Oxidation of commercial (α-type) zein with hydrogen peroxide improves its hydration and dramatically increases dough extensibility even below its glass transition temperature

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Water holding,

ABSTRACT

To improve the rheological properties of zein doughs, α-type zein and zein-starch doughs were prepared with the oxidising agents, hydrogen peroxide and peroxidase, which strengthen gluten-based doughs by cross-linking. Hydrogen peroxide and peroxidase increased zein dough extensibility compared to preparation with water. Hydrogen peroxide prepared zein doughs were extensible and cohesive below zein's glass transition temperature. The doughs did not exude water and maintained flexibility when stored. Confocal laser scanning microscopy revealed that in zein-starch doughs prepared with hydrogen peroxide a thin continuous zein matrix was formed around the starch granules, whereas doughs prepared with water exhibited clumps of granules. SDS-PAGE of zein doughs and films treated with the oxidising agents showed no evidence of zein polymerisation, nor did Fourier transform infrared spectrometry reveal any significant changes in secondary structure. Further, hydrogen peroxide treatment did not increase zein film glass transition temperature, but it did increase transition enthalpy, and film water uptake increased with hydrogen peroxide concentration. The greatly increased extensibility of hydrogen peroxide prepared zein doughs and their improved water-holding are not due to oxidative cross-linking. It is proposed that the effects are primarily due to hydroxylation of amino acid aliphatic side chains, improving hydration through hydrogen bonding.

1. Introduction

Zein has potential as a replacement for wheat gluten in leavened gluten-free products such as bread. Commercial type zein (essentially α-zein) can form a viscoelastic dough in an aqueous system at 35^{o}C and higher (Lawton, 1992; Schober et al., 2008, 2010), above its hydrated glass transition temperature (T_g). Several different ways have been investigated to improve the dough rheological properties of zein, including addition of hydrocolloids (Schober et al., 2008) and co-proteins (Mejia et al., 2012). Some hydrocolloids, for example hydroxypropyl methylcellulose, have also been shown to improve gas cell stability in zein breads (Schober et al., 2008). This is possibly by creating a mechanism for gas bubble stability, as suggested by Gan et al. (1995) and Sroan et al. (2009). Regarding chemical modification of zein, we have shown that dough preparation with dilute acetic and lactic acids, as opposed to water, results in a more extensible dough that forms uniform fibrils when stretched (Sly et al., 2014). Moreover, with the inclusion of maize starch or rice flour, dough can be formed that will expand and hold air when subjected to alveography.

However, the functional properties of aqueous zein doughs are very temperature sensitive. When the doughs are cooled to 25^{o}C and below, the zein rapidly squeezes water out and reverts to its amorphous state, and the doughs become hard (Mejia et al., 2007; Sly, 2014). However, Schober et al. (2008) found that cooling below the T_g of zein does not destroy zein fibres unless they are exposed to mechanical impact, when the fibres break into small pieces. This temperature sensitivity is one of the reasons that zein is not yet a viable replacer for wheat gluten.

It is very well established that oxidation of wheat gluten dramatically alters its dough functional properties (Wieser, 2007). Oxidation primarily results in polypeptide polymerisation through the conversion to SH groups to disulphide cross-links. Dityrosine cross-links can also be formed (Tilley et al., 2001). A great number of reagents can be used to bring about oxidation of gluten, including chemical oxidising agents such as ascorbic acid (in the form of dehydroascorbic acid), potassium bromate and hydrogen peroxide (Hanft and Koehler, 2005; Rodriguez-Mateos et al., 2006; Wieser, 2007) and oxidising enzymes such as lipoxygenase, peroxidase, glucose oxidase, tyrosinase and laccase (diphenol oxidase) (Goesaert et al., 2005; Hanft and Koehler, 2005; Takasaki et al., 2005).

Strangely, the effects of oxidising agents on the prolamins of other cereals have been little investigated. Treatment of maize and sorghum flours with glucose oxidase has been shown to increase bread loaf volume, and data indicated that the cause was protein polymerisation (Renzetti and Arendt, 2009). Tyrosinase was found to cross-link isolated oat globulins but not oat prolamins (Flander et al., 2011). Recently, it has been shown the barley C-hordein can be degraded and undergo side-chain modification through treatment with metal catalysed hydrogen peroxide induced oxidation (Huang et al., 2016).

The aim of this study was determine whether oxidising agents can modify the functional properties of aqueous commercial (α-type) zein and zein-starch doughs. Zein films cast from aqueous ethanol were also studied as zein's structure and glass transition as well its as rheological properties have been found to be influenced by thermal treatment and chemical cross-linking (Magoshi et al., 1992; Madeke and Kokini, 1996).

2. Experimental

2.1 Materials

Commercial zein (Sigma Z3625) and horseradish peroxidase (199 U/mg, Sigma P8250) was obtained from Sigma-Aldrich. Maize starch was obtained from local suppliers.

2.2 Preparation of zein doughs

Zein doughs were prepared according to Schober et al. (2010), except that the zein was not defatted. Triplicate samples of zein and either water, hydrogen peroxide, horseradish peroxidase, or acetic acid were placed in separate tubes and pre-warmed to 50ºC (40ºC for horseradish peroxidase to conserve enzyme activity) in a water bath. The ratio of zein to liquid was 1:2. The liquid was then added to the zein and the mixture vortexed at high speed for 30 s. The doughs were then manipulated with a spatula to form a dough ball, then allowed to rest for approx. 40 min at 50ºC (40ºC for horseradish peroxidase).

2.3 Preparation of zein-starch doughs

Triplicate samples of zein (1 part by weight) and starch (4 parts by weight) were vortexed together to thoroughly mix. The zein-starch mixture (10 parts by weight) and either water, hydrogen peroxide solution, or acetic acid solution (6 parts by weight) were placed in separate tubes and pre-warmed to 50ºC in a water bath. The liquid was then added to the zein-starch and the mixture vortexed at high speed for 30 s. The doughs were then manipulated with a spatula to form a dough ball then allowed to rest for approx. 40 min at 50ºC.

