality.

There are various forms of non-covalent interactions that can occur within and between proteins. Of these, hydrogen bonding and hydrophobic interactions have been widely studied. Both urea and ethanol have been used to examine the effect of hydrogen bonding in proteins (Mitchell & Littman, 2000; Zhang & Cremer, 2010). However, high concentrations of urea have been shown to promote the unfolding of proteins because it is thermodynamically

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favourable due to the lack of bulk water in contact with the protein. Urea is also capable of direct interactions with the peptide backbones of proteins (Bennion & Daggett, 2003; Zhang & Cremer, 2010), which has to be taken into account when using urea to study the role of non-covalent interactions in proteins.

One traditional method for studying the hydrophobic interactions of proteins is through the use of salts ranked on their ability to salt in or salt out proteins. This ranking or series of salts is known as the Hofmeister series (Jungworth & Winter, 2008; Melander & Horvath, 1977; Peterson & Saykally, 2006; Selling, Maness, Bean, & Smith, 2013; Zhang & Cremer, 2010). While the exact mechanism of the salting in or salting out is not completely understood, the addition of these salts is thought to change the three dimensional structure of the proteins and/or their interaction with water and the salts (i.e. co-solvents). This can disrupt or promote hydrophobic or hydrophilic interactions within a protein depending on the salt used and the protein in question (Jungworth & Winter, 2008; Melander & Horvath, 1977; Peterson & SayKally, 2006; Zhang & Cremer, 2010).

A number of researchers have used salts from the Hofmeister series to investigate the role of non-covalent protein interactions on the mixing properties of gluten (He, Roach, & Hoseney, 1992; Melnyk, Dreisoerner, Bonomi, Marcone, & Seetharaman, 2001; Preston, 1989). Wellner, Bianchini, Mills, and Belton (2003) studied the impact of anions from the Hofmeister series on the secondary structure of gluten.

Thus, the purpose of this research was to investigate the role of non-covalent interactions in commercial zein functionality using a similar approach that has been successfully used with wheat gluten. Reagents such as ethanol and urea were added during mixing to determine the effect of denaturants on zein mixing properties. Similarly, to determine the role of hydrophobic interactions in zein's functionality, aqueous solutions of salts spanning the entire spectrum of the Hofmeister series were added to zein while being mixed in a farinograph at elevated temperatures. The results of this research will provide greater insight into how and why zein functions and allow for future manipulation of zein to increase its use in wheat-free food products and industrial products.

2. Materials and methods

2.1. Materials

Zein isolate (90.6% protein on a dry matter basis and 5.2% moisture) was purchased from Sigma–Aldrich, Co. (St. Louis, MO). Vital wheat gluten (70.8% protein on a dry matter basis and 6.8% moisture) was obtained from Midwest Grain Products (Atchison, KS). Na_2SO_4, NaCl, NaI, NaSCN, urea and beta mercaptoethanol (β-ME) were purchased from Sigma–Aldrich, Co. (St. Louis, MO). Ethanol under the trade name CHROMASOLV (95% ethanol + 5% isopropanol) was purchased from Sigma–Aldrich, Co. (St. Louis, MO). Molecular weight standards lysozyme from chicken egg white (14 kDa), carbonic anhydrase (29 kDa), and bovine serum albumin (66 kDa) were purchased from Sigma–Aldrich, Co. (St. Louis, MO).

2.2. Visco-elastic material formation

In order to determine the importance of the different bonding interactions on zein VEM formation, zein was mixed by a Farinograph-AT (Duisburg, Germany) at 73 rpm for 20 min at 40 °C. For all mixing experiments, 40 g of zein was placed in the farinograph's 50 g mixing bowl. After 1 min of calibration time, 20 ml of de-ionised (DI) water or solution of interest was added and mixing continued. Treatments included aqueous solutions of Na_2SO_4, NaCl, NaI and NaSCN at concentrations of 0–2 M, urea at concentrations 0–4 M, and ethanol at concentrations of 5–15% (v/v). In addition, 2% (β-ME) (v/v) was also used as a treatment. Vital wheat gluten was examined under the same conditions as zein, except gluten was run at 30 °C with 30 ml of water.

After mixing in the farinograph for 20 min, zein VEMs were immediately frozen in liquid nitrogen, stored at −80 °C, and lyophilized. Lyophilized VEM was ground using a mortar and pestle and stored at −20 °C on desiccant for subsequent analyses. For visual representation of the materials formed during mixing, in some cases, zein and gluten were immediately removed from the farinograph and photographed.

2.3. Molecular weight distribution

To determine if there were any changes to the molecular weight distribution of the zein proteins during mixing, lyophilized samples collected from the farinograph-AT were solubilised at a concentration of 4 mg sample to 1 ml of a 50 mM Tris–borate, pH 10.0 buffer containing 2% SDS for 30 min with continuous vortexing. Samples were centrifuged for 5 min at 9300*g* and the aliquots were collected and heated for 5 min at 95 °C. Molecular weight standards were solubilised and analysed in the same manner and used to indicate molecular weight distribution of zein samples. Molecular weight standards were lysozyme from chicken egg white (14 kDa), carbonic anhydrase (29 kDa), and bovine serum albumin (66 kDa). To determine if any disulfide linked polymers were present, 2% β-ME (v/v) was added to an aliquot of the solubilised proteins prior to analysis by size exclusion chromatography (SEC). Samples were analysed via SEC using an Agilent 1100 HPLC system equipped with a Biosep-3000 column (Phenominx, Torrance, CA) and guard column using 50 mM sodium phosphate, pH 7.0 buffer containing 1% SDS (w/v) as a mobile phase. Proteins were detected at 214 nm with a UV detector over a 30 min span with a flow rate of 1 ml/min and an injection volume of 20 µl. Column temperature was fixed at 40 °C.

