Correlation Between Viscoelasticity, Microstructure, and Molecular Properties of Zein and Pennisetin Melts

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ABSTRACT: Cereals are a large source of biopolymers, where mainly the starch is used for food and feed. A rapidly growing cereal application is the production of biofuel, mainly produced from corn in the US. The starch is fermented to ethanol leaving spent grain rich in cereal proteins as a by-product. The corn protein zein is currently extracted on a large scale and used in, for example, material applications. Similarly, pennisetin can be extracted from pearl millet, a crop critical for food security in sub-Saharan Africa. The formation of viscoelastic melts is crucial for (bio)plastics production and the viscoelasticity, microstructure, and molecular properties of zein and pennisetin melts were determined here. The proteins were mixed with plasticizers (polyethyleneglycol or glycerol/citric acid) to form melts. The melts displayed a phase separated microstructure with protein-rich and plasticizer-rich regions with distinctly separate T_gs. The pennisetin melts formed cross-links at temperatures above 60°C, which could be related to the high content of cysteine and methionine, as compared to zein. As a consequence, pennisetin melts showed a more thermocomplex behavior than zein melts. For zein melts, the mixture of glycerol and citric acid interacted with protein in addition to being a plasticizer causing a high-molecular weight shoulder in the molecular weight distribution. The study showed that, although both zein and pennisetin form viscoelastic melts, the choice of plasticizer strongly affects both melt structure and physical properties. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 125: 2245–2251, 2012

Key words: zein; pennisetin; bioplastic; rheology; TEM; Van Gurp-Palmen plot; molecular weight determination

INTRODUCTION

In recent decades, biodegradable materials from renewable agricultural resources such as starch and proteins have attracted much attention for sustainable development and environmental conservation. Plant proteins such as corn protein, wheat gluten, soy proteins, and so on have been used to manufacture bioplastics.[1,2] These proteins have the advantages of being thermoplastic, abundant, relatively inexpensive, biodegradable, and at the same time hydrophobic and insoluble in water.

Cereals are large volume commodities mainly used for food and feed. Starch has been well-researched in the material and foam areas (see e.g., the review by Liu et al.[3]), whereas cereal proteins are much less developed. Biofuel (ethanol) is starting to compete for the grain at a large scale. The by-products of both biofuel production and food (brewers spent grain and bran) are mainly used for feed, yielding no or low profit. The by-products are rich in protein, however, which can be extracted for conversion to, for example, high-value cereal proteins.

Maize is the largest crop produced globally and ~ 40% is produced in the US. It is mainly used for feed but a rapidly growing amount is used for biofuel and materials applications.[4] The maize prolamin protein zein is commercially available as a food additive and the large amount available from the by-products of biofuel production is presently being extracted at a large scale, resulting in decreasing prices.

Although the indigenous African cereal pearl millet accounts for a marginal fraction of the world grain production, millets are extremely important for the subarid and subhumid zones as staple crops.[5] Pearl millet and similar millets are cereals that are uniquely drought-tolerant and produce crops when other cereals such as maize fail, which contributes to their importance for food security. As we enter the greenhouse age, many agricultural

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areas will become dry; the importance of drought-tolerant crops, such as pearl millet, will grow in a global perspective and these will to an increasing extent replace other crops for food and biofuel applications. The prolamin protein of pearl millet is pennisetin and it is considered similar to kafirin from sorghum, which is even more hydrophobic than zein.[6] There are no further published results of the material properties of pennisetin.

The amino acid sequence of zein is well known and has been published.[7] Pearl millet is less studied but contains typically higher quantities of the essential amino acids methionine and cysteine than maize.[8] Zein and pearl millet can be further divided into the slightly differing protein fractions as α-, β-, and γ-species.[5] The α-zein shows two broad bands in gel electrophoresis with molar masses of 19 and 22 kDa, and there are various models for the tertiary structure of α-zein.[9] The latest research by Momany et al.[10] describes the molecule as being a coiled-coil of alpha helices with four residues per turn in the central helical section. Nonpolar residues form a hydrophobic surface inside a triple superhelix. The nine helical sections that have been previously postulated[11] are modeled as three sets of three interacting coiled-coil helices.