2.4 Preparation of zein films

Film preparation involved weighing zein (1.4 g protein equiv.) into 100 ml Erlenmeyer

flasks. Twenty g aqueous ethanol (70% (w/w)) was added. The flask was closed with foil and heated on a hotplate for 10 min at 70^0C with rapid stirring to completely dissolve the zein. The solution was cooled to ambient temperature. Then, hydrogen peroxide solution was added to obtain a range of final concentrations (0, 12.5, 100 and 150 mg hydrogen peroxide/100 g protein) and mixed by stirring for 1 min. Aliquots of the solution (2.16 g) were transferred into rectangular silicone baking trays (69 mm × 28 mm) and dried in an oven (without force draught) at 50^0C overnight. The cast films were approx. 80 µm thick. Zein films with the inclusion of hydrogen peroxide were yellow in colour, with a smooth surface and flexible. Zein films with no peroxide were yellow- brown in colour, with a rough surface and friable.

2.5 Preliminary extensibility tests

For zein doughs, water, hydrogen peroxide (3.5%), horseradish peroxidase (100 U) and a mixture of hydrogen peroxide (3.5%) plus horseradish peroxidase (100 U) were used as the liquid component in these initial experiments. The pH was adjusted to pH 6-7 with 0.2 M NaOH and the doughs prepared. After resting, the dough pieces were rinsed briefly in water (40ºC) and manipulated by hand for 30 seconds, pressing the dough pieces into a cylindrical, longitudinally split polyethylene mould (60 mm long and 4 mm diam.) to obtain a uniform size and shape. The moulded samples were removed from the mould and placed over the vertical struts (30 mm apart) of a Kieffer rig mounted on a TA-XT2 type texture analyser (Stable Micro Systems, Godalming, UK) and clamped in place at both ends. Within 2 minutes of dough preparation, the doughs were then stretched at a test speed of 3.3 mm/s to a distance of up to 150 mm. The extended dough pieces were folded in two and photographed.

Zein-starch doughs were prepared as described using either water or hydrogen peroxide (7.5,

15, and 30%) as the liquid component of the doughs but stretched by hand.

2.6 Zein and zein-starch dough tensile properties

Zein and zein-starch doughs were prepared as described above. Tensile properties of all these doughs were measured as described by Sly et al. (2014) using a Kieffer rig. Zein dough pieces were pressed into cylindrical, longitudinally split rubber moulds (85 mm long and 8 mm diam.). Texture analyser parameters were as described above. The dough temperature during measurement was 32°C. Peak force (N) and extensibility until rupture (mm), and distance to maximum force (mm) were measured. The experiment was repeated on moulded dough pieces after cooling to ambient temperature (24°C).

2.7 Confocal laser scanning microscopy (CLSM)

Zein and zein-maize starch doughs were prepared as described above. Thin pieces of these freshly prepared doughs (approx. 200 μm thick) were placed on a glass slide. Doughs were examined by CLSM using a Nikon A1+ system (Tokyo, Japan) with a Plan Apo 20 x objective (N.A. 0.75) under natural fluorescence at an excitation wavelength of 488 nm and emission filter 450/50 with a depth up to 50 μm (1.4 μm z-stacks). Images were analysed using Nikon NIS-Elements Advanced Research software (Tokyo, Japan).

2.8 SDS-PAGE

Zein doughs prepared as described above with water, hydrogen peroxide, peroxidase and hydrogen peroxide plus peroxidase were air-dried at ambient temperature. Additionally, zein with hydrogen peroxide addition was cast into films (plasticiser was not added) as described above.

SDS-PAGE was performed under non-reducing and reducing conditions using 4-12%

polyacrylamide gradient gels (8 x 8 cm x1.0 mm thick with 15 wells) (NuPAGE® Novex, Invitrogen. Carlsbad, CA). Invitrogen Mark12 Unstained Standard was used. Samples were loaded to 10 μg constant protein. Staining was with Coomassie Brilliant Blue R-250. After de-staining, the gels were photographed by scanning on a flatbed scanner.

2.9 Fourier Transform Infrared Spectroscopy (FTIR)

Zein doughs (prepared with either water, hydrogen peroxide, peroxidase or hydrogen peroxide plus peroxidase) and films (prepared with 0, 12.5, 100 and 150 mg hydrogen peroxide/g protein) were prepared as described under section 2.3 and 2.4. The dried zein doughs and films were then crushed into powder using a pestle and mortar and dried in a desiccator over silica gel for 72 h. FTIR spectroscopy was performed as described by Taylor et al. (2009). A Vertex 70v FTIR spectrophotometer (Bruker Optik, Ettlingen, Germany) was used in the attenuated total reflectance (ATR) mode with 64 scans, an 8 cm^{-1} band width, and an interval of 1 cm^{-1} at a wave number 400-4000 cm^{-1} At least four replicates were analysed per treatment. Spectra were Fourier deconvoluted with a Lorentzian filter with a band width of 12 and a resolution enhancement factor of 2.

2.10 Differential scanning calorimetry (DSC)

Zein film powder samples (15 mg) which had been dried in a desiccator, as described in2.9, were weighed into 100 μL aluminum pans and sealed immediately. DSC was performed using a Mettler Toledo HP DSC827^e Differential Scanning Calorimeter (Schwerzenbach, Switzerland). The instrument was calibrated with pure indium and an empty pan was used as the reference. DSC scans were performed at a heating rate of 10 ^{0}C/ min from 25 to 280^{0}C under nitrogen pressure (40 bar) with a flow rate of 60 ml/min. Glass transition onset (T_i) and midpoint (T_g) temperatures and enthalpy of transition ΔH_m were measured. The data were

plotted and analysed using STAR^e software version 9.20.

2.11 Phosphate buffer uptake

Weighed dried zein film rectangles (preparation as described under 2.9) were immersed in 0.2 M pH 6.8 sodium phosphate buffer for 12 h at 39°C in an oven. The films were taken out of the buffer and surface water removed by placing the films between paper towels and then weighed. Water uptake was calculated as a percentage of film dry weight.

2.12 Statistical analysis

Dough tensile, DSC and buffer uptake data were subjected to one-way analysis of variance using XLSTAT Pro software (Addinsoft SARL, Paris) to determine the effect of oxidising agents. Means were compared at $p = 0.05$ using Fisher's Least Significant Difference Test (LSD). All experiments were replicated.

