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A One-Step Approach for Esterification of Zein with Methanol

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ABSTRACT: Zein (corn protein) was reacted with methanol in the presence of *para*-toluenesulphonic acid under mild conditions to give a material formed by esterification of predominantly the amide groups of the protein. The formation of methyl zeinate was confirmed by proton NMR. The new signal appeared at 3.67 ppm in zein methylation product, which is absent for pure zein can be assigned to the protons of the CH_3 group of an ester. The strong C=O stretching vibration due to the presence of ester group in the region of 1739 cm^{-1} in case of methylated zein was also noticed from FTIR studies. The increase in the C : N atom ratio in the zein methylation product obtained from elemental analysis results further indicates the conversion of a significant proportion of the $-CONH_2$ groups in zein to $-COOCH_3$ groups in the esterified product. The methylated product had glass transition temperature about 20°C lower than that of the unmodified zein. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: biopolymers; zein; proteins; modification; plasticization

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INTRODUCTION

There is a growing interest on using biopolymers to replace petroleum based synthetic polymers. Zein, the main storage prolamin of corn, has been a widely used popular natural polymer due to its useful properties.[1] Most of the applications depend on the ability of zein to form hydrophobic coatings, and on its resistance to microbial action being biodegradable at the common natural conditions. A commercially available source of zein is the corn gluten meal (CGM) that is a by-product of starch extraction from corn and can be utilized as a raw material to produce thermoprocessable polymeric materials based on zein.[2]

Zein is soluble in mixtures of ethanol, isopropanol, and other organic solvents with water, and zein films with relatively high tensile strength can be made by solution casting. The principal disadvantage of films made from zein (and other proteins) is their brittleness. The conventional approach to imparting flexibility to zein films is plasticization with compounds such as fatty acids, glycols, and many others.[3–5] The plasticizers are typically small molecules that weaken the intermolecular bonds between protein chains. Analysis of the extensive literature on plasticization of zein shows that in almost all cases the plasticizers used as additives do not have specific chemical interactions with zein molecules. This kind of plasticization has some disadvantages due to formation of a heterogeneous system that is not stable in certain cases. For example, the plasticising effect of a small molecule such as glycerol on zein film is lost over a period of time by migration of glycerol to the surface of the film. Consequently, it is important to find ways to accomplish "internal plasticization" of zein.

Internal plasticization involves chemical combination of a plasticizer and a polymer[6] in such a fashion that, ideally, the plasticizer retains its ability to reduce the glass transition temperature of the host polymer. From the point of view of the host polymer, e.g., zein, it is chemical modification, because a new chemical substance is formed via a covalent bonding between the plasticizer and zein.

A variety of functional groups are found in zein. They are summarized in Figure 1, which was compiled on the basis of published data.[1,7] The names of amino acids are given in full, in three- and one-letter systems. The size of letters reflects relative amount of each amino acid in zein molecule. Amino acids shown in bold have functions different from those of hydrocarbons (proline also) and can potentially provide sites for zein modification, using mild chemical and physical conditions. The literature data are inconsistent in case of minor amino acids whose proportions vary in the different forms of zein and also in zeins from different suppliers.

