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Physicochemical and Structural Properties of Oat Globulin Polymers Formed by a Microbial Transglutaminase

NAI-CHI SIU,† CHING-YUNG MA,*,† AND YOSHINORI MINE‡

Food Science Laboratory, Department of Botany, The University of Hong Kong, Hong Kong, and Department of Food Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Oat globulin was polymerized by a microbial transglutaminase (TG), and some physicochemical and functional properties of polymers were studied. Reversed-phase HPLC revealed that the number of ϵ-(γ-glutamyl) lysine isopeptide bonds formed after 4 h of enzyme incubation was 2.21 μmol/g of protein. SDS-PAGE showed that the oat globulin acidic polypeptides (AP) were more susceptible to polymerization than the basic polypeptides (BP), and the reactivities of both AP and BP were enhanced by the addition of other substrate proteins. Differential scanning calorimetry showed that both the denaturation temperature and denaturation enthalpy were decreased after TG treatment. Fourier transform infrared spectroscopy revealed marked increases in the intensity of two intermolecular β-sheet bands associated with aggregate formation but little conformational changes in the polymerized protein. TG incubation led to progressive changes in flow properties of oat globulin dispersions, indicating enhanced pseudoplasticity and increased viscosity and yield stress.

KEYWORDS: Oat globulin; transglutaminase; protein polymerization; structural properties; DSC; FTIR spectroscopy; flow properties

INTRODUCTION

Oat globulin, the major oat protein fraction, has structural properties very similar to those of the 11S globulins of soybean and other legumes (*1*−*3*). These hexameric globulins, with estimated M_r values of 320−370 kDa, contain six subunits, and each subunit is made up of an acidic and a basic polypeptide with M_r values of 32−37 and 20−22 kDa, respectively, linked by disulfide bonds (*3*). A thorough understanding of the relationship between structure and function is essential to better utilize these plant proteins as food ingredients. Whereas there are many techniques that can be used to study the molecular structure and conformation of proteins, relatively few methods are available to probe the structural organization of oligomeric proteins such as the 11S globulins. In previous studies, the conformation and molecular structure of oat globulin have been studied by differential scanning calorimetry (DSC) (*4*), UV and fluorescence spectrophotometry (*5*), and Fourier transform Raman (*6*) and infrared spectroscopy (*7*). The analysis of polymerized products catalyzed by transglutaminase has been shown to provide valuable information on the structure of complex proteins such as legume globulins (*8*, *9*).

Transglutaminase (TG; EC 2.3.2.13) catalyzes the cross-linking between protein and other molecules, including the same or different proteins via the formation of ϵ-(γ-glutamyl) lysine covalent bonds (*10*). TG has been used to alter the molecular structure and improve the functional properties of food proteins such as whey protein, soy proteins, gluten, and meat proteins (*11*−*14*). In most of these studies, the formation of polymerized proteins was followed by SDS-PAGE or gel filtration chromatography, and the functional properties of the polymers were evaluated. For better control of the polymerization process, and to enhance the functionality of the polymers, the changes in physicochemical and structural properties of protein during TG treatment should be monitored. In this study, a microbial TG was used to polymerize oat globulin, and some physicochemical properties, including thermal and flow characteristics, were examined by DSC and steady flow viscometry, respectively. As shown in the previous investigations (*4*, *15*), the study of thermal and flow properties can provide valuable information on the structure and conformation of oat globulin under the influence of various buffer environments and treatments. The conformation of the TG-polymerized oat globulin was also monitored by Fourier transform infrared (FTIR) spectroscopy and compared to that of heat-aggregated protein.

MATERIALS AND METHODS

Preparation of Oat Globulin. Oat seeds (variety Hinoat) were grown at the Central Experimental Farm, Agriculture and Agri-Food Canada, Ottawa, Canada. They were dehulled and ground in a pin-mill and defatted by Soxhlet extraction with hexane. Oat globulin was extracted from the defatted oat groats with 1 M NaCl (*16*). The protein content of oat globulin, determined according to the micro-Kjeldahl method (*17*) using a nitrogen to protein conversion factor of 5.80, was 98.9% (*18*). Succinylated oat globulin was prepared according to the

* Address correspondence to this author at the Department of Botany, The University of Hong Kong, Pokfulam Road, Hong Kong (telephone +852-22990318; fax +852-28583477; e-mail macy@hkucc.hku.hk).
† The University of Hong Kong.
‡ University of Guelph.