3. Results and discussion

3.1 Dough rheological properties and structure

In preliminary work, mixing zein doughs with water or oxidising agents at 40 or 50^{o}C, temperatures above the T_g of hydrated zein, showed that cohesive doughs could be formed in all cases (Fig. 1A). Addition of the oxidising agents resulted in doughs which were considerably more extensible than those mixed with water alone. Of the treatments used, addition of 3.5% hydrogen peroxide solution (approx. 17.5 mg hydrogen peroxide/g zein) resulted in doughs that were approximately twice as extensible as doughs prepared in water. Doughs containing peroxidase plus hydrogen peroxide were slightly less extensible, approximately 1.5 times the length of water-prepared doughs. On extension, doughs

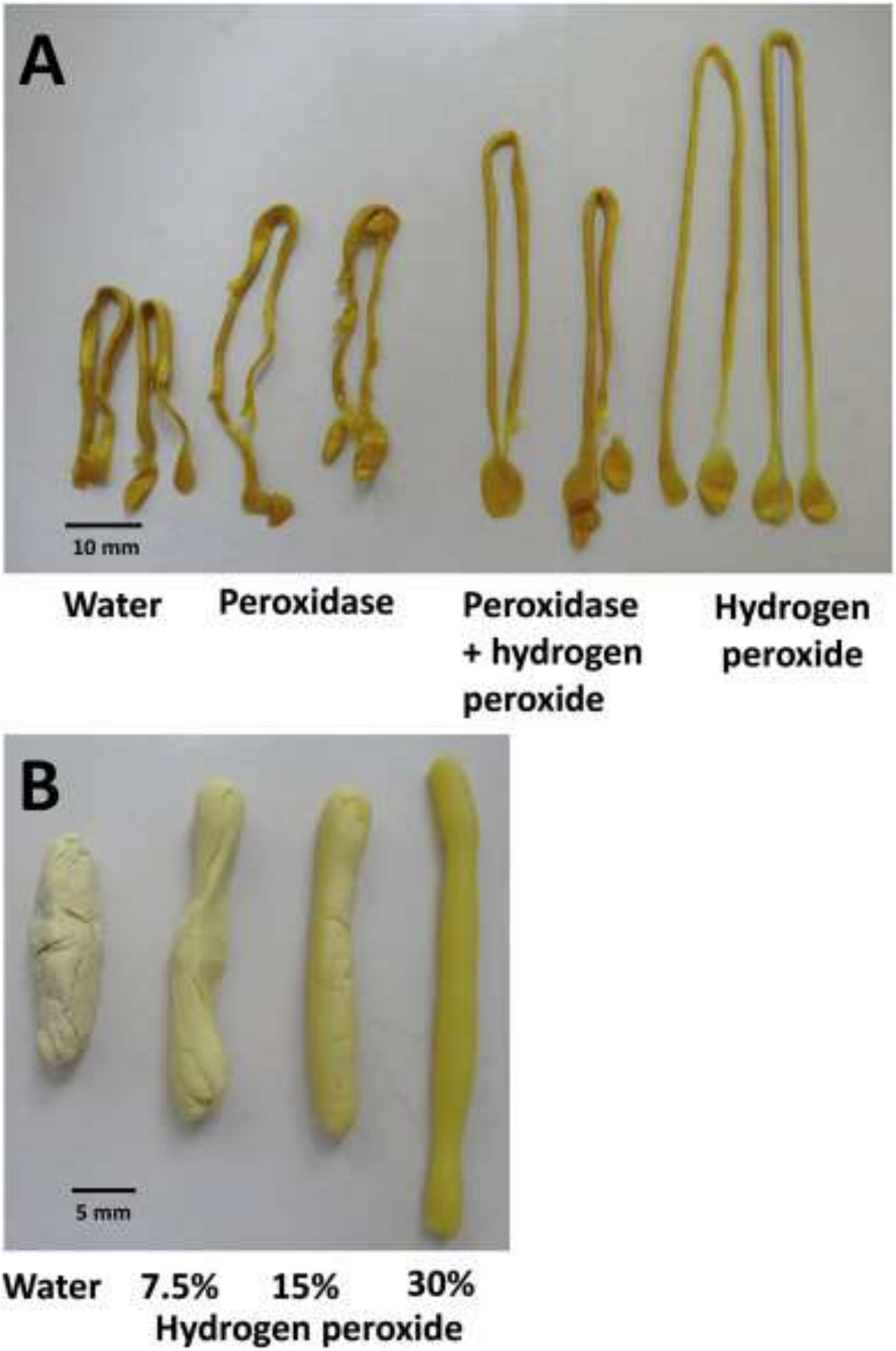

Fig. 1. Effects of preparation with hydrogen peroxide, peroxidase and hydrogen peroxide + peroxidase on the extensibility of zein doughs stretched on a texture analyser. **A**) Zein only doughs**. B**) Zein-starch doughs (1:4 ratio).

prepared with peroxidase were only slightly longer than the water-prepared doughs. Due to the greater observable changes brought about by hydrogen peroxide treatment compared to peroxidase, further work on the rheological properties of zein and zein-starch doughs was continued using hydrogen peroxide only.

Fig. 1B illustrates the maximum extension before dough piece breakage obtained by hand preparation of zein-starch doughs. The addition of corn starch to zein mixed above the T_g of hydrated zein, also resulted in doughs being formed. The zein was fully incorporated into the dough with water and at all hydrogen peroxide concentrations. However, higher levels of hydrogen peroxide were needed in order to form a cohesive and extensible zein-starch dough than with zein alone (Fig. 1A). Zein-starch doughs prepared with water were very crumbly and could hardly be extended by hand without breaking. The 7.5% hydrogen peroxide prepared dough was less crumbly than the water dough and could be extended slightly. With 30% hydrogen peroxide (approx. 1 g hydrogen peroxide/g zein) a very soft, smooth, cohesive dough was formed that could be easily extended by hand. Unlike water prepared doughs, none of the zein-starch doughs prepared with hydrogen peroxide exuded water on standing, either when still warm or on cooling. These doughs remained flexible on cooling and remained so when stored in sealed polyethylene bags for several days at ambient temp.