Using cereal proteins for nonfood applications may be a promising way to produce biodegradable materials with a large range of functional properties owing to their unique structure.[12] A protein-based material can be defined as a stable three-dimensional (3D) macromolecular network stabilized and strengthened by hydrogen bonds, hydrophobic interactions, and disulfide bonds.[1] However, as cereal protein materials are fragile, a plasticizer is required. Plasticizers are low-molecular weight molecules that modify the 3D structure of proteins by increasing their flexibility. The most commonly studied plasticizers are water, glycerol, sorbitol, mannitol, diglycerol, triethylene glycol, polyvinyl alcohol, polyethylene glycol, and lipids.[13–19]

These plasticizers plasticize all the cereal proteins, but there are no known perfect plasticizers for zein[18] and even less is known about pennisetin plasticization. When the amount of plasticizer is increased, the plasticization effect will increase but phase separation into protein-rich and plasticizer-rich regions will occur.[20]

Molecular weight and its distribution is an important parameter in commercial polymer production and processing. There are many publications that relate the molecular weight distribution (MWD) to viscoelastic properties of melts, at least for synthetic polymers.[21,22] However, this relationship has been little discussed for biopolymer melts. Not a great deal is known about the structure of protein melts. Thus, the combination of MWD data obtained from the mechanical spectrum and microscopy will give new insight into the protein melt structure. In this work, the model by Cocchini and Nobile[21,23] has been used to obtain molecular information for zein and pennisetin in melts. The Cocchini and Nobile model predicts the MWD from the mechanical spectrum ($G^*(\omega)$) of the melt.

The aim of this study was to determine the melt properties of pennisetin as compared to zein, to obtain molecular data on the proteins and to correlate these data to melt microstructure and physical properties. These relationships can be used to predict melt behavior and thermoforming. Thermoplastic cereal proteins have a wide range of applications, from injection molding of disposables and extruded foams to wheat-free bread.

EXPERIMENTAL

Zein was obtained from Sigma-Aldrich (Schnelldorf, Germany). Pennisetin was provided by Dr. Dlmani CSIR, South Africa. It was extracted from pearl millet grain using aqueous ethanol plus sodium metabisulphite at elevated temperature by a procedure similar to the industrial processes described for zein by Shukla and Cheryan.[17] Before melt preparation, the proteins (zein and pennisetin) were defatted in *n*-hexane as described by Oom et al.[24] The protein content for zein and pennisetin were 95 and 99% (w/w), respectively. Polyethyleneglycol 400 (PEG 400), citric acid, glycerol, and the other chemicals used were of analytical reagent grade and obtained from Sigma-Aldrich.

Zein and pennisetin were hand mixed with aqueous ethanol (70%, v/v ethanol) at a ratio of solvent/protein = 0.3 to form a melt. The solvent/plasticizer liquid was quickly absorbed into the protein rendering a melt. Hand mixing was used to mimic the potential application of bread making. Then, the plasticizer was added under further mixing at room temperature to form a homogeneous melt. Two types of plasticizers were used for plasticization, citric acid plus glycerol (2 : 1) and PEG 400 at a ratio of plasticizer/protein = 0.1.

The dynamic rheological properties of the melts were determined on a controlled strain rheometer ARES G2 (TA Instruments, New Castle) using parallel-plate geometry (20 mm diameter and 2 mm gap). The blend was placed between plates immediately after mixing, and the test was started after the melt had rested for 10 min. The exposed edges were covered with paraffin oil to reduce water loss from the sample. Strain sweep tests at 10 rad/s were performed beforehand on separate samples to identify the linear viscoelastic region of the melts. Measurements of each experimental point were made at least in triplicate. Temperature scan tests, from 10 to 80°C, were carried out at constant frequency (10 rad/s) and strain (0.1) at a heating rate of 2°C/min. Time scans of viscoelastic properties were made at constant frequency and

strain. The shift in loss tangent and storage modulus were recorded during 3600 s at 60°C. Mechanical spectra were recorded from 100 to 0.1 rad/s in oscillatory shear, at a constant strain of 0.01 and at constant temperatures of 10, 20, 30, 40, 50, and 60°C.