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procedure of Ma (*19*), and the extent of modification, determined by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method (*20*), was found to be 75.2%.

Polymerization of Oat Globulin by TG. Microbial TG, derived from the culture of *Streptovericillium* sp., was supplied by Ajinomoto Co. Inc.. Oat globulin dispersions (5 mg/mL) were prepared in 0.1 M phosphate buffer (pH 7.5) containing 0.2 M NaCl and were incubated with TG at 0.01 unit/mg of protein in a water bath kept at 37 °C. At specific time intervals, aliquots of reaction mixture were mixed with equal volumes of sample buffer and heated for 5 min in a boiling water bath to inactivate the enzyme. The reaction mixtures were dialyzed exhaustively against distilled water at 4 °C and freeze-dried. The extent of polymerization was determined by following the formation of high molecular weight polymers and the disappearance of the oat globulin acidic and basic polypeptides by SDS-PAGE. Gel filtration chromatography was not used to follow enzyme reaction because the polymerized oat globulin has very low solubility, and complete solubilization of the protein samples cannot be achieved even with buffers containing 0.5% SDS or 6 M urea. Control samples were prepared by incubating the protein dispersions in the absence of TG. To study the structural properties of TG-polymerized oat globulin, protein dispersions were incubated with the enzyme for 4 h, heated at boiling temperature for 5 min, and then dialyzed exhaustively against distilled water at 4 °C and recovered by freeze-drying.

SDS-PAGE was performed in 12.5% linear gradient gels according to the method of Laemmli (*21*). Protein samples (≈5 mg/mL) were treated with sample buffer containing 0.063 M Tris-HCl, 10% SDS, 5% β-mercaptoethanol, and 0.01% bromophenol blue. The polymerized protein was almost completely dissolved in the buffer, and the sample solutions were centrifuged to ensure that only soluble protein was analyzed. The amount of protein loaded was ~10−20 μg in 4 μL. The standard protein markers were from an LMW electrophoresis calibration kit (Pharmacia Biotech, Piscataway, NJ). The molecular weights of protein bands were determined using a Phoretix densitometric image analysis system (Phoretix International, Newcastle Upon Tyne, U.K.).

Determination of ϵ-(γ-Glutamyl) Lysine Cross-Links. Exhaustive proteolytic digestion of the polymerized oat globulin was carried out according to the method of Sato et al. (*22*). The proteolytic digests were derivatized with phenylisothiocyanate (PITC) according to the method of Bidlingmeyer et al. (*23*). The resulting phenylthiocarbamyl (PTC) amino acids and peptides were fractionated by RP-HPLC using a Zorbax XDB-C8 reversed-phase column and a Hewlett-Packard 1100 HPLC system equipped with a diode array UV−vis detector (Hewlett-Packard, Waldbronn, Germany).

DSC. The thermal properties of oat globulin and the polymers were studied by DSC according to the method of Harwalkar and Ma (*4*), using a TA 2920 modulated DSC thermal analyzer (TA Instruments, New Castle, DE). Approximately 1 mg of protein was weighed into the aluminum pan, and 10 μL of 0.01 M phosphate buffer, pH 7.4, was added. The pan was hermetically sealed and heated from 25 to 140 °C at a rate of 10 °C/min. A sealed empty pan was used as a reference. Peak transition temperature or denaturation temperature (T_d), enthalpy of denaturation (ΔH) and width at half-peak height ($\Delta T_{1/2}$) were computed from the thermograms by a Universal Analysis Program, version 1.9D (TA Instruments). All experiments were conducted in triplicate, and the coefficient of variation ranged from 0.3 to 0.6% for T_d and $\Delta T_{1/2}$ and from 5 to 10% for ΔH.