Table 1A shows the tensile properties of zein doughs mixed at 50°C with water, acetic acid (5.4%) or hydrogen peroxide (10%) and measured above (32°C) and below (24°C) the T_g of hydrated zein. All doughs were highly extensible, as they all reached maximum extension of the Kieffer rig (270 mm) without breaking. However, the strength of the doughs differed considerably depending on the treatment. For doughs measured above the T_g of hydrated zein, the resistance of the doughs to extension ranked from highest to lowest: water (2.0 N),

acetic acid (0.35 N) and hydrogen peroxide (0.1 N) as measured by peak force. At 24^{o}C, below the hydrated zein T_g, the resistance of the doughs to extension ranked in the same order but the values were higher, approx. nine times higher for the water treatment, six times for acetic acid and four times for hydrogen peroxide. Notably, the water-prepared dough at 24^{o}C, although still extensible, had substantially higher ($p < 0.05$) dough strength (peak force and peak stress) than all the other treatments. Thus, with the acetic acid and particularly the hydrogen peroxide treatments the increase in dough strength with reduction in dough temperature from 32 to 24^{o}C was far less than with the dough prepared with water

With the hydrogen peroxide dough preparation, there was a large (approx. 5-6 fold) and significant ($p<0.05$) reduction in dough strain at maximum force and Hencky (true) strain at 32^{o}C compared to dough preparation with water and acetic acid. This was a consequence of the hydrogen peroxide-prepared dough being much softer (low dough stress) but maintaining extensibility. However, only the zein prepared in water and extended at 24^{o}C exhibited substantial elasticity, with a Young's modulus 0.34 kPa. With all the other treatments the Young's moduli were very low.

Considering the tensile properties of the zein-starch doughs (Table 1B) the general pattern was similar to that of the zein only doughs. The doughs prepared with acetic acid and hydrogen peroxide had lower peak stress than doughs prepared with water, both above and below the hydrated zein T_g. Unlike the zein only doughs there was no significant difference ($p \geq 0.05$) in dough strength between the hydrogen peroxide and acetic acid doughs. Cooling of the doughs from 32°C to 24°C resulted in an increase in dough strength for all treatments. Also, in all cases the peak stress of the zein-starch doughs was significantly higher ($p<0.05$) than that of the zein only doughs. Importantly, however, unlike zein only doughs there was a

Table 1

Tensile properties of zein doughs prepared with water, dilute acetic acid and hydrogen peroxide above (32°C) and below (24°C) zein's hydrated glass transition temperature

A. Zein only doughs

Treatment	Temperature	Peak Force (N)	Extension (mm)	Peak stress (kPa)	Strain at max. force (%)	Hencky strain (ε_H)	Extensional Viscosity (η_E, kPa.s)	Young's Modulus (*E*, kPa)
Distilled	32°C	2.00a±0.21[1,2]	270[3]	0.040a±0.004	1004.4c±0.1	2.31d±0.00	1.82b±0.19	0.091a±0.010
water	24°C	17.33b±5.39	270	0.345b±0.107	262.7ab±59.2	0.95bc±0.21	4.62c±2.22	0.341b±0.176
Acetic	32°C	0.35a±0.04	270	0.007a±0.000	857.3c±263.3	2.12d±0.31	0.28ab±0.08	0.015a±0.002
acid	24°C	2.79a±0.20	270	0.056a±0.004	356.1b±30.9	1.27c±0.09	0.94ab±0.11	0.071a±0.008
Hydrogen	32°C	0.10a±0.01	270	0.002a±0.000	159.3a±51.2	0.44a±0.24	0.02a±0.00	0.001a±0.001
peroxide	24°C	0.46a±0.01	270	0.009a±0.000	194.4ab±2.1	0.66ab±0.01	0.10a±0.00	0.006a±0.000

[1] Means ± Standard Deviation, n = 4

[2]Values in columns with different letters differ significantly ($p < 0.05$)

[3]Dough did not break before hook maximum vertical displacement (150 mm)

B. Zein-starch doughs

Treatment	Temperature	Peak Force (N)	Extension (mm)	Peak stress (kPa)	Strain at max. force (%)	Hencky strain (ε_H)	Extensional Viscosity (η_E, kPa.s)	Young's Modulus (*E*, kPa)
Distilled	32°C	0.71ab±0.17[1,2]	160.1c±38.2	0.014ab±0.003	420.2b±128.2	1.41c±0.29	0.29b±0.135	0.020c±0.008
water	24°C	2.40d±0.68	26.0a±6.8	0.048d±0.013	134.1a±16.9	0.29a±0.12	0.45c±0.134	0.013b±0.001
Acetic	32°C	0.23a±0.16	67.2b±30.1	0.005a±0.002	162.9a±33.0	0.47ab±0.21	0.05a±0.035	0.003a±0.003
acid	24°C	1.24c±0.03	50.5ab±6.0	0.025c±0.001	169.3a±12.9	0.52ab±0.08	0.24b±0.011	0.013b±0.002
Hydrogen	32°C	0.27a±0.05	270.0[3]d±0.0	0.005a±0.001	195.6a±5.0	0.67b±0.02	0.05a±0.011	0.004a±0.001
peroxide	24°C	1.20bc±0.14	270.0d±0.0	0.024bc±0.003	201.7a±1.9	0.70b±0.01	0.25b±0.030	0.017bc±0.002

[1] Means ± Standard Deviation, n = 4

[2]Values in columns with different letters differ significantly ($p < 0.05$)

[3]Dough did not break before hook maximum vertical displacement (150 mm)

marked effect of dough preparation treatment on the extensibility of zein-starch doughs. Doughs prepared in water and acetic acid broke well before maximum extension both above and below the T_g of hydrated zein and at lower temperature of 24^{o}C neither dough was substantially extensible. In contrast, the dough prepared in hydrogen peroxide solution was highly extensible, to the maximum extension of the Kieffer rig (270 mm), both at 24^{o}C and 32^{o}C.