2.4. Solubility

To determine how the different reagents affected zein solubility, 5 mg of zein was vortexed for 30 min in 1 ml of a given treatment solution and centrifuged at 9300*g* for 5 min. The supernatant was collected and analysed via SEC as described above. Salt treatments solutions included Na_2SO_4, NaCl, NaI and NaSCN at a concentration of 2 M. Other treatments included ethanol at a concentration of 15% (v/v). Urea was also analysed at concentrations of 1 M, 2 M, and 4 M. Controls included a negative control consisting of DI H_2O and a positive control of 50 mM Tris–borate pH 10.0 buffer containing 2% SDS. Zein was completely dissolved in later solvent. Treatments were compared by integrating the total peak area from the SEC chromatograms with peak area for the sample dissolved by 2% SDS equalling 100% solubility. Solvent blanks were analysed for each treatment to insure that no sample matrix peaks were included in the integration of results.

2.5. Surface hydrophobicity

Surface hydrophobicity was determined as described by Chelh, Gatellier, and Sante-Lhoutellier (2006) with modifications. Zein (5 mg protein) was vortexed for 30 min at 40 °C in 20 mM Na-Phos buffer pH 6.0 with 2 M concentrations of Na_2SO_4, NaCl and NaSCN. A control mixed only with the 20 mM Na-Phos buffer was also measured. After vortexing, samples were allowed to cool to room temperature (~22 °C) and 200 µl of 1 mg/ml bromophenol blue (BPB) in DI H_2O was added. Samples plus BPB were then vortexed for 10 min. After vortexing the samples were centrifuged for 15 min at 2000*g*. Then, 900 µl of 20 mM Na-Phos buffer pH 6.0

was added to 100 μl of the supernatant (1:10 dilution). The diluted samples were vortexed and read at an absorbance of 595 nm on a Beckman DU 530 Life Science UV/Vis Spectrophotometer (Beckman, Coulter, CA). The instrument was blanked with 20 mM Na-Phos buffer pH 6.0. Samples were compared against their corresponding controls that contained only the sample matrix (i.e. the salts) at the appropriate concentration. Surface hydrophobicity was calculated using the equation:

$$\text{BPB bound } (\mu g) = 200\ \mu g\ (\text{Abs control} - \text{Abs sample})/\text{Abs control}$$

2.6. Experimental design

All HPLC analyses were run in duplicate. For reproducibility, regression analysis was completed for duplicated of treatments. It was found than all duplicates had an $R^2 \geqslant 0.98$.

Samples were run in triplicate for the solubility and surface hydrophobicity tests and analysis of variance was completed with a $P < 0.05$ using Statistical Analysis Software (SAS 9.1, SAS Institute Inc., Cary, NC). A comparison of means using Tukey's studentized range test was used to determine differences in hydrophobicity.

3. Results and discussion

3.1. Visco-elastic material formation

To determine the role of non-covalent interactions during zein mixing 1 M and 2 M solutions of Na_2SO_4, NaCl, NaI and NaSCN were added to zein–water mixtures during mixing in a farinograph at a temperature above the glass transition of zein. Glass transition of commercial zein can vary due to variations between lots. However, for these experiments, mixing at 40 °C was considered optimum and above the zeins glass transition as defined by Schober et al. (2008, 2010). The same lot of zein was used in this research as in the work of Schober et al. (2008, 2010). The salts were selected to be representative of the Hofmeister series, where salts are ranked on their ability to "salt in" or "salt out" proteins. The addition of Hofmeister salts is known to change the secondary and tertiary structure of the proteins by altering the interaction among proteins, water and the co-solvent (salts). In some cases Hofmeister salts have been shown to bind directly to the protein, thus disrupting or promoting hydrophobic interactions within a protein depends on the salt used and the protein in question. Anions have a greater effect on proteins than cations (Jungworth & Winter, 2008; Melander & Horvath, 1977; Peterson & SayKally, 2006; Zhang & Cremer, 2010). The typical order of anions to salt out proteins is:

$$CO_3^{2-} > SO_4^{2-} > H_2PO_4^- > Cl^- > Br^- > I^- > ClO_4^- > SCN^-$$

Sulfate is considered to be on the extreme end of salting out (sometimes referred to as kosmotropic) whereas iodide and thiocyanate are on the extreme end for salting in proteins (sometimes called chaotropic). Chloride is considered to be in the middle of the spectrum of the Hofmeister series based on its ability to salt in or salt out proteins. While cations have a lesser effect on proteins than anions, they still have an effect on protein structure and solubility (Jungworth & Winter, 2008; Melander & Horvath, 1977; Peterson & SayKally, 2006; Zhang & Cremer, 2010). Based on the salts' ability to salt out proteins, cations follow the general order:

$$NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+}$$

Sodium is considered to be in the middle of the Hofmeister series for the cations' ability to salt in or out proteins. For this reason, sodium was chosen as the cation for all experiments used in this research.

The addition of salts from the Hofmeister series to zein mixed at 40 °C was found to have a profound effect on VEM formation. At the extreme end of the kosmotropic salts (salting out), Na_2SO_4 completely disrupted zein's ability to form a VEM when compared to the control (Fig. 1(A), (B) and (O)). This is evident by the complete lack of resistance to mixing on the *Y* axis of the farinograms (Fig. 1(A) and (B)) for both the 1 M and 2 M treatments. It should be noted that these concentrations were chosen based on past research that has reported concentrations below 1 M, salts most likely impact electrostatic interactions in proteins and are not at sufficient levels to impact hydrophobic interactions (Melnyk et al., 2001; Preston, 1989).