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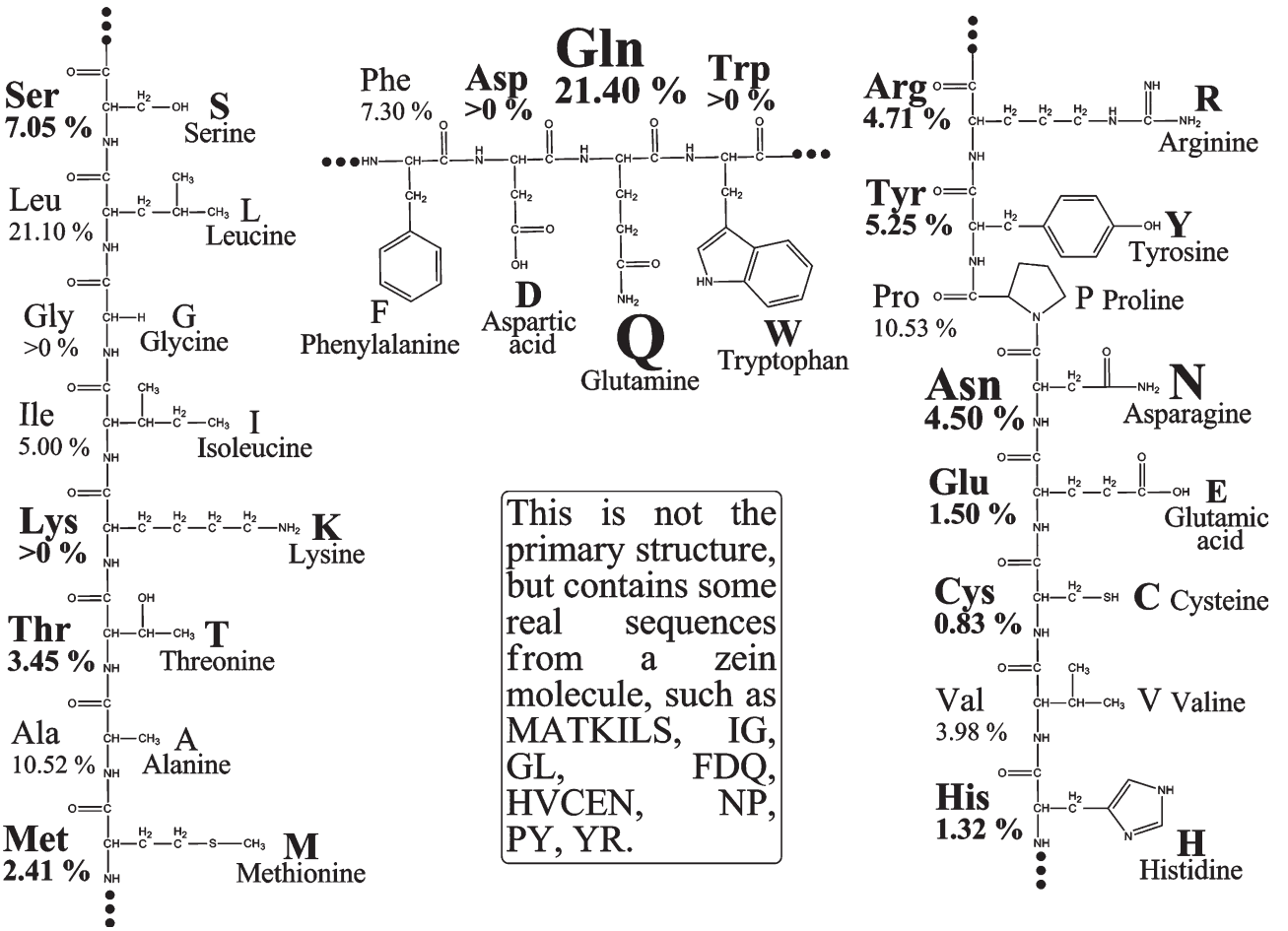

Figure 1. Typical proportions of amino acids in a commercially available zein.

From the typical chemical composition of zein it can be concluded that the best chance of chemical change is esterification of the free carboxylic groups of glutamic and aspartic acids. The ester radical could be the terminal group of a plasticizer molecule connected covalently to zein. It was reported that the alcohols with two, three, and more carbon atoms in the molecule can react with the carboxylic group of proteins, including zein, with lower yields and in a longer time.[8] This could be due to the steric hindrance effect. The best results have been achieved only with methanol, which is not an effective plasticizer for zein. Another obstacle is the very small amount, of the order 2%,[1] of free carboxylic groups in commercial zein.

Since the amide groups of glutamine and asparagine zein make up more than 25% of the functional groups in zein, a possible chemical modification can be focused on those amide groups, using hydrolysis reaction or another kind of deamidation. A suitable method for such a procedure using nitrous acid has been reported.[9,10] Zein was esterified with methanol in the presence of a catalytic amount of concentrated hydrochloric acid (or hydrogen chloride) at room temperature to increase the yield of the methylated zein in comparison with nondeamidated reactant.[8] It was also noted[8] that amide groups of peptide bonds were not affected. Direct esterification of free NH_2 groups of amides in proteins has not been reported, because this transformation due to the properties of amide groups, needs harsh conditions that are not always suitable for proteins. However, direct esterification of such groups has been reported for lysergic acid and its derivatives with the objective of obtaining methyl esters.[11] A mixture of lysergic acid (or its derivative, e.g. isolysergic acid) and anhydrous methanol was treated at different temperatures (from 0 to 65°C, the boiling point of methanol) in the presence of a solution of hydrogen chloride or *para*-toluenesulfonic acid.