Zein-starch dough was also prepared using 15% hydrogen peroxide at 60% liquid addition, as described. Further warm water could be added incrementally to this dough with hand working to a total of 80% liquid content, to obtain a highly extensible dough. Even when cooled to ambient temperature, the extended dough could be reformed and stretched again. The dough was extremely soft and extensible but exhibited very little strength (Fig. 1S supplementary material). Further, the dough did not exude water on standing at ambient temp., below the T_g of hydrated zein. The dough also remained flexible for several days when stored in a sealed polyethylene bag.

CLSM of zein only doughs prepared with water above the T_g. of zein revealed that before stretching, the dough appeared as an uneven mass of aggregated particles of hydrated zein (solid arrows) (Fig. 2Aa). On stretching, the particles aligned into linear fibrils in the direction of stretching (Fig. 2Ab). Within the fibrils there were some discontinuities (dotted arrows), which we have attributed to zein that had not full transformed into dough (Sly et al., 2014), presumably due to inadequate hydration of the zein. The transformation of zein into fibrils/fibres has been identified as critical to zein's visco-elastic dough behaviour (Lawton, 1992; Schober et al., 2010). With dough preparation with hydrogen peroxide before stretching, some of the zein particles had coalescenced into a continuous dough matrix

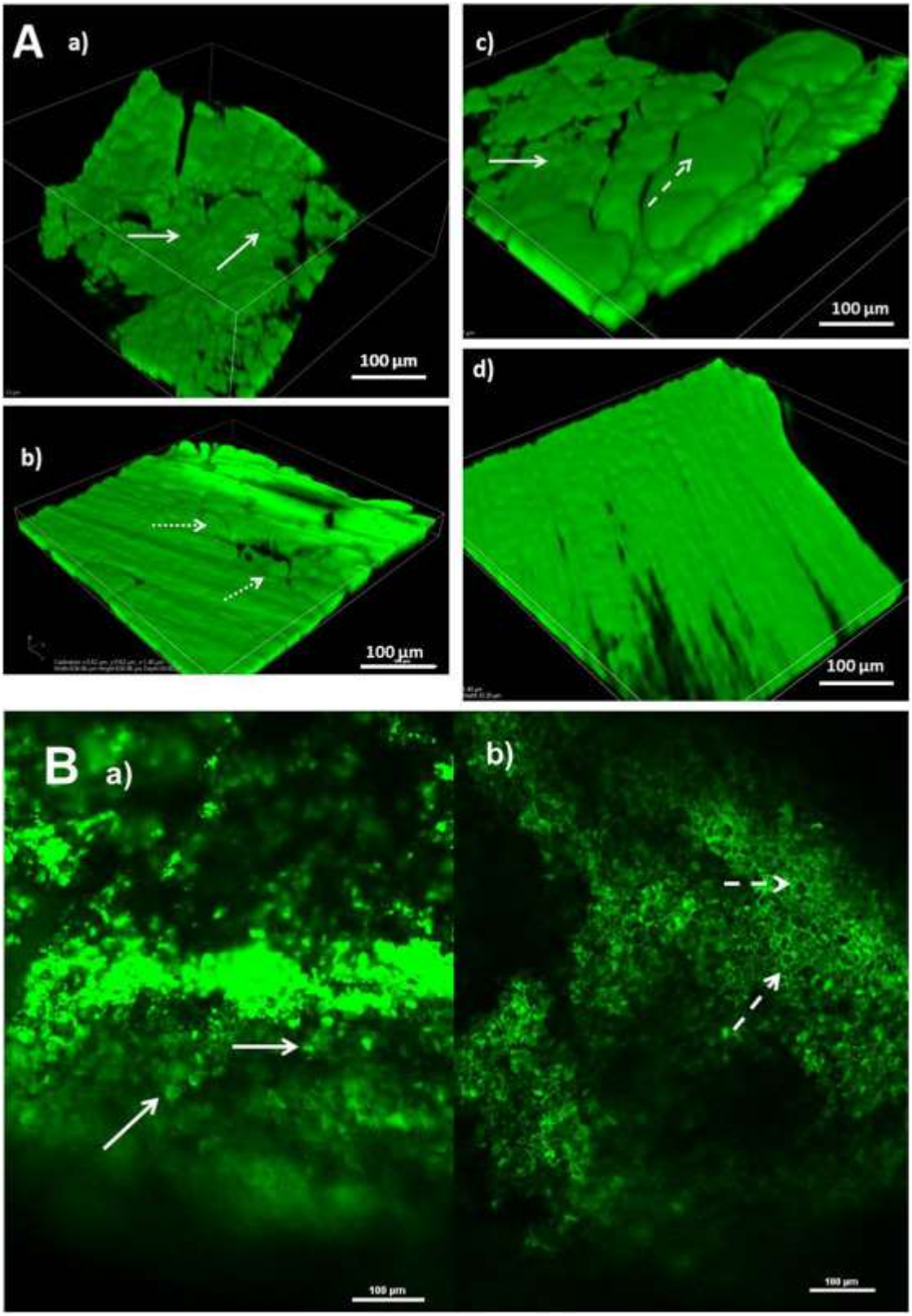

Fig. 2. Confocal laser scanning microscopy images of zein doughs prepared with water and hydrogen peroxide. A) Zein only doughs (z stack images). a. Water prepared - not stretched; b. Water prepared - stretched; c. Hydrogen peroxide prepared - not stretched; d. Hydrogen peroxide prepared - stretched. Solid arrows = linear zein fibrils, dotted arrows = discontinuities in fibrils, dashed arrows = continuous dough matrix. B) Zein-starch doughs (slice images). a. Water prepared; b. Hydrogen peroxide prepared. Solid arrows = starch granules surrounded by zein, Dashed arrows = continuous matrix of zein dough around starch granules.

(dashed arrow) (Fig. 2Ac). On stretching of the hydrogen peroxide prepared zein dough, fine very uniform aligned fibrils were formed with only small discontinuities (Fig. 2Ad).