The molecular properties of zein and pennisetin were characterized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions as described by Da Silva and Taylor,[25] but using a 4–12% acrylamide gradient gel prepared as described by Byaruhanga et al.[26]

Modulated differential scanning calorimetry (MDSC) measurements were carried out in Q100 (TA Instruments, New Castle) using 5–10 mg samples in aluminum pans. An oscillation period of 60 s, an amplitude of ±0.5°C, and a heating rate of 10°C/min was used. The sample was purged with a nitrogen flow of 50 mL/min.

Thermogravimetric curves were obtained in a TA Q50 analyzer (TA Instruments). Evaporation tests, between 40 and 150°C, were carried out on 5–10 mg samples under a nitrogen atmosphere at a heating rate of 10°C/min.

The microstructure of different blends was analyzed with a transmission electron microscope (TEM). Small pieces of blends were chemically fixated in 2% glutaraldehyde and postfixated with 1% OsO_4 in the same salt solution. Samples were dehydrated in grade ethanol series, followed by an infiltration in resin, LR White, and then polymerized. Thin sections of ~ 70 nm were cut on a ultratome Reichert-Jung Ultracut E (Reichert-Jung, Germany) using a diamond knife and stained with uranyl acetate and lead citrate. The sections were examined in a TEM, LEO 906 E (LEO Electron Microscopy, Cambridge, England) at an acceleration voltage of 80 kV.

RESULTS AND DISCUSSION

Microstructure of pennisetin- and zein-based melts

The microstructure of pennisetin- and zein-based melts was determined using transmission electron microscopy (TEM) as shown in Figure 1. The micrographs show that the plasticizer and protein phase separate in all samples into one phase rich in protein (dark) and one phase rich in plasticizer/solvent (bright). This effect was more pronounced in the pennisetin melts than in the zein-based melts. Thus, there were many spherical droplets of several sizes of plasticizers in the zein-based melts. The small droplets of plasticizer were also likely to be able to migrate in the melt and merge together creating the larger droplets. Conversely, there were more large droplets of plasticizers of irregular shape in the pennisetin-based melts, which indicates a higher heterogeneity. The larger droplets are forming a chain-like structure in both zein melts. This, may originate from tip streaming caused by extensional flow during mixing.[27] At higher magnifications, it can be seen that the protein network of zein melts is infiltrated by very small droplets of plasticizer, which is not seen in the pennisetin melts. These droplets are round and have a well-defined interface between the protein network and the plasticizer inside the droplets. The pennisetin melt with glycerol/citric acid is the most phase separated sample of these four protein melts and has the