FTIR Spectroscopy. Protein dispersions (10% w/v) were prepared in D_2O instead of H_2O because D_2O has greater transparency in the infrared region of interest (1600−1700 cm^{-1}). To ensure complete H/D exchange, samples were prepared 1 day before, kept at 4 °C, and equilibrated to room temperature prior to infrared measurements. Infrared spectra were recorded in a Bio-Rad Excalibur FTIR spectrometer (Bio-Rad Laboratories, Cambridge, MA) equipped with a deuterated triglycine sulfate (DTGS) detector. Samples were held in an IR cell with a 25 μm path length CaF_2 window. The scans were performed at 4 cm^{-1} resolution, and 32 scans were averaged. Preliminary data showed that increasing the number of scans from 32 to 512 did not significantly improve the resolution of the IR spectra. Deconvolution of the infrared spectra was performed using Bio-Rad software (Merlin version 1) and according to the method of Kauppinen et al. (*24*). Band assignment of oat globulin in the amide I region (1600−1700 cm^{-1}) followed the system of Susi and Byler (*25*). All FTIR experiments were performed in duplicate, and reproducible data (with standard deviation < 10%) were obtained.

Flow Tests. The flow behavior of dispersions (10% w/v in 0.01 M phosphate buffer, pH 8.0, containing 0.2 M NaCl) of oat globulin and polymerized protein formed after different TG incubation periods was examined by steady flow experiments. A Stresstech controlled stress rheometer (Rheologica Instruments AB) equipped with a cone and plate measuring system (3 cm diameter, 4° angle) was used. Dissolved air from the protein dispersions was removed by vacuum evacuation prior to viscometric measurements. Protein dispersion containing TG was incubated in situ in the measuring system at 37 °C by connecting it to a temperature-controlled recirculating water bath. Light parafin oil (BDH, Poole, U.K.) was applied to the cone and plate system to prevent the loss of moisture from the samples during incubation. After specific incubation periods, the protein mixture was cooled rapidly by connection to a constant-temperature water bath maintained at 23 °C, and the flow experiment was carried out at the same temperature after equilibration for a few minutes. Preliminary data showed that the relative activity of TG at 23 °C was <20% when compared to that at 37 °C, and because the flow test took only 1−2 min to complete, the residual enzyme activity during measurements should be minimal. The shear stress was varied to provide a shear rate range of 0− ~500 s^{-1}. The rheological data were fitted to the power-law model

$$\sigma = m\dot{r}^n$$

where σ is shear stress, $\dot{r}$ is shear rate, and m and n are the consistency coefficient and flow behavior index, respectively. The apparent viscosity was recorded at 100 s^{-1}. The yield stress values of the dispersions were estimated using the Casson equation as described previously (*15*).

RESULTS AND DISCUSSION

Polymerization of Oat Globulin by TG. The polymerization of oat globulin incubated with TG at 37 °C was followed by SDS-PAGE (**Figure 1A**). The control exhibited two groups of protein bands with estimated average molecular weights of around 22 and 36 kDa, corresponding to the basic (BP) and acidic (AP) polypeptides of the oat globulin monomer (*2*). With increase in incubation time, there were progressive increases in the amount of polymers (those bands with molecular weights >40 kDa), including high molecular weight polypeptides or aggregated proteins that cannot enter the separating gels. There were concomitant decreases in AP and BP (**Figure 1A**). **Figure 2** shows the changes in the content of polymers and polypeptides during TG incubation. The concentration of polymers increased rapidly during the initial 60 min and then leveled off at ~30%. The amount of AP decreased more rapidly than did BP, with about 40 and 25% losses in AP and BP, respectively, at the end of the incubation (**Figure 2**).