Mixing zein in the presence of NaCl produced similar results as Na_2SO_4. From (Fig. 1(C) and (D)) it is evident that treatment with NaCl had a deleterious effect on the visco-elastic properties of zein. Clumps of zein large enough to wedge between the paddle and the mixing bowl of the farinograph were formed with the 1 M NaCl treatment. The spikes in mixing resistance seen in Fig. 1(D) were due to the clumping of zein. Visually, when compared to a control zein VEM mixed with DI H_2O, the zein mixed with either Na_2SO_4 or NaCl was easily distinguished and remained in discrete particles and would not form a VEM (Fig. 2(A)–(C)). It should be noted that increasing the amount of water while mixing zein in the presence of Na_2SO_4 and NaCl did not change these results, the zein would not absorb additional water and excess water simply ran out of the farinograph mixing bowl.

In contrast to the Na_2SO_4 and NaCl, the addition of 1 M and 2 M solutions of NaI was found to have a slight softening effect on the VEM when compared to the control (Fig. 1(E), (F) and (O)). This is evident by the overall decrease in farino units over the course of the mixing which is indicative of a softer VEM. The zein mixed with 1 M NaI took longer to hydrate than that of 2 M NaI (Fig. 1(F)). The reason for increased hydration time during mixing of 1 M NaI is unknown, but could be related to differences in the degree to which NaI altered the protein structure of the zein.

Overall, mixing zein in the presence of NaSCN produced similar results to that of NaI (Fig. 1(G) and (H)). As with NaI, mixing zein with NaSCN produced a VEM at lower peak farino units. Thus, both NaI and NaSCN made the VEM softer and slightly more extensible. The upper and lower limits (upper and lower lines) of mixing resistance were closer to the mean resistance to mixing force (middle line). This is indicative of a smoother more homogeneous dough or VEM. Although subjective, the softening of zein VEMs during mixing with NaI and NaSCN was readily apparent when VEMs were stretched by hand and compared to the control VEM mixed with DI H_2O. These findings support those found by Selling et al. (2013). Here addition of thiocyanate salts were found to plasticize zein, lowering its glass transition temperature and reducing zein's tensile strength and increases zein's elongation properties.

To further investigate the roles of non-covalent interactions on zein isolate functionality, the addition of urea and ethanol during mixing was evaluated. While urea and ethanol are known to impact protein structure, the exact mechanism of urea and ethanol's ability to change protein functionality is not well understood. Recent research suggests that urea non-specifically binds directly to the peptide backbone increasing protein solubility as well as changing the amount of bulk water coming into contact with proteins (Bennion & Daggett, 2003; Zhang & Cremer, 2010). The structural changes made to water by urea may also affect the hydration properties of the protein and affect hydrophobic interactions (Zhang & Cremer, 2010). As concentrations of urea (Fig. 1(I)–(K)) and ethanol (Fig. 1(L)–(N)) added during mixing increased, the zein VEMs resistance to mixing decreased. This is indicative of a softer VEM and it was noted that these VEMs could be extended farther by hand before breaking when compared to the control (Fig. 1(O)). The shape and resistance (*Y* axis) of the farinograms