We envisaged that a similar method might be applicable to a protein molecule, in particular zein, which is stable at elevated temperatures. In this communication we report the initial results of a novel one-step method of zein methylation that is essentially a combination of conventional esterification of —COOH groups and (mainly) direct amide esterification. The objective was to effect a significant extent of methylation of the amide groups, in particular, as a first stage toward internal plasticization of zein chains due to introduction of a much more reactive ester functionality, which could be replaced by many other moieties with higher plasticization properties, e.g., via trans-esterification reactions where methoxy group seems to be more desirable moiety, in particular due to a low boiling point of methanol.

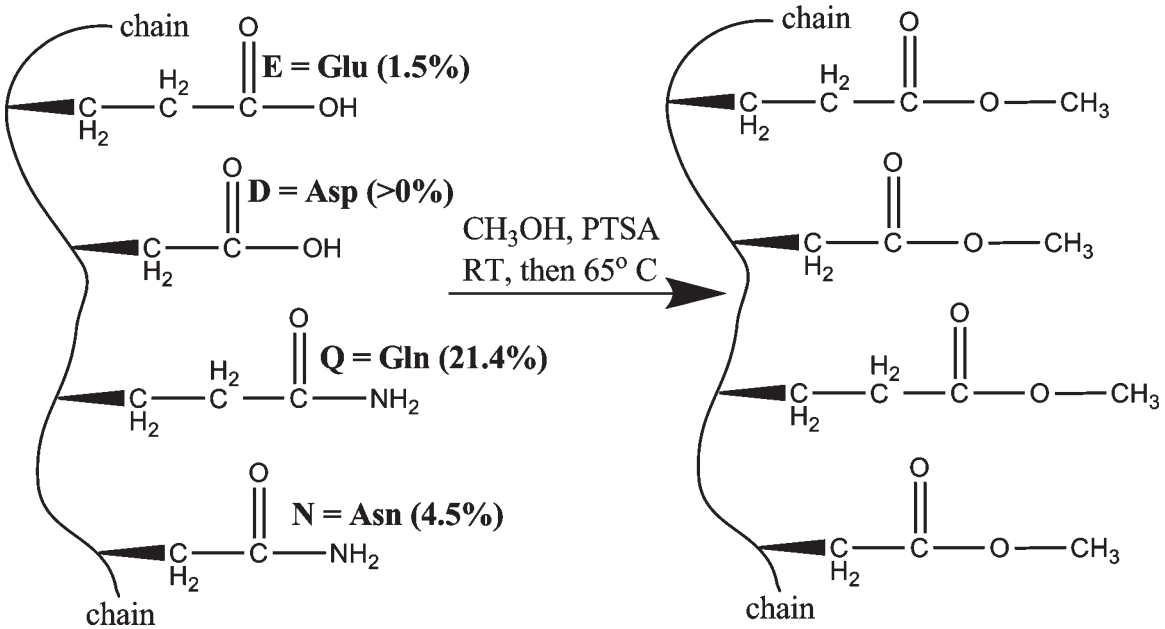

Figure 2. Schematic representation of zein methylation.

EXPERIMENTAL

Materials

Zein was supplied by MP Biomedicals. Two different batches were used (Lot Nos.6246H and 8479H), which is significant because the two batches of zein had slightly different solubility in 99.9% methanol. The first lot was 100% soluble (at the desired concentration), but the second lot was less soluble at room temperature. However, in the experimental conditions that were used, they were both sufficiently soluble. The FTIR and ^{1}H NMR spectra of the two lots of zein were identical. Methanol (99.9%, HPLC Grade) was supplied by Burdick & Jackson. *para*-Toluenesulfonic acid monohydrate (pTSA; 97.5%) was supplied by Acros Organics. All chemicals were used as received.

Methylation of Zein

A mixture of 2 g of zein and 20 g of pTSA was dissolved in 100 mL of 99.9% methanol in a 500-mL flask. The flask containing the slightly opalescent solution was stoppered and left at room temperature for 120 h, and then the reaction mixture was heated under reflux for 4 h at 65°C. Following reflux 250–300 mL of water was added to the reaction mixture, whereupon a white precipitate was immediately formed. The mixture was allowed to stand until the precipitate settled on the bottom of the flask, then most of the supernatant liquid was decanted and replaced by aqueous Na_2CO_3 solution to neutralize residual acid. The precipitate was isolated by vacuum filtration, washed on the filter with a small quantity of cold water and then dried in vacuum for 24 h at 40°C. Totally, 1.2 g of slightly off-white solid product (60% yield) was recovered after drying.