The higher reactivity of AP than of BP was also reported in soy glycinin, broadbean, and pea legumin (*8*, *9*, *26*), which have oligomeric structures very similar to that of oat globulin. In the native glycinin and broadbean legumin, 70−80% of AP disappeared after TG reaction, whereas most of the BP remained (*9*, *26*). Ikura et al. (*27*) reported that the BP of glycinin were not involved in polymer formation catalyzed by TG from guinea pig liver. According to the structural model proposed by Plietz et al. (*28*), AP is mainly located at the surface of the protein and, hence, more accessible for polymerization. The TG reaction sites in BP must be inaccessible to the enzyme in this oligomeric structure due to the shielding effect of AP. The present data show that despite common quaternary structure, minor structural differences between oat globulin and other legume 11S globulins may lead to different reactivity to TG. Oat globulin AP has

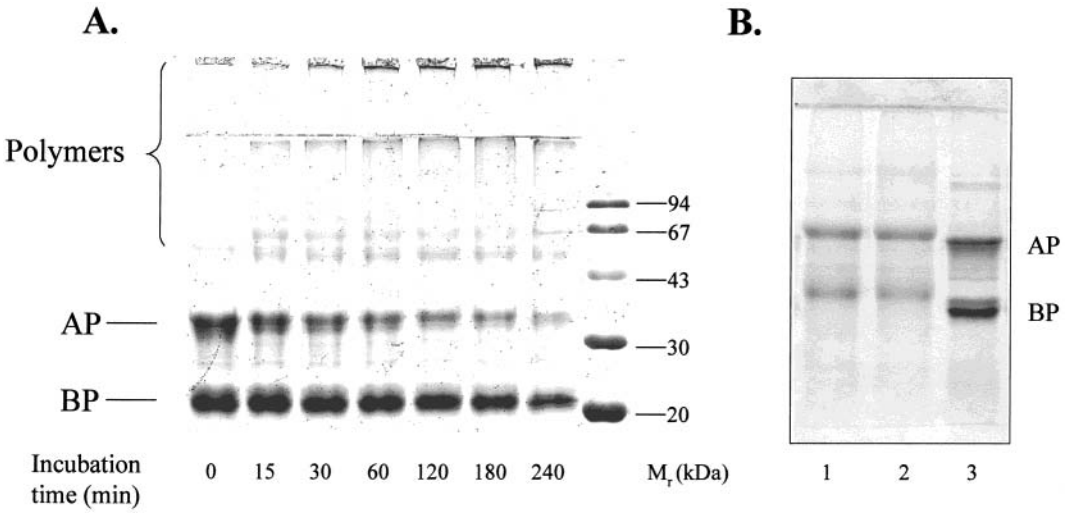

Figure 1. (A) SDS-PAGE profiles of transglutaminase-polymerized oat globulin: (lanes 1–7) oat globulin treated with transglutaminase for various time periods; (lane 8) protein markers. (B) SDS-PAGE profiles of native and succinylated oat globulin: (lane 1) succinylated oat globulin; (lane 2) succinylated oat globulin incubated with transglutaminase for 4 h; (lane 3) native oat globulin.

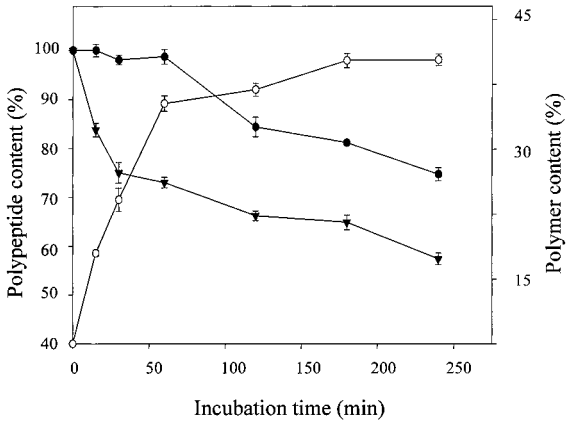

Figure 2. Changes in the content of polymerized oat globulin (○) and acidic (▼) and basic (●) polypeptides during transglutaminase treatment. The protein contents were determined by densitometric scanning of the SDS-PAGE profiles.

much lower reactivity than legume AP (42 vs 70−80%), whereas BP seems to retain some reactivity to TG, suggesting that not all of the TG reaction sites are shielded. The difference in reactivity might also be due to differences in protein solubility. Oat globulin has relatively low solubility (*15*), and dispersions were used instead of completely solubilized protein, leading to incomplete reaction with the enzyme.