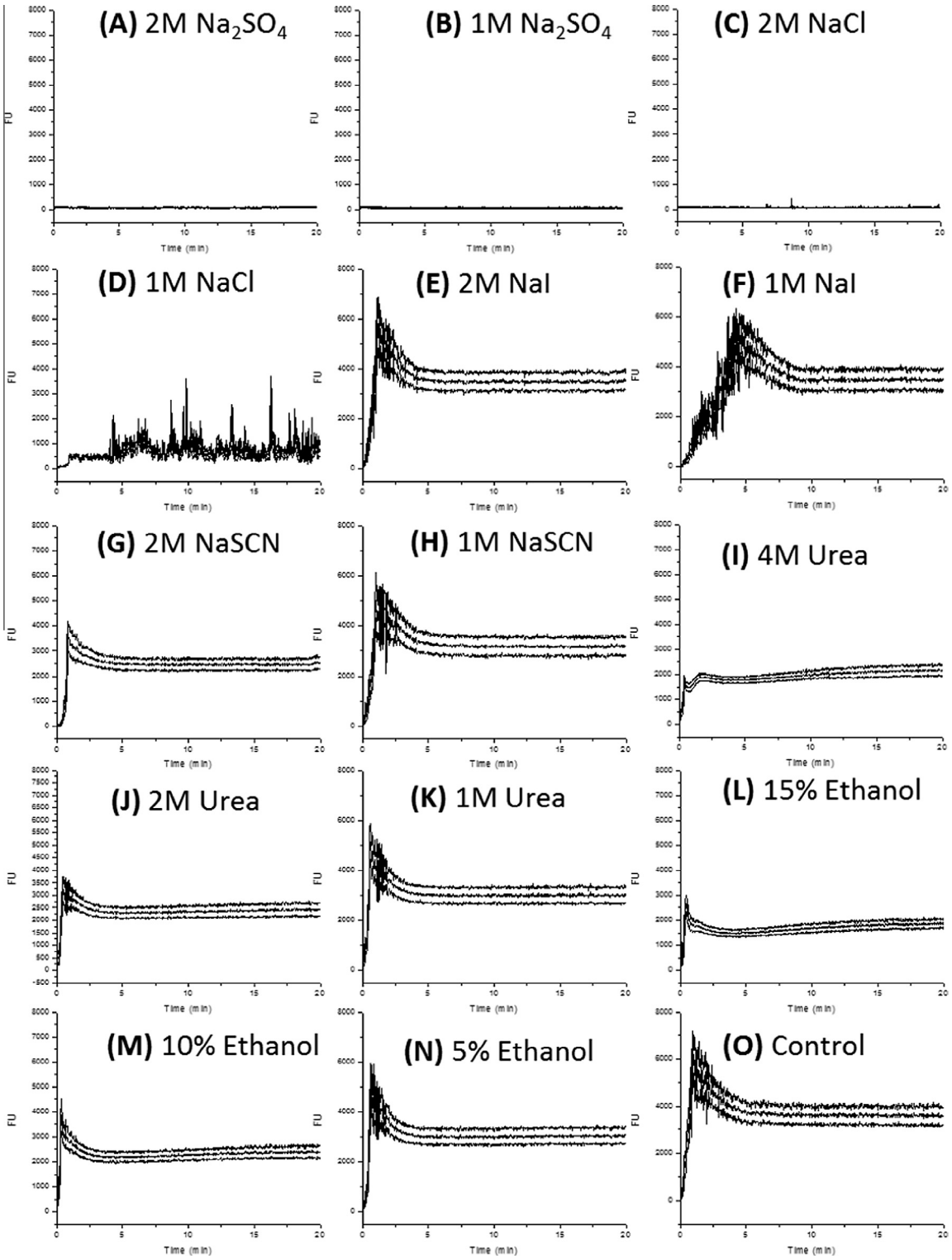

Fig. 1. Farinograms of zein treated with 2 M Na_2SO_4 (A), 1 M Na_2SO_4 (B), 2 M NaCl (C) and 1 M NaCl (D), 2 M NaI (E), 1 M NaI (F), 2 M NaSCN (G), 1 M NaSCN (H), 4 M urea (I), 2 M urea (J), 1 M urea (K), 15% ethanol (L), 10% ethanol (M), 5% ethanol (N), and control zein treated with DI water (O). All samples were mixed for 20 min at 40 °C in a farinograph. The middle farinogram line represents the mean resistance from mixing. The two outermost lines represent the minimum and maximum deviations from the mean mixing line during mixing.

were nearly identical between 4 M urea and 15% ethanol (v/v), 2 M urea and 10% ethanol (v/v), and 1 M urea and 5% ethanol (v/v) (compare Fig. 1(I)–(K) to Fig. 1(L)–(N)).

While the above results show that non-covalent interactions play a role in the formation of visco-elastic zein materials, they provide no information on the role of covalent disulfide bonds. In order to determine if disulfide bonds are important for zein VEM formation, a solution of 2% β-ME was added to zein during mixing in the farinograph. Fig. 3(A) and (B) shows that the addition of 2% β-ME did not disrupt zein's ability to form a VEM when mixed at 40 °C. This is very different from the behaviour of gluten in that cleavage of disulfide bonds completely disrupts gluten's ability to

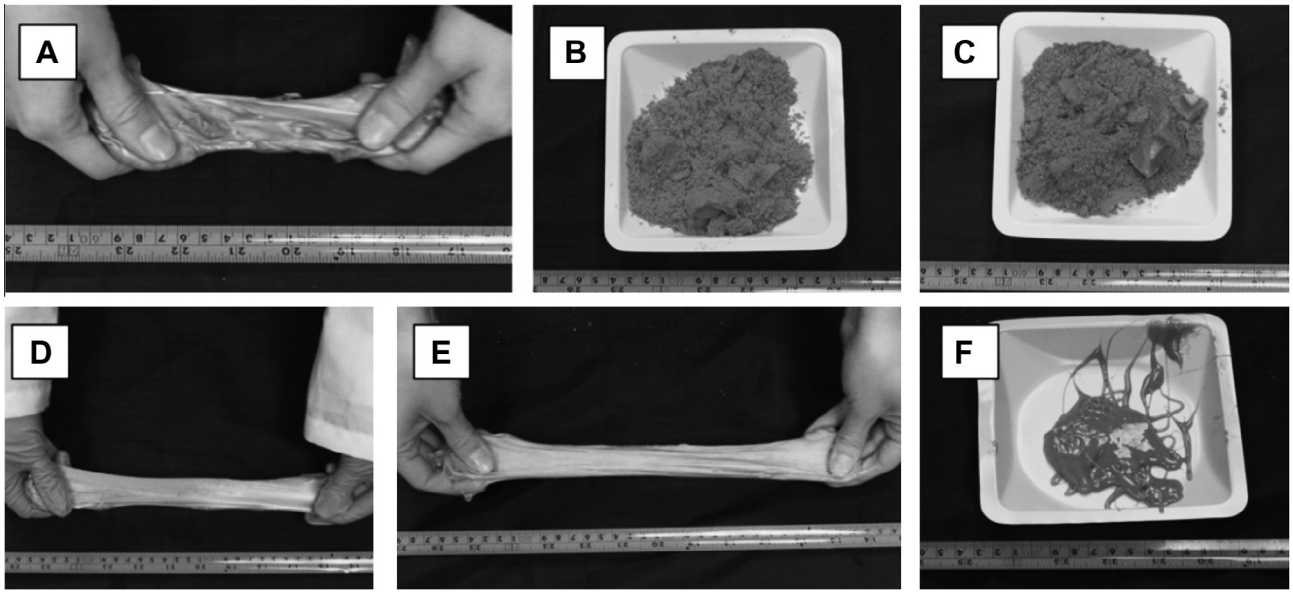

Fig. 2. Visual representation of control zein VEM (A), zein treated with 2 M Na_2SO_4 (B), zein treated with 2 M NaCl (C), zein treated with 2% β-ME (D), control wheat gluten (E), and wheat gluten treated with 2% β-ME (F). Samples in photographs (A)–(D) were mixed for 20 min at 40 °C in a farinograph. Samples in photographs (E) and (F) were mixed for 20 min at 30 °C in a farinograph. All samples were photographed immediately after mixing.