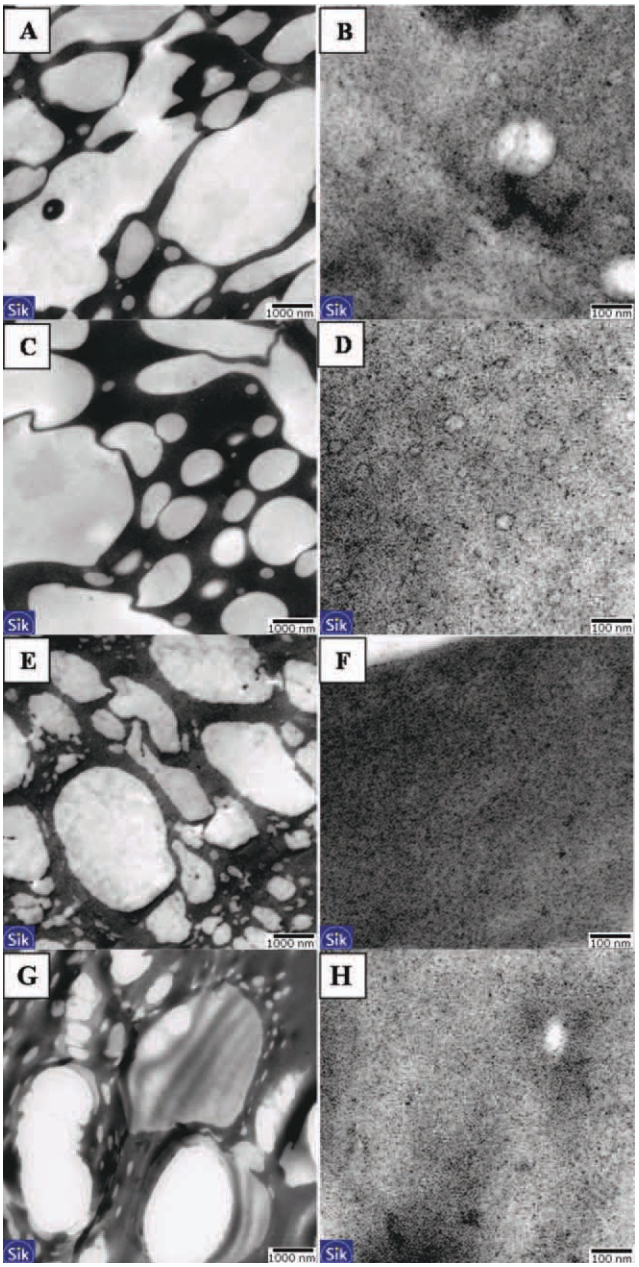

Figure 1 TEM micrographs of protein-based melts [scale bar is 1000 (right) and 100 nm (left)]: zein melt with (A, B) PEG and (C, D) glycerol/citric acid; pennisetin melt with (E, F) PEG and (G, H) citric acid/glycerol. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

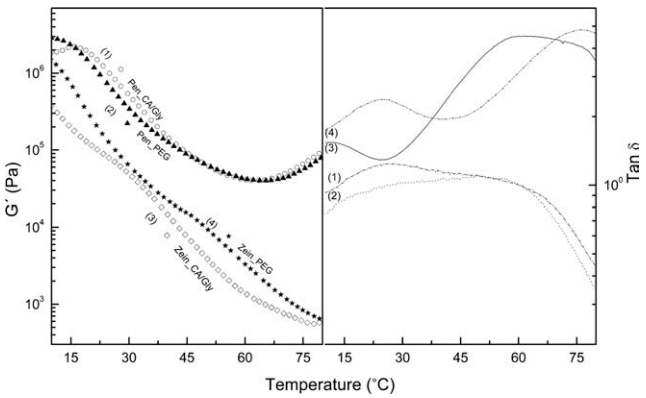

Figure 2 Temperature ramp test in oscillatory shear for zein- and pennisetin-based melts plasticized with PEG and citric acid/glycerol: storage modulus (G') (A) loss tangent (tan δ) (B).

largest droplets of plasticizer, with the protein in thin lamellas between the plasticizer containing droplets. The high plasticizer/protein ratio is the main cause of the phase separation. The mixing could also influence the phase separation, as it involved only low mechanical energy thus involving less exposure of the protein to the plasticizer.

Thermo-mechanical behavior of pennisetin- and zein-based melts

Figure 2 shows the changes in G' (storage modulus) and tan δ (loss tangent) with temperature, at constant frequency, for the protein melts plasticized with PEG and citric acid/glycerol. Zein exhibited the expected, uniform decrease in G' with temperature that is typical of a melt, whereas pennisetin showed distinctly different regions as the temperature was raised. G' decreased between 10 and 60°C down to a plateau region, resulting from increased protein–protein interactions. Between 60 and 80°C, G' underwent an unexpected increase and tan δ a dramatic drop, which could be attributed to protein cross-linking reactions or to solvent evaporation.[28] The solvent evaporation is shown in Figure 3(A); the weight loss is the same for both zein and pennisetin melts and the loss is only 6% at 80°C. Cross-linking reactions in the pennisetin melts above 60°C are, therefore, the likely explanation of the increase in G'. Figure 3(B) shows DSC thermograms of the denaturation of pure zein and pennisetin.[29] The peaks are found around 90°C and are broad. This means that the denaturation already starts below 60°C and could be the cause of the cross-linking in the pennisetin melts. The molecular differences between the two proteins are still sufficient not to cause the same cross-linking in the zein melts.

As observed in Figure 2, the pennisetin melts obtained with both plasticizers displayed more structured systems with higher values of G' and lower values of tan δ. It is evident that the tan δ values of pennisetin are always lower than zein, which corresponds to a predominantly more elastic microstructure. Nevertheless, the temperature dependence of the storage modulus was always similar for zein and pennisetin melts in the case of both plasticizers.