Characterization

Fourier Transform Infrared (FTIR) spectra were recorded for the reaction product in the form of a powder using a Thermo Electron NICOLET 8700 FTIR spectrometer with diamond crystal ATR attachment. Sixty-four scans at resolution 4 cm^{-1} were averaged for each sample. The signals were processed using OMNIC software.

^{1}H-NMR spectra were recorded at 400 MHz using a Bruker DRX400 spectrometer. Samples were dissolved in a mixture of acetone-d_6 with 99.9 atom % D (containing 1% v/v TMS; supplied by Sigma-Aldrich) and deuterium oxide (acetone : D_2O = 6 : 1; i.e., 85.7% v/v acetone in D_2O). The spectral data were processed using the TOPSPIN and MestReC 4.7.0.0 software packages.

Differential scanning calorimetry (DSC) was carried out using a DSC Q1000 instrument (TA Instruments, USA). The test samples were contained in aluminium pans and heated at 10°C

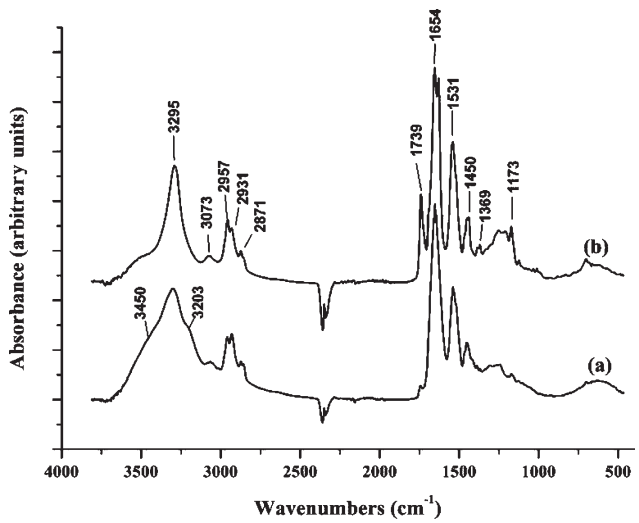

Figure 3. FTIR spectra of (a) zein and (b) zein methylation product.

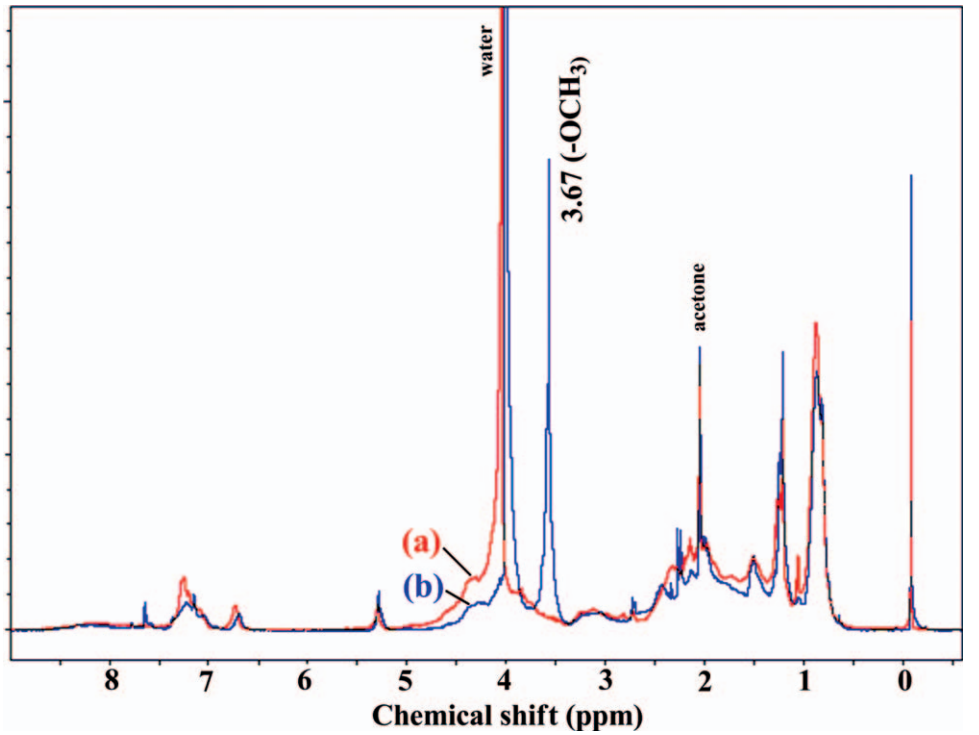

Figure 4. ^{1}H NMR spectrum of (a) zein and (b) zein methylation product. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