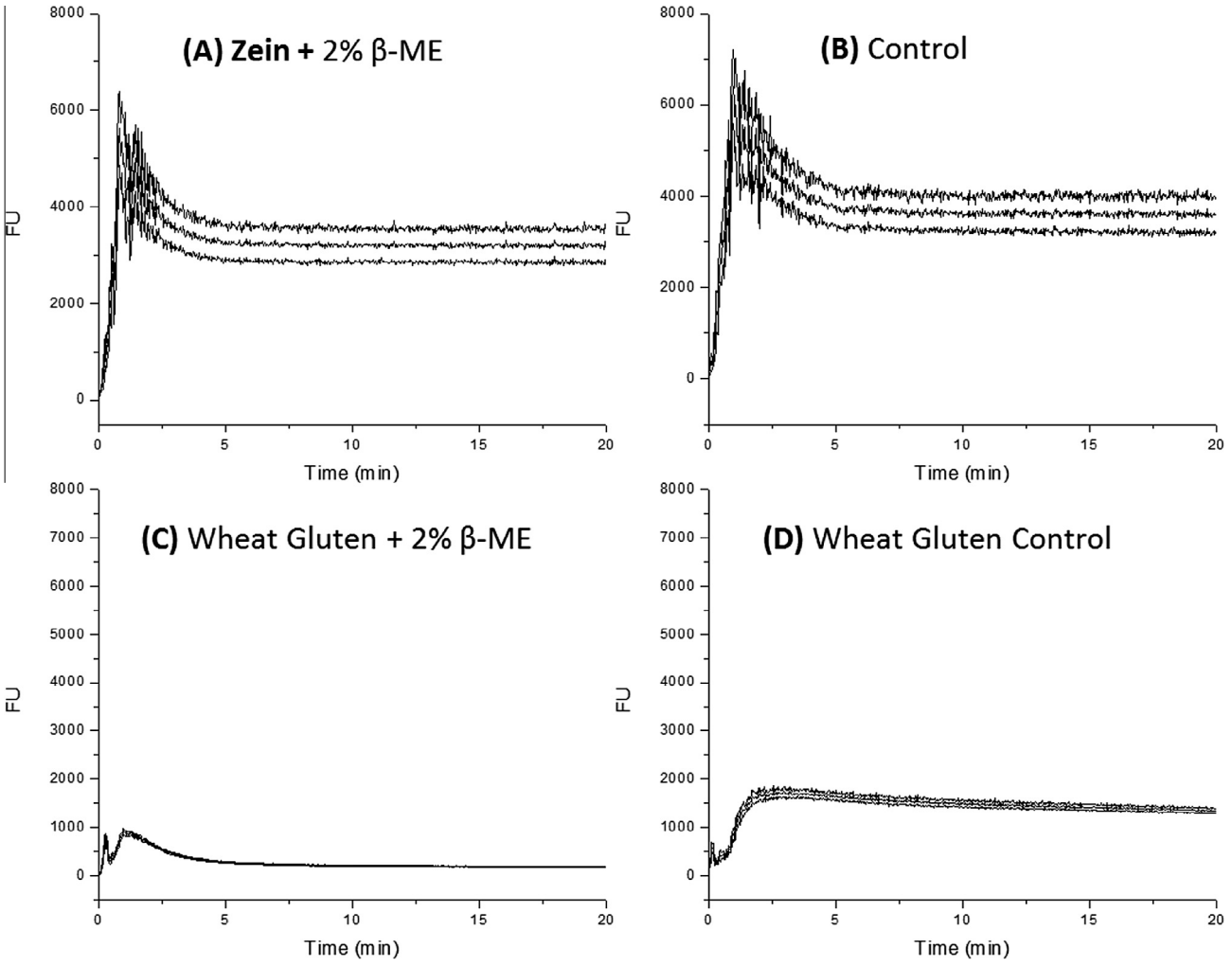

Fig. 3. Farinograms of zein treated with 2% β-ME (v/v) (A), control zein treated with DI water (B), wheat gluten treated with 2% β-ME (v/v), and control wheat gluten treated with DI water. Zein samples were mixed for 20 min at 40 °C in a farinograph. Wheat gluten samples were mixed for 20 min at 30 °C. The middle line of the farinograms represents the mean resistance from mixing. The two outermost lines represent the minimum and maximum deviations from the mean mixing line during mixing.

form a visco-elastic material (Shewry & Tatham, 1990). Commercially isolated zein is comprised almost entirely of α-zein that is known to have very little cysteine (Lawton, 2002). Thus, the small amount of disulfide bonding present should have a minimal influence on zein's functionality. Addition of 2% β-ME did slightly decrease mixing resistance and minimise deviation from the mean mixing force (Fig. 3(A) and (B)). This may have been an effect of the cleaving of some intra-disulfide bonds that may have been present or may have been due to 2% β-ME having a similar affect as the chaotropic salts of the Hofmeister series. There was no observable difference between β-ME treated VEMs and the control VEM when pulled apart by hand (Fig. 2(A) and (D)).

While many different hypotheses for how gluten functions to form a visco-elastic material have been put forward, a pre-requisite for all these ideas is the presence of large polymeric proteins held together via disulfide bonds. It has been demonstrated numerous times that these large polymeric proteins are critical for the functionality of wheat dough (Carceller & Aussenac, 2001; Shewry & Tatham, 1997). In the presence of β-ME, these disulfide bonds are cleaved, rendering wheat gluten unable to form a visco-elastic substance. To demonstrate this, 2% β-ME was added to a commercially available wheat gluten isolate and mixed in the farinograph. It was found that cleavage of disulfide bonds did indeed prevent gluten from forming a VEM (Fig. 3(C) and (D)). When comparing the 2% β-ME treated wheat gluten to the β-ME treated zein, the key differences noted were that zein was able to produce a VEM that maintained its integrity throughout mixing, while gluten produced a sticky paste that had little resistance to mixing (compare farinograms from Fig. 3(A) and (B) to Fig. 3(C) and (D) and photos in Fig. 2(A) and (D) to those in Fig. 2(E) and (F)).