Two tan δ peaks in the zein and pennisetin with PEG and citric acid/glycerol as plasticizer can be observed [Fig. 2(B)]. These glass transition temperatures (T_g) of zein plasticized with PEG occurred at 25 and 76°C, whereas T_g of zein plasticized with citric acid/glycerol were found at 10 and 59°C. The T_g for PEG plasticized melts appeared in the case of pennisetin, at 25 and 60°C and, for the citric acid/glycerol plasticized melts at 17 and 60°C. This observation can be related to the lower molecular weight of citric acid and glycerol as compared to PEG and that they thus exert a more pronounced plasticizing effect on the proteins as compared to PEG. In a previous study, Oom et al.[24] observed a $T_g = -3$°C for zein resins plasticized with oleic acid and aqueous ethanol. These resins were prepared by precipitation in excess of solvent, and the low T_g compared to the present study can be explained by higher levels of plasticizer in these resins.

SDS-PAGE under reducing condition showed (Fig. 4) that the zein used in the melts contained a group of monomers at 19 and 22 kDa identified as α_1 and α_2, that are less rich in histidine, arginine, proline, and methionine.[11] Zein also showed bands at 10 and 18 kDa, identified as β and δ, which are less abundant but rich in methionine.[28,30] In addition, three oligomers were found at 38, 49, and 60 kDa. Pennisetin exhibited two major bands at 19 and 22 kDa in the same region as α-zein but homologous with δ-zein, which are rich in methionine and

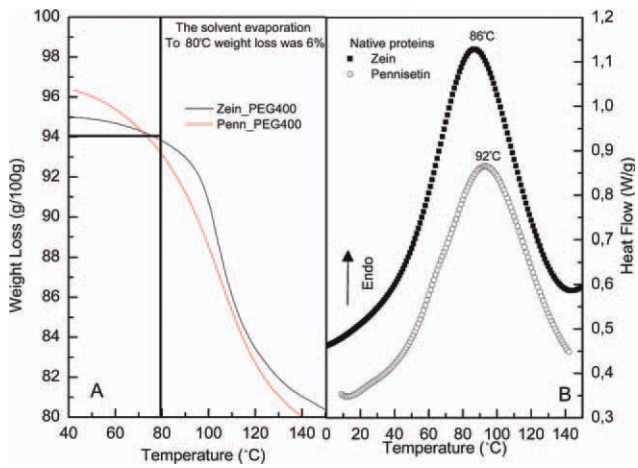

Figure 3 The solvent evaporation of zein- and pennisetin-based melts with PEG400 (A) DSC thermograms for pure zein and pure pennisetin protein (B). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

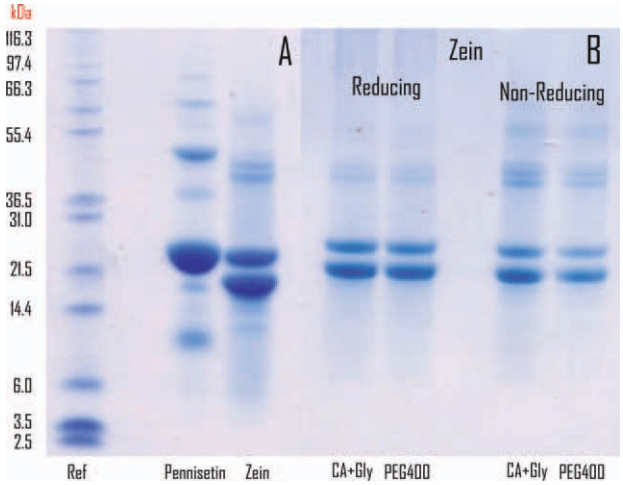

Figure 4 SDS-PAGE for pure pennisetin and pure zein, under reducing conditions. Lane 1: marker, lane 2: pennisetin, lane 3: zein (A), zein-based melts under reducing and nonreducing conditions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cysteine,[31] the sulfur-containing amino acids. Pennisetin also contained a band at 9 kDa, called α-setarins, which are relatively rich in methionine[32,33] and can be related to the δ-zein. In this sense, the higher values of G' found for pennisetin melts as a result of protein cross-linking reactions (Figs. 2 and 5) could be associated to disulfide bridge breaking as these were not present to the same extent in zein.