Concerning the zein-starch doughs, doughs prepared with water exhibited clumps of starch granules, each surrounded by zein (solid arrows) (Fig. 2Ba). In contrast, the doughs prepared with hydrogen peroxide solution, the zein formed a uniform thin (approx. 2 μm) continuous matrix around the starch granules embedding them all (dashed arrows) (Fig. 2Bb). The ability of the hydrogen peroxide treated zein to form this thin continuous matrix of dough around the starch granules is presumably responsible for the great extensibility and better hydration of the hydrogen peroxide prepared zein-starch doughs (Table 1B and Fig. 1S).

3.2 Zein dough and film physico-chemical properties

SDS-PAGE confirmed that the commercial zein was essentially only α-zein (α-zeins 19 and 22), with no evident bands corresponding to β-zein (M_r 17-19 kDa) or γ-zein (M_r 27 kDa), i.e. bands less than or greater than those of α-zein (Esen, 1987) (M_r 21-25 kDa) (Fig. 2S, supplementary material). Further, there was no evidence of zein polymerisation of the doughs or films with any of the oxidising treatments, either under non-reducing or reducing conditions. Since the addition of oxidising agents can form both disulphide and ditryosine cross-links in wheat gluten (Wieser 2007; Tilley et al., 2001), it had been expected that any polymerisation of the zein due to disulphide and dityrosine cross-links would have been detected under non-reducing conditions and cross-linking due to dityrosine under reducing conditions. The absence of polymerisation due to the treatments under non-reducing conditions can be attributed to the fact that commercial zein is lacking cysteine groups for substantial disulphide bonding. As also found in this present work, commercial zein is

essentially α-zein (Oom et al., 2008) which contains only one near terminal end cysteine residue (Belton et al., 2006) and lacks the cysteine-rich γ- and β-zein sub-classes. The absence of visible induced polymerisation with SDS-PAGE under reducing conditions was probably due to the level of dityrosine cross-links being very low. Hanft and Koehler (2005) found that the level of dityrosine as a proportion of total tyrosine in wheat dough was less than 0.1% of the tyrosine residues, even at relative high levels of hydrogen peroxide addition (approx. 90 mg/g protein). Therefore any very low level of dityrosine cross-linking that might have influenced dough properties may not have been visible in the electrophoretograms.

FTIR spectroscopy of the dried zein doughs and aqueous-ethanol cast films both gave a very similar pattern in the Amide I region, with the band indicating α-helical conformation being predominant over that indicating β-sheet conformation (Fig. 3A, B). Alpha-zein is well-known to be predominantly α-helical with only a small amount of β-sheet conformation (Shewry and Tatham, 1990). There was no change in the relative proportions of the α-helical or β-sheet conformations of the zein doughs prepared with hydrogen peroxide or peroxidase when compared with the zein dough control (Fig. 3A). However, zein dough treated with both hydrogen peroxide and peroxidase showed a slight increase in the relative proportion of β-sheet conformation (Fig. 3A). Aqueous ethanol cast zein films treated with hydrogen peroxide also showed no conformational change compared with the water control, even when increasing levels of hydrogen peroxide were used (Fig. 3B).

The Amide II band pattern was somewhat different, with α-helical conformation predominating with the aqueous ethanol cast zein filmstreated with water or hydrogen peroxide (Fig. 3B) and β-sheet conformation apparently being predominant in doughs for all