This is significant in that few proteins are capable of forming a visco-elastic substance like wheat gluten. Zein from corn and caroubin from carob germ flour have also been reported to contain proteins known to behave like gluten (Schober et al., 2011; 2010; 2008; Lawton, 2002; Mejia et al., 2007; Oom et al., 2008; Smith, Bean, Schober, Tilley, Herald & Aramouni, 2010). Caroubin, a non-wheat protein was found to function for similar reasons as wheat gluten (i.e. high M_w disulfide bonded proteins) (Smith et al., 2010). This makes zein unique in that it is capable of forming a gluten-like VEM from relatively small molecular weight proteins (~21–22 kDa) that are not dependent on disulfide linkages like caroubin and wheat gluten (Carceller & Aussenac, 2001; Lawton, 2002; Smith, Bean, Herald, & Aramouni, 2012; Smith et al., 2010).

Similarly, the fact that the control zein VEMs did not break down with mixing over time as wheat gluten did (Fig. 3(B) and (D)) may be attributed to the fact that zein has very little disulfide bonding. Wheat gluten breaks down with mixing over time and is dependent on large molecular weight disulfide bonded proteins. Zein, on the other hand, can be mixed almost indefinitely without breakdown because it is not relying on large molecular weight disulfide linked proteins for functionality. In fact, zein was mixed in access of four hours in the farinograph with no decrease in farino units during preliminary studies. Instead there was a gradual increase in farino units with mixing over time which was most likely attributed to moisture loss (data not shown).

From the farinograph data and the subjective observations from stretching the VEMs by hand after mixing, these results show that chaotropic salts of the Hofmeister series had some beneficial effect on zein VEM formation, possibly by promoting the unfolding of zein proteins. This unfolding of proteins, typically seen with the addition of chaotropic salts (i.e. NaI and NaSCN), may allow for increased interactions between proteins by exposing regions of the protein that were previously buried. Such non-covalent protein–protein interactions may be one reason why zein is capable of forming a VEM. Zein VEM formation is sensitive to non-covalent interactions. This is evident by the strong negative disruptive effects of Na_2SO_4 and NaCl treated zein compared to the slight increase in extensibility seen when NaI and NaSCN were present.

Interestingly, the mixing properties of zein were impacted by the salts from the Hofmeister series differently than gluten has been reported to. For example, He et al. (1992) and Preston (1989) found that kosmotropic salts increased the mixing strength of wheat flour and chaotropic salts (at high levels) resulted in reduced mixing strength of wheat flour.

3.2. Molecular weight distribution

To determine if changes to the molecular weight distribution of zein occurred during mixing with the various treatments, SEC was used. The commercial zein used in this work did not contain significant amounts of disulfide bonded protein complexes (Fig. 4). If disulfide bonds were present, a shift to smaller molecular weight proteins would have been observed when comparing the non-reduced to reduced (through addition of β-ME) sample. This would have been evident by chromatogram peaks shifting to a later elution time from non-reduced zein to reduced zein. This further supports the hypothesis that commercial zein isolate does not form a VEM due to the presence or formation of disulfide bonded protein complexes (or through any other covalent linkages). Furthermore, none of the treatments had an effect on covalent interactions of zein over the course of mixing, as no changes to the molecular weight distribution occurred during mixing (Fig. 5). All peaks eluted at the same time with no shifts in peak areas for any treatment. Because changes in covalent interactions would cause shifts in peaks indicative of changes to molecular weight distribution, zein does not form VEMs due to changes in covalent interactions (i.e. the formation of larger covalently bonded polymeric proteins) during mixing. Note that these samples were dissolved and

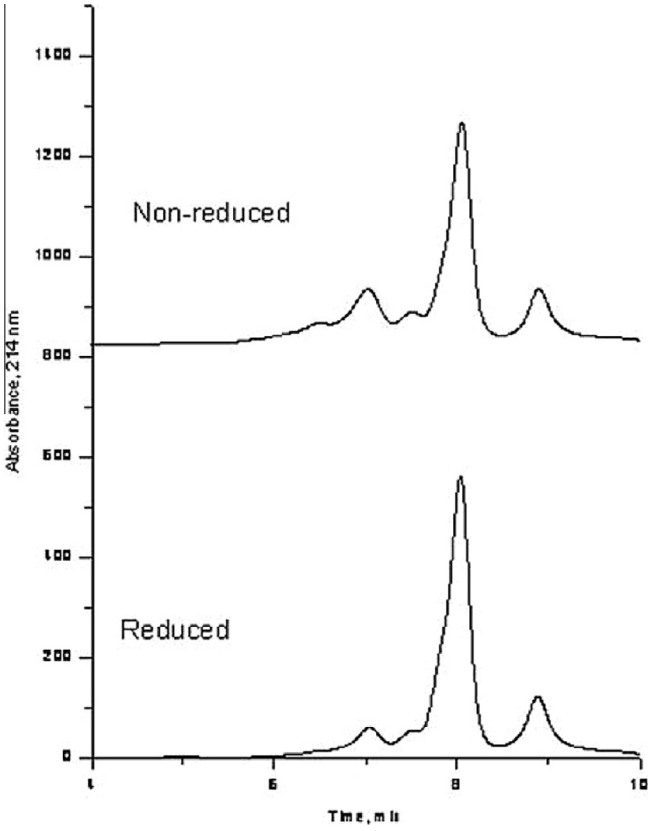

Fig. 4. Size exclusion chromatograms of reduced (treated with beta mercaptoethanol) (bottom) and non-reduced (top) solubilised commercial zein isolates.