Figure 1B shows the effect of succinylation of oat globulin on the polymerization process. No change in SDS-PAGE pattern was observed when the highly succinylated protein was incubated with TG for 4 h, indicating that polymerization did not occur. The data suggest that TG-catalyzed polymerization of oat globulin involves the cross-linking between the ϵ-amino groups of lysine residues, acting as the acyl acceptor, and the γ-glutamyl groups of glutamic acid residues. Blocking of the ϵ-amino groups of lysine by succinylation therefore stops the formation of either intra- or intermolecular ϵ-(γ-glutamyl) lysyl cross-links. A similar effect of acetylation on TG-catalyzed polymerization was observed in soy glycinin (*26*) and whey proteins (*29*, *30*).

The slight shift in band mobility when compared to the unsuccinylated protein (**Figure 1B**, lane 3) is probably due to the increases in hydrodynamic volume of modified protein (*31*).

Table 1. Effect of Mixing Oat Globulin with Other Protein Substrates on Transglutaminase Reactivity of the Acidic (AP) and Basic (BP) Polypeptides of Oat Globulin

protein mixture (1:1)[a]	AP content (% of original)	BP content (% of original)
oat globulin	57.4[b]	74.7[b]
oat globulin/Supro 610	0	37.7
oat globulin/casein	0	51.4
oat globulin/β-lactoglobulin	0	71.1

[a] The total protein concentration of the reaction mixtures was 1% (w/v). All protein mixtures were treated with transglutaminase at 37 °C for 4 h, and the AP and BP contents of oat globulin were estimated from SDS-PAGE profiles of the protein mixtures. [b] Average of duplicate determinations.

The decrease in staining intensity in the succinylated samples could be attributed to the elimination of positively charged groups in the native oat globulin (*32*).

The amount of ϵ-(γ-glutamyl) lysyl cross-links formed in the polymerized oat globulin was determined by RP-HPLC of the proteolytic digest. The calculated dipeptide content was 2.21 $\pm$ 0.09 μmol/g of protein, and the numbers of lysine and glutamate residues modified per molecule were estimated to be 2.07 and 3.48, respectively. A similar level of ϵ-(γ-glutamyl) lysyl dipeptide was also detected in the TG-catalyzed polymerization of soy protein isolate (*33*).

The reactivity of oat globulin to TG was found to increase when other protein substrates were mixed with the oat protein. When oat globulin was mixed 1:1 with a commercial soy protein isolate, Supro 610 (Protein Technologies International, St. Louis, MO), or two milk proteins, casein and β-lactoglobulin (Sigma Chemical Co., St. Louis, MO), there were marked changes in the reactivity of both AP and BP, as determined by SDS-PAGE (**Table 1**). There was complete disappearance of AP, whereas the reactivity of BP was slightly increased when β-lactoglobulin was used as a cosubstrate and markedly increased when casein or Supro 610 was present. The oat globulin polymers formed in the reaction mixtures cannot be determined because the other substrates were also polymerized and shown as high molecular weight bands (data not shown). The results suggest that the TG-catalyzed polymerization of oat globulin can be greatly enhanced when other TG substrates are added to the reaction mixture. This may be attributed to the higher solubility of these proteins