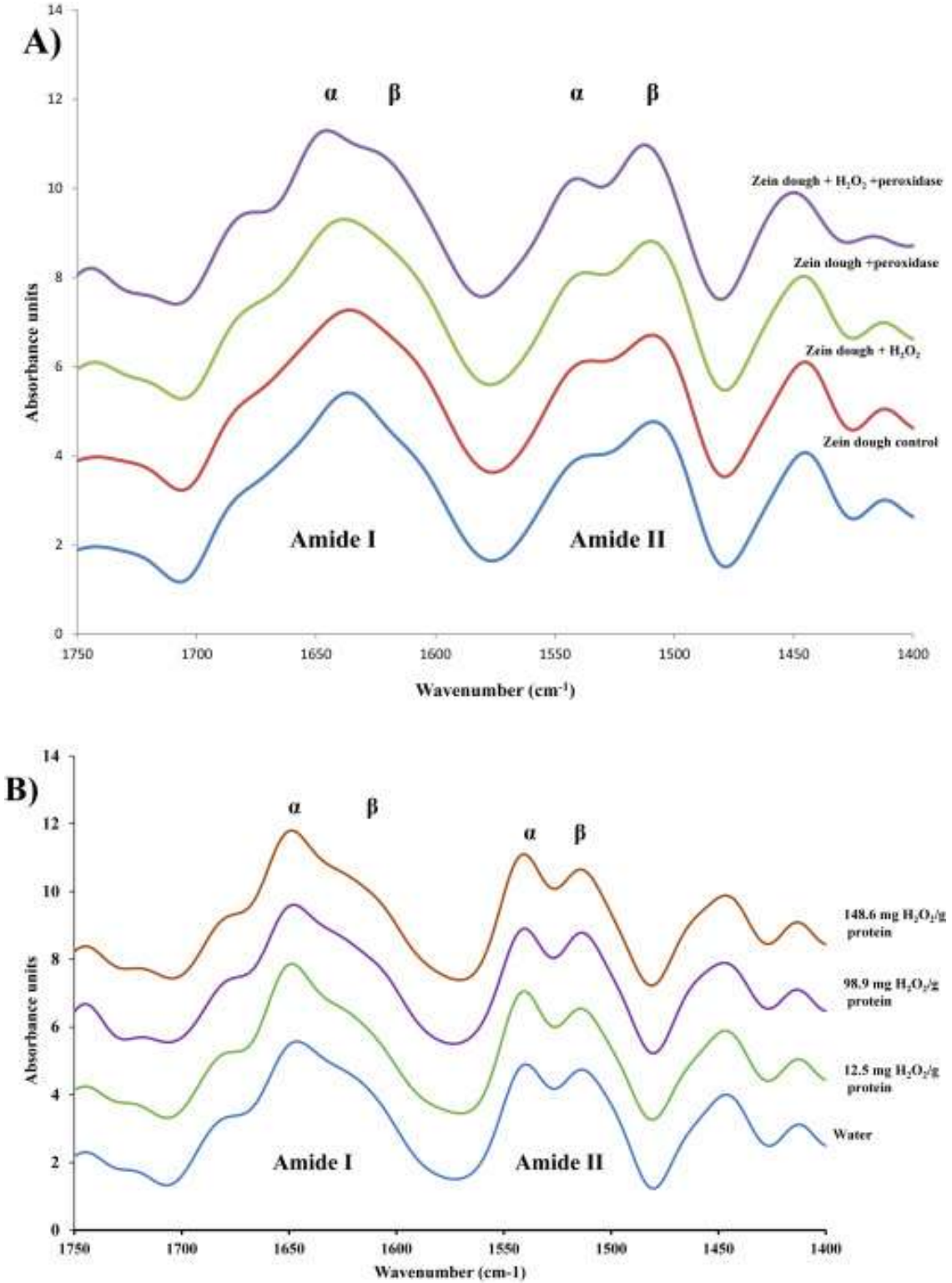

Fig. 3. Fourier transform infra-red spectroscopy traces of zein doughs and films prepared with water and oxidising agents. α = band associated with α-helical conformation, β = band associated with β-sheet conformation. A) Doughs prepared with water, hydrogen peroxide, peroxidase and hydrogen peroxide + peroxidase. B) Films prepared with water and hydrogen peroxide.

the treatments (Fig 3A). However, it is doubtful whether the latter was a correct reflection of the zein's conformation. The Amide II band has much lower protein conformational sensitivity than the Amide I band (Kong and Yu, 2007) and for this reason the Amide I region is normally used in studies of the secondary structure of zein and other prolamins (Mejia et al., 2007).

Mejia et al. (2007) found that when zein dough was stretched at 35^{o}C (above zein's hydrated T_g) there was a substantial increase in β-sheet conformation as determined by FTIR spectroscopy. However, β-sheet conformation declined when the zein was cooled to 25^{o}C, below the T_g. Hence, in this present work it appears that none of the oxidising treatments on either aqueous zein dough or zein dissolved in aqueous ethanol resulted in a permanent change in protein secondary structure, despite the fact the doughs retained extensibility below the T_g of hydrated zein. This finding is consistent with absence of observed zein polymerisation with the treatments (Fig. 2S), since substantial covalent bonding would probably have stabilised the changes in secondary structure, as takes place with wheat gluten (Weegels et al., 1994).

Concerning the physical properties of the zein films prepared with hydrogen peroxide, there was a progressive significant ($p<0.05$) increase in phosphate buffer uptake by the films with increasing hydrogen peroxide concentration, from 53% for films prepared with water to 70% with 150 mg hydrogen peroxide/g protein (Table 2). This is in contrast to our findings with kafirin films when covalent cross-linking took place as a result of aldehyde treatment, there was a reduction in buffer uptake by the films (Anyango et al., 2011). It is, however, in agreement with the observation in the present study that the zein doughs prepared with hydrogen peroxide solution did not exude water on standing.

Table 2

Effect of different concentrations of hydrogen peroxide on the phosphate buffer uptake and T_i, T_g and ΔH_m of zein films[1]

Hydrogen peroxide concentration (mg/g protein)	Phosphate buffer uptake (%)	T_i (^{0}C)	T_g (^{0}C)	ΔH_m (J/g/K)
0	52.9a±0.3[2,3]	176.5a±0.4	178.3a±0.5	0.61a±0.04
12.5	57.9b±1.5	176.5a±0.1	178.3a±0.1	1.94d±0.01
100	63.3c±0.3	176.4a±0.0	178.3a±0.1	1.06b±0.04
150	70.3d±1.9	176.6a±0.1	178.2a±0.1	1.56c±0.11

[1]T_i = glass transition onset temp., T_g = glass transition midpoint temp., ΔH_m = enthalpy of transition

[2]Mean values ± standard deviation, n=2

[3]Mean values in the same column but with different letters differ significantly ($p<0.05$)

The T_g of the dried zein film zein, taken as the midpoint transition temperature, was 178^oC (Table 2). This is considerably higher than the 139^oC reported by Madeka and Kokini (1996) also for Sigma zein (but not prepared into films), but more similar to the 160^oC reported by these authors for purified zein and the 165^oC reported for laboratory prepared zein by Magoshi et al. (1992). The dry film zein T_g was not affected by the hydrogen peroxide treatments. This is in contrast to work which showed that covalent cross-linking substantially increased the T_g of films cast from gliadin in the presence of cross-linking agents (Hernández-Muñoz et al., 2005). The absence of an effect of hydrogen peroxide treatment on zein T_g is nevertheless consistent with the SDS-PAGE results (Fig. 1S) that there was no observable polymerisation of the zein by hydrogen peroxide. Casting the zein films in the presence of hydrogen peroxide did, however, substantially significantly increase ($p<0.05$) ΔH_m, the enthalpy of transition, although there was no trend in the magnitude of ΔH_m with increasing hydrogen peroxide concentration (Table 2). As there was no evidence of hydrogen peroxide induced polymerisation this effect could have been a result of increased molecular entanglement.

4. Conclusions

Preparation of zein doughs in the presence of hydrogen peroxide radically alters their rheological properties. The doughs become softer and highly extensible and retain this extensibility when cooled below the T_g of hydrated zein. Further, the doughs hold water below the zein T_g, whereas without hydrogen peroxide the zein rapidly exudes water and reverts back its amorphous state and hardens. The absence of observable polymerisation, no increase in T_g nor clear change in secondary structure indicates that the hydrogen peroxide

treatment did not result in substantial oxidative cross-linking of the commercial zein, unlike that which occurs with gluten (Hanft and Koehler, 2005; Rodriguez-Mateos et al., 2006). This can be attributed to the fact that commercial zein has limited capacity for disulphide bonding as α-zein, its predominant component, has only one terminal cysteine residue.