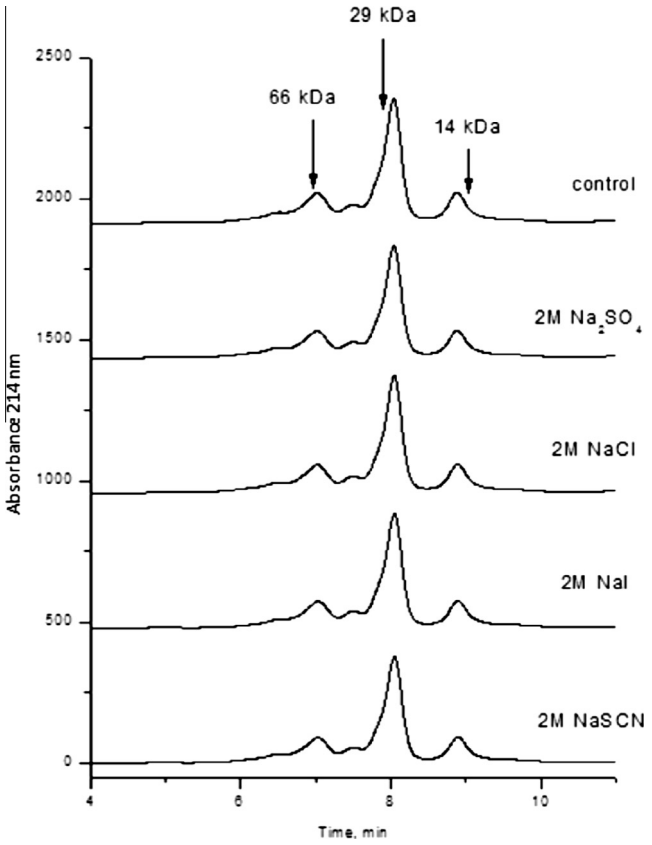

Fig. 5. Size exclusion chromatograms of zein treated with 2 M concentrations of Na_2SO_4, NaCl, NaI and NaSCN compared to a control zein treated with DI water. Treatments are labelled on the right side of size distribution plots. M_W standards were used to provide reference M_W and were obtained from lysozyme from chicken egg white (14 kDa), carbonic anhydrase (29 kDa), and bovine serum albumin (66 kDa).

analysed in the presence of SDS, a strong protein denaturant. Any protein complexes held together via non-covalent interactions would be expected to be disrupted by the SDS. These findings are further supported by those found by Selling (2010), where increases of zein M_W were not observed during extrusion until temperatures of 140 °C or greater were reached.

Since covalently bound large protein complexes are not found in commercial zein and did not form during mixing, zein must form dough via a different mechanism than wheat. The role of non-covalent interactions must be considered in the case of zein as being the primary mechanism for the formation of visco-elastic materials.

3.3. Solubility

The Hofmeister series is commonly used to salt in or salt out proteins. This means that the salts are used to change protein solubility to either promote flocculation or solubilisation. In order to determine if the effects of the salts, urea and ethanol on zein protein functionality were due solely to changes in non-covalent interaction or if changes to solubility played a role as well, the changes to zein solubility with the various treatments was examined. Effects on zein solubility were examined at 2 M concentration for each salt, 15% (v/v) concentration for ethanol, and 1 M, 2 M, and 4 M urea. These treatments were compared against a negative control (DI H_2O) and a positive control (50 mM Tris–borate pH 10.0 buffer containing 2% SDS), which solubilised all of the protein. It was found that none of treatments had significant differences ($P < 0.05$) in solubilising the zein isolate except for 4 M urea compared to the positive control, which solubilised ~20% of the zein. Thus, the overall softening effects of the 4 M urea treatment on zein seen with the farinogram (Fig. 1(I)) may have been due to both an increase in zein solubility and changes to non-covalent protein interactions. The effects of all other treatments can be attributed to changes in non-covalent interactions and not changes to zein solubility. It was surprising that the chaotropic salt treatments did not have any effect on zein solubility since salts have been shown to solubilise some gluten (Preston, 1981). This was probably due to the hydrophobic nature of zein. With zein, the disruption of hydrophobic interactions by the chaotropes probably promoted the interaction of zein with water only enough to promote zein VEM formation (Fig. 1(E)–(H)), but not enough to solubilise zein.

3.4. Surface hydrophobicity

To confirm changes to zein isolate protein structure in the presence of the Hofmeister salts used in this study, surface hydrophobicity was determined by the proteins ability to react with BPB. Previous work with BPB has been shown to bind to the hydrophobic side chains of amino acids (Chelh et al., 2006). More importantly, the use of BPB allowed the surface hydrophobicity of proteins to be determined without the need to solubilise the proteins first. More widely used surface hydrophobicity assays require the proteins to be solubilised. Zein can only be solubilised in the presence of high levels of detergents such as SDS or aqueous alcohols, both of which would alter the tertiary structure of the protein and thus alter surface hydrophobicity.

Fig. 6 shows that in the presence of 1 M and 2 M concentrations of Na_2SO_4 and NaCl, zein had significantly less ($P < 0.05$) surface hydrophobicity. At a concentration of 0.5 M, Na_2SO_4 and NaCl were not significantly different when compared to the control. This is probably because low concentrations of salts seem to have an inverse Hofmeister effect, which is dependent on electrostatic interactions based on size and hydration properties of the ions (Zhang & Cremer, 2010). Only when the salts have neutralized the overall charge distribution of the protein will they begin to have a Hofmeister effect (Zhang & Cremer, 2010). The neutralization of charge distribution is also dependent on the protein and the salt used (Zhang & Cremer, 2010). Only the 0.5 M treatment of NaSCN had significantly different hydrophobicity than the control, which again may have been due to changes in electrostatic interactions rather than hydrophobicity. Both the 1 M and 2 M concentrations of NaSCN did not significantly change surface hydrophobicity relative to the control. This means that there was not a significant increase in hydrophobic amino acids at the surface of the zein proteins when zein was exposed to the NaSCN. It should be noted that NaI could not be used in this experiment because the BPB assay is a photometric assay. Solutions of NaI turn brown in colour with time, so the subtraction of absorbance of the treated zein from the salt control would change as readings were being taken.