min^{-1} in an atmosphere of flowing, oxygen-free nitrogen. For all samples the following cycle was used: heat from 0 to 170°C, cool to 0°C, heat from 0 to 170°C. The data were analyzed using TA software.

Elemental analysis was carried out using Carlo Erba Elemental Analyser EA 1108. The analytical method was based on the complete and instantaneous oxidation of the sample by "flash combustion" at 1020°C which converts all organic and inorganic substances into combustion products.

RESULTS AND DISCUSSION

The aim of the methylation was to attach methyl ester groups to a number of radicals in the zein chains, as depicted schematically in Figure 2. The method affects the carboxylic groups of glutamic and aspartic acids and the amide groups of glutamine and asparagine in the zein molecule.

The esterification of free carboxylic groups in a protein molecule, including zein, involves a conventional one-step procedure (protein, alcohol, acid catalyst). By contrast, replacement of the NH_2 group of an amide by an alkoxy group is more difficult but can be done in a two-step procedure that includes deamidation and further esterification of a protein.[8–10] Direct esterification of an amide[11] is potentially a better way to accomplish the desired change to zein chains. Our method is similar to the procedure reported previously[11] for preparation of esters of lysergic acid.

FTIR Spectroscopy

Comparison of the FTIR spectra of the pure zein [Figure 3(a)] and zein methylation product [Figure 3(b)] reveals new bands at 1739 $cm^{-1}$and 1173 cm^{-1} in the spectrum of the methylated product. These are assigned to the C═O and C─O stretching vibrations, respectively, of methyl ester groups.[12] There is also a significant increase in the intensities of the CH_3 stretching modes at 2957 and 2871 cm^{-1} and bending mode at 1369 cm^{-1} relative to the CH_2 modes at 2931 and 2857 cm^{-1}. This indicates that formation of methoxy groups has occurred during the methylation process. The other major change is the substantial reduction in the area of the NH stretch mode centred at 3295 cm^{-1}. This multicomponent band includes contributions from the primary amides of the amino acid moieties at 3203, 3295, and 3450 cm^{-1}, and from secondary amides of the main

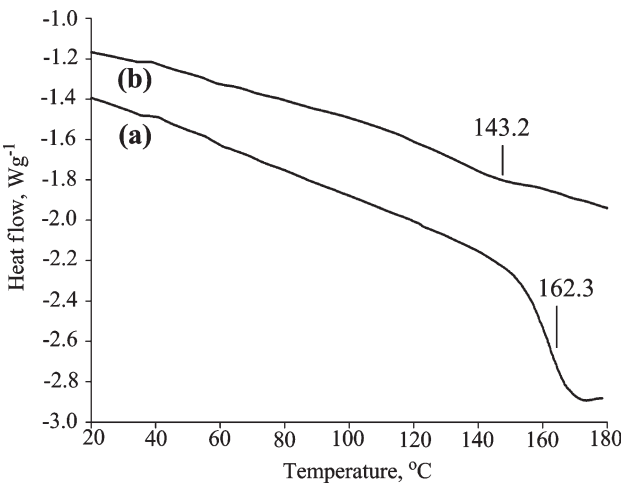

Figure 5. DSC second scans of pure (a) zein and (b) methylated zein.