Oxidation of proteins with hydrogen peroxide can also bring about formation of hydroxyl and carbonyl groups on hydrophobic amino acid residues with aliphatic side chains such as leucine, isoleucine, valine and alanine, and oxidise proline to hydroxyproline and pyroglutamic acid (Xu and Chance, 2007). These amino acids are predominant in commercial zein, accounting for some 46 g% of its composition (Shukla and Cheryan, 2001). Huang et al. (2016) found that metal catalysed hydrogen peroxide induced oxidation caused backbone fragmentation and carbonyl side chain formation in C-hordein. Since under the oxidation conditions employed in this work there was no evidence of zein fragmentation, it seems likely that the major reaction taking place was hydroxylation of the zein amino acid aliphatic side chains. Such hydroxylation would account for the improved water holding of zein dough and increased water uptake of the zein films that could occur through hydrogen bonding.

The change in zein dough rheology with oxidation by hydrogen peroxide is far less temperature dependant than that found using dilute organic acid treatment (Sly et al., 2014) and does not require addition of hydrocolloids (Schober et al., 2008) or of co-proteins (Mejia et al., 2012). Although many challenges still remain, oxidative modification of zein rheological properties represents a further step forward in development of technology to enable zein to replace gluten in dough systems.

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Appendix A. Supplementary data

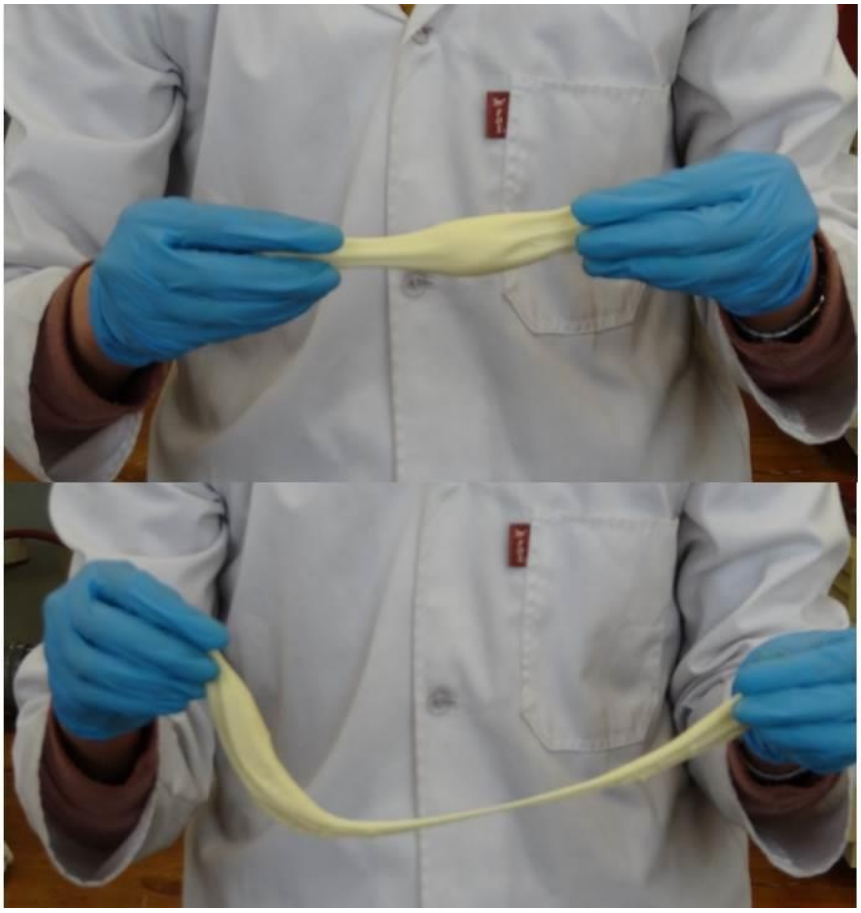

Fig. 1S. Images demonstrating extensibility of hydrogen peroxide prepared zein-starch dough

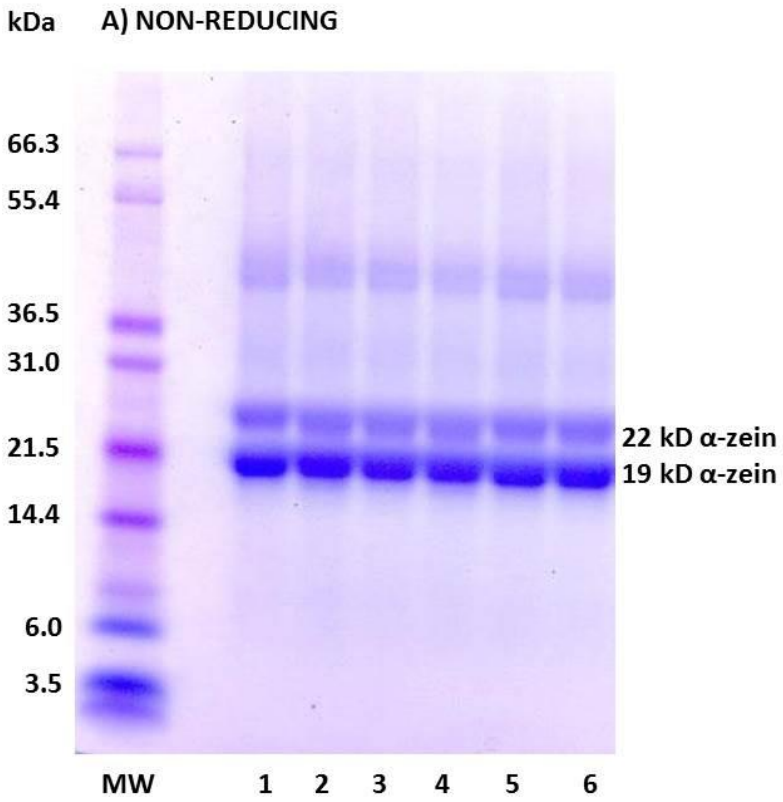

Fig. 2S. Sodium dodecyl sulphate-polyacrylamide gel electrophoregrams of zein doughs and films prepared with water and oxidising agents.
A) Non-reducing, B) Reducing. MW standards; 1. Dough water; 2. Dough hydrogen peroxide; 3. Dough peroxidase; 4. Dough hydrogen peroxide + peroxidase; 4. Film water; 6. Film hydrogen peroxide.