When mixed in the presence of NaCl and Na_2SO_4, zein was unable to form a VEM. While it is not clear exactly how kosmotropic salts work to "salt in" proteins, it is not just related to ordering or disordering water (Zhang & Cremer, 2010). It is clear that zein functionality was severely impacted in the presence of NaCl and Na_2SO_4. This lack in functionality was accompanied by a decrease in surface hydrophobicity when zein was exposed to these salts. The lack of functionality was not related to solubility as zein was not soluble in any of the salts used for the mixing experiment. Zein showed decreased surface hydrophobicity in the presence of the kosmotropic salts, suggesting that hydrophobic regions of the proteins were buried in the interior of the protein under these conditions. Non-functional zein aggregates may have formed under kosmotropic conditions which are supported by the observation

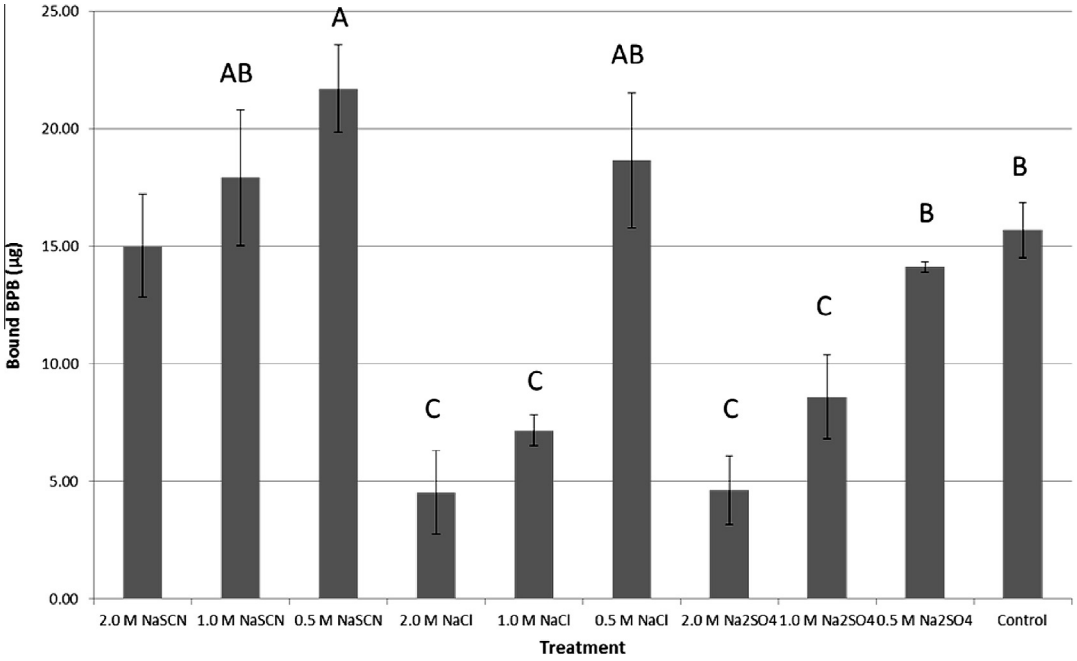

Fig. 6. A graphical representation of zein's surface hydrophobicity determined by zein's ability to bind bromophenol blue (BPB) expressed as µg BPB bound (*y* axis). Zein was treated with NaSCN, NaCl and Na_2SO_4 at concentrations 2 M, 1 M and 0.5 M, vortexed and compared against a control (*x* axis). The upper case letters represent differences of means where similar letters identify no significant differences between the means ($P < 0.05$).

that zein mixed in the presence of NaCl and Na_2SO_4 formed a powder like material and excess water was excluded from this material. The loss of surface hydrophobicity combined with the loss in functionality may point to hydrophobic regions of zein that may be important in zein's ability to form VEMs. If these regions bury themselves in the presence of the kosmotropic salts and are thus not available for inter protein–protein interaction, visco-elastic materials are not formed. This may also be the reason why removal of surface lipids was found to improve the functionality of zein in a dough system (Schober et al., 2008), i.e. surface lipids reduced the ability of proteins to interact possibly by either blocking the hydrophobic regions of the proteins and thus preventing protein–protein interactions or by changing the tertiary structure of the proteins and preventing non-covalent protein–protein interactions.

4. Conclusions

The research presented here shows that non-covalent interactions between low molecular weight proteins plays an important role in the ability of zein to form visco-elastic materials rather than chemical and physical interactions of extremely high molecular weight protein complexes as found in gluten. Zein may form VEM through non-covalent protein–protein interactions. In the presence of chaotropes, these interactions are modified which results in slight increases in extensibility of the VEM. However, in the presence of kosmotropes the proteins bury hydrophobic regions internally and this may disrupt the ability of protein–protein interactions necessary for VEM formation. This research also demonstrates that large disulfide linked protein complexes are not necessary for zein to form a material with visco-elastic properties. Thus, changes in secondary structure of zein during mixing must be influenced by non-covalent interactions between the proteins, possibly relating to changes in α-helices to β-sheet structures, as reported by Mejia et al. (2007). Modifications to zein proteins, either by additives or modifications to the proteins themselves to enhance these interactions, may improve the ability of zein to form visco-elastic materials either alone or in the presence of co-proteins. This research will aid in the future utilisation of zein in food and other industrial applications.

There are no large polymeric protein complexes present in zein and yet zein is still capable of forming a visco-elastic material during mixing. This is exciting as it has always been held as a dogma that to form a visco-elastic material or dough, the presence of extremely high M_W protein complexes was needed. This raises many questions on what other protein sources that have been overlooked in the past might be able form visco-elastic materials similar to wheat gluten through manipulation of non-covalent interactions.

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