Rheological behavior of pennisetin- and zein-based melts

The linear viscoelastics mechanical spectra for a given protein melt can be empirically superposed on a "van Gurp-Palmen-Plot" (vGP-plot) as in Figure 5. This plot can be used as an indicator of the applicability of time-temperature superposition (tTS). It is represented by the phase angle, δ, of the rheological data plotted as a function of the corresponding absolute value of the complex shear modulus $|G^*|$. For tTS to be applicable, the isothermal frequency curve should merge into one common line.[34] It is worth mentioning that this plot also provides a time-dependence curve that is very sensitive to change in microstructure.[35] Figure 5 shows the vGP-plot of the mechanical spectra of the proteins at different temperatures (10–60°C).

As can be observed in the Figure 5(C,D) for pennisetin blends, there is an apparent failure of the results to merge into one line, in particular at temperatures above 40°C, where the effect of protein

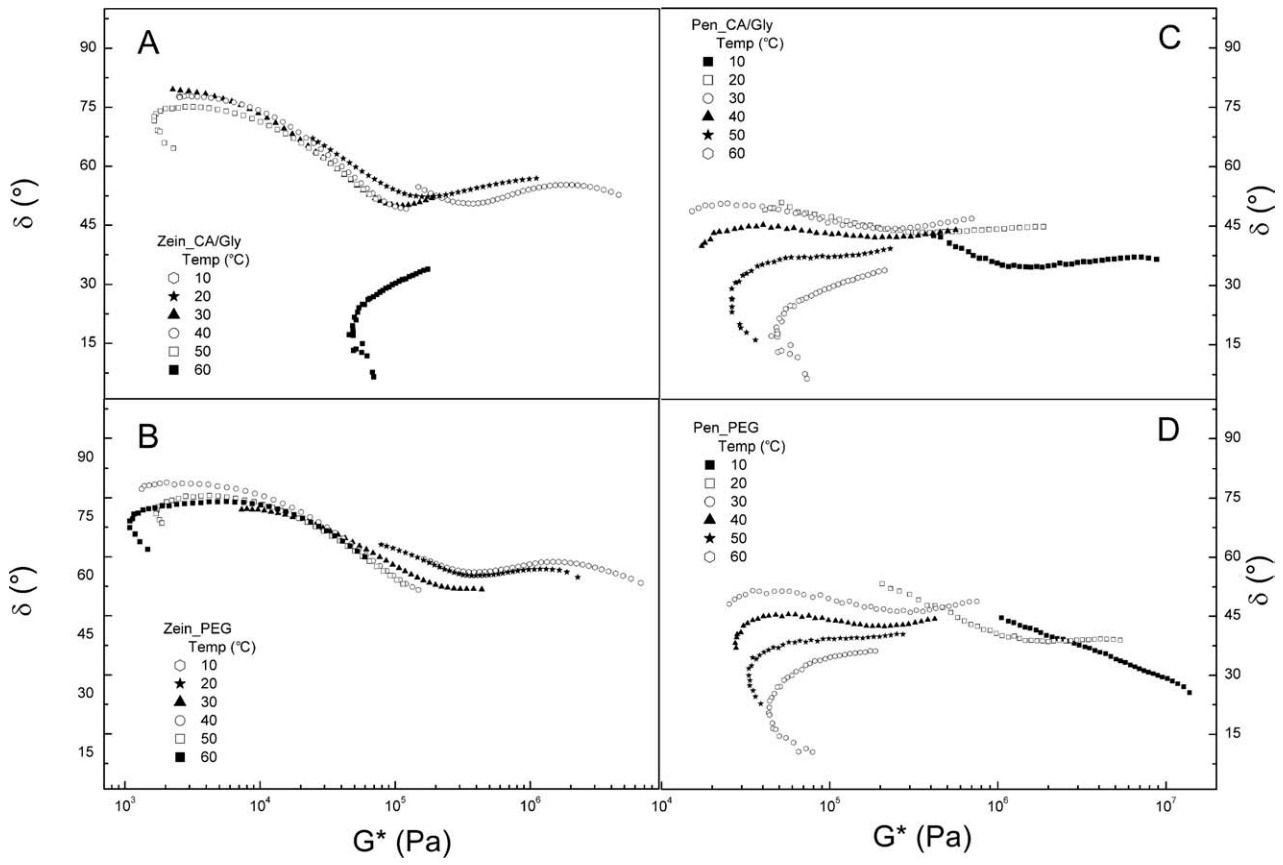

Figure 5 The vGP plots of protein-based melts. Zein-based melts with PEG (A), zein-based melts with citric acid/glycerol (B), pennisetin-based melts with PEG (C), and pennisetin-based melts with citric acid/glycerol (D).

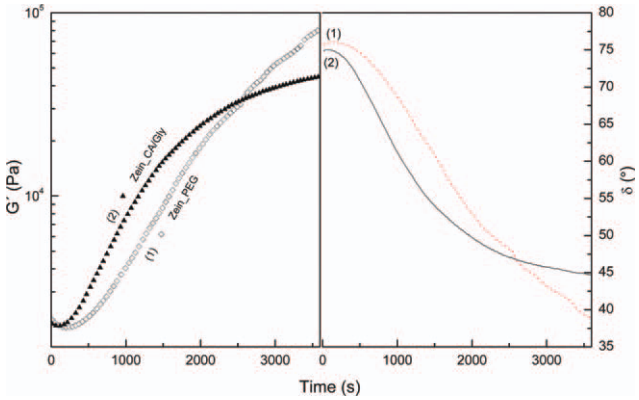

Figure 6 Dynamic time scan at 60°C for zein-based melts plasticized with PEG and citric acid/glycerol. Storage modulus (A) change of the phase angle (B). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

denaturation is more pronounced (cf. Fig. 3). The pennisetin melts has a complex thermorheological behavior, which, for example, could depend on changes in the physical microstructure with temperature, which in turn can be confirmed by performing temperature ramp tests (Fig. 2). Thus, the pennisetin melts did not obey the tTS principle.