Table I. Elemental Analysis Results for Zein and Methylated Zein

Name	C, %	H, %	N, %	C : N atom ratio
Zein	54.4	7.7	14.6	4.3 : 1
Methylated zein	56.9	7.9	11.4	5.8 : 1

chain at 3300 cm^{-1} as well as a smaller band at 3073 cm^{-1}.[13] The intensities of the primary amide components at 3203 cm^{-1} and 3450 cm^{-1} are reduced during the esterification process, causing the overall decrease of the mentioned NH stretch mode. The intensities of the secondary amide components remained unchanged. A similar dynamic at a lower scale is observed in the Amide I and II bands. The Amide I band at 1654 cm^{-1} is due to the C=O stretch mode of both primary and secondary amides, however, it also contains some contribution from the Amide II band of the primary amides.[13,14] This band was slightly reduced after the esterification process, whereas the Amide II band at 1531 cm^{-1} and the band at 1450 cm^{-1} remained unchanged, as these bands are due to C—N stretch and N—H deformation of the secondary amides. The secondary amides of the peptide bonds from the protein chains were therefore not affected by the esterification method.

The changes observed in the spectra confirm that replacement of primary amide groups with methoxy groups has occurred during the methylation process, indicating that the esterification was successful.

^{1}H-NMR Spectroscopy

^{1}H-NMR spectroscopy was used to provide a further spectroscopic comparison of zein and the methylation product. Initial experiments with samples dissolved in deuterated methanol revealed a broad intense solvent signal in the vicinity of 3 ppm that overlapped the methoxy CH_3 signal that was expected at 3.67 ppm.[15] Changing to the acetone-d_6 : D_2O (6 : 1 v/v) solvent, gave the spectra shown in Figure 4, for the zein methylation product and pure zein, respectively, with the spectrometer locked for acetone-d_6. The signal at 3.67 ppm in the spectrum of the zein methylation product can be confidently assigned to the protons of the CH_3 group from an ester group on zein.[15] That signal is absent from the spectrum for pure zein, and it correlates well with the predicted chemical shift for the CH_3 protons. In general, the full spectra of both zein and methylated zein possess typical signals of protein protons with characteristic chemical shifts. Apart from the signals of the protons of acetone (δ 2.05 ppm) and water (δ about 4.00 ppm) many other signals can be distinguished (δ ppm):[12,16,17] CH_3 (0.88–0.90); CH_2 from the aliphatic radicals (1.23–2.19 region); CH_2-CO from the amino acids radicals (2.21–2.32 region); single protons attached to the α-carbon atom of the main chain (αCH 4.00–5.00 region partially overlapped by the strong water signal). The protons of the primary amide groups of the amino acid moieties (7.20–7.30) and the secondary amide protons from the chain (a very broad and weak band centered at 8.30) are seen just after the proton signals of the aromatic rings (6.75–6.81). The intensity of the NH_2 protons is slightly reduced due to their replacement by the ester groups. However, the most reliable diagnostic signal of the successful esterification is the singlet of OCH_3 group, present only in the spectrum of the methylated zein.

DSC

The first heating scan for the methylated zein showed loss of absorbed methanol, and the first scan for pure zein showed loss of absorbed water. The second heating scans gave T_g = 143°C for the methylated zein, and T_g = 162°C for pure zein which is within the range of the many reported values for zein.[18] The glass transition zone of the samples obtained from the second scans are shown in Figure 5. The zein methylation product thus had a significantly smaller glass transition temperature than pure zein, which is consistent with the attachment of —OCH_3 groups to the protein chains.

Elemental Analysis

The results of the elemental analysis are shown in the Table I. The C:N atom ratio was calculated on the basis of atom percentage of the elements. Zein: (54.4/12)/(14.6/14) = 4.5/1.04 = 4.3 carbon atoms to 1 nitrogen atom. Methylated zein: (56.9/12)/(11.4/14) = 4.7/0.81 = 5.8 C atoms to 1 N atom. This reflects an increased amount of carbon in the product of reaction and a reduced amount of nitrogen. The differences in the C and N contents of zein and the zein methylation product are consistent with conversion of a significant proportion of the —$CONH_2$ groups in zein to —$COOCH_3$ groups in its methylated analogue.

CONCLUSIONS

From the results of the characterization experiments that were carried out on the product of the new zein methylation procedure, it is clear that zein was successfully modified by formation of the methyl ester, principally via the amide groups of the protein. It should be noted, however, that the reaction conditions have not been optimized, and a greater extent of methylation may be possible with an optimized procedure. Nonetheless, the objective of developing a method for functionalization, as the first step of internal plasticization of zein, was achieved. Even this first step gave significant reduction of T_g. The method is a one-pot procedure that requires relatively mild conditions and should be applicable to other proteins with a similar primary structure (amino acids composition).

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