Contrary for zein melts, the isothermal frequency curve fitted fairly well, which points to a much simpler thermorheological behavior than for pennisetin melts, especially for temperatures below 50°C. Figure 5(A,B) show results obtained at temperatures between 10 and 60°C.

As apparent in Figure 5(A) for zein melts plasticized with citric acid/glycerol, zein showed a change in microstructure at 60°C. This phenomenon was further corroborated by a time dependence test in oscillatory shear at 60°C for a period of 3600 s. As can be seen in Figure 6, zein exhibited a pronounced change with a marked increase in storage modulus (G') and a decrease in phase angle (δ) from 75 to 35°. This increase in the elastic component occurred within 500 s. The effect is believed to be similar to the previously observed aging in zein dough systems.[24] The aging was explained by oxidation of the protein. Despite sealing the edges of the melt during the measurement, the oxygen level inside the sample is apparently sufficient to cause oxidation at this elevated temperature.

tTS was used to expand the time or frequency regime of the mechanical spectrum. Figure 7 shows the resulting master curves after tTS for mechanical spectra between 10 and 40°C, presented at a reference temperature of 10°C, for G' and G''. As can be observed, the loss modulus in both samples is always slightly higher than the storage modulus in this temperature range at high and intermediate frequency, whereas at low frequency, the difference between G'' and G' increases, as expected for an amorphous melt. There is a hook at low frequency for the zein melt plasticized with citric acid/glycerol [Fig. 7(B)] that comes from the mechanical spectra obtained at 40, 50, and 60°C. This is the same effect of oxidation that is observed in Figure 6. This mechanical spectrum was not included in the calculation of the master curve.

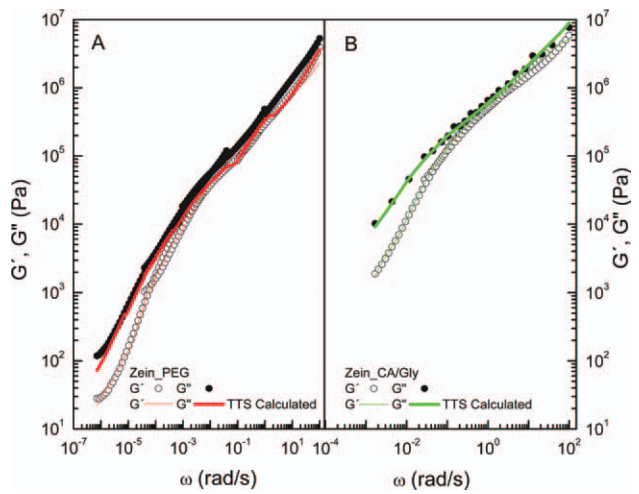

Figure 7 Master curve of the frequency dependence of the linear viscoelasticity functions for zein-based melts plasticized with PEG and citric acid/glycerol. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Molecular weight distribution and microstructure

The numerical prediction of the MWD data using the model proposed by Cocchini and Nobile[23] for zein based melts is shown in Figure 8. The plateau modulus was calculated from the relaxation time spectrum for each sample and the model was calibrated using the molecular weight determined by SDS-PAGE (Fig. 4) in the absence of other calibration data. To render absolute predictions of the molecular

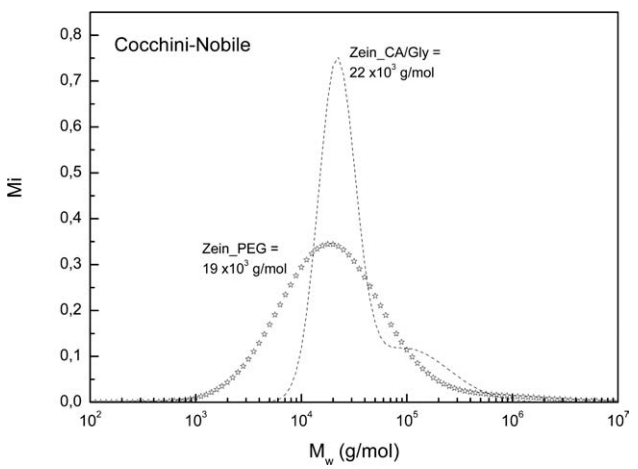

Figure 8 The numerical prediction of MWD for zein-based melts plasticized with PEG and citric acid/glycerol.

weight, the model must be calibrated with some reference data on the melt under study or by literature data concerning a melt with a behavior similar to the one studied. This means that, while the model did not give absolute molecular weight data, the effect of the two plasticizer systems could be compared. The same calibration constant k was used in both cases. A distinct influence of the plasticizer system could be observed on the MWD. The melts plasticized with PEG yielded a broader distribution. The melts plasticized with citric/glycerol acid had a narrower distribution and displayed a distinct shoulder at 10 times the main peak caused by polymer aggregation. This indicates that citric acid/glycerol has a more pronounced effect on the protein conformation. We can speculate that the reducing effect of citric acid is the main factor and that this is also the effect of the different melt structure observed in the microstructure (Fig. 1). The SDS-PAGE results [Fig. 4(B)] of zein plus plasticizers in solution also show a formation of oligomers at higher molecular weight.

CONCLUSIONS

Zein and pennisetin in the presence of plasticizers can form viscoelastic melts. Both protein melts form a phase separated microstructure at the present ratio of plasticizer-solvent/protein of 0.5. The different phases have clearly different T_gs indicating phases rich in protein or rich in plasticizer. The pennisetin melts, as compared to zein melts, formed cross-links at temperatures above 60°C, which could be related to the high content of cysteine and methionine. As a consequence, pennisetin melts showed a more thermocomplex behavior than zein melts. The mixture of glycerol and citric acid interacted with zein in addition to being a plasticizer. This mixture caused a polymerization of the protein with a high-MW shoulder in the MWD, contrary to what was observed when PEG was used as a plasticizer. These melts showed a uniform, broad MW distribution.

A general conclusion is that as protein-based bioplastics need plasticization in all practical applications, the choice of plasticizer will significantly affect the behavior of the melt. It may not only cause a phase separated structure but may also induce polymer–polymer interactions.

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