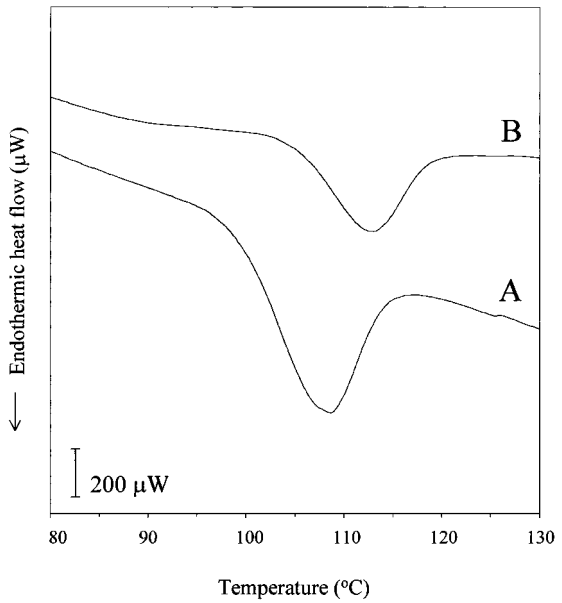

Figure 3. DSC thermograms of native (A) and transglutaminase-polymerized (B) oat globulin.

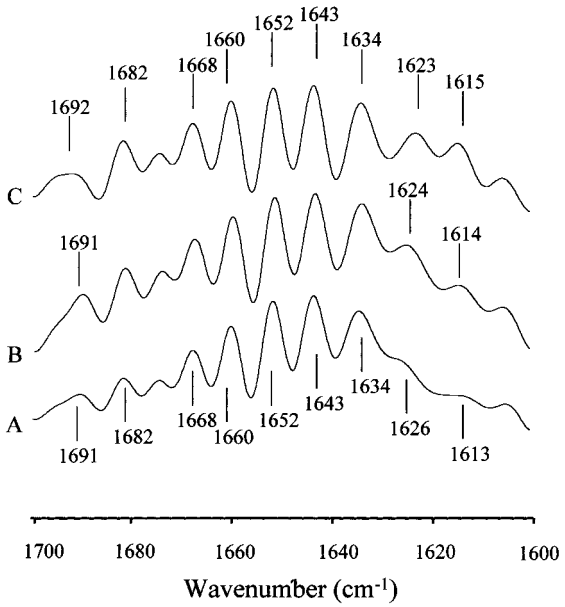

Figure 4. Stacked plots of deconvoluted infrared spectra of oat globulin: (A) native oat globulin; (B) transglutaminase-polymerized oat globulin (4 h of incubation); (C) heat-induced oat globulin aggregates.

when compared to oat globulin. Han and Damodaran (*34*) suggested that the thermodynamic compatibility of substrate proteins plays an important role in TG reaction. Similar types of proteins can form heterologous polymers due to favorable free energy of interactions. All three proteins seem to be compatible with oat globulin in forming heterologous polymers, including good TG substrates such as casein (*27*) and poor substrates such as β-lactoglobulin (*35*).

Thermal Characteristics. Figure 3 shows the DSC thermograms of oat globulin (**Figure 3A**) and the TG-polymerized protein (**Figure 3B**). Although the TG-treated sample has been heated to inactivate the enzyme, a previous study (*36*) showed that heating at 100 °C for up to 10 min did not lead to marked changes in the DSC characteristics of oat globulin. The data show that TG treatment led to a slight increase in denaturation temperature (from 108.2 to 111.5 °C) and a marked reduction in enthalpy (from 23.7 to 14.1 J/g). The width at half-peak height ($\Delta T_{1/2}$) was slightly decreased (from 9.04 to 8.09 °C).

An increase in T_d normally indicates higher thermal stability (*4*), whereas a decrease in ΔH suggests protein denaturation because a partially unfolded polypeptide would require less heat energy (lower ΔH) to denature completely (*37*). The increased transition temperature may be due to the formation of high molecular weight complexes with a compact and ordered conformation, which would have higher thermal stability than the unassociated protein. The half-peak width is an index to evaluate the cooperativity of protein unfolding (*38*), and the decrease in this value in the TG-treated oat globulin suggests that the polymers would denature in a more highly cooperative fashion than the control. Similar increases in thermal stability and decreases in $\Delta T_{1/2}$ were observed when oat globulin was heat-coagulated at temperatures below its T_d and have been attributed to the formation of aggregates with compact network structure (*5*). Because the enzyme reaction was carried out at 37 °C, it is unlikely that the decrease in ΔH in the polymerized oat globulin is due to protein unfolding, as observed in the heat-aggregated protein (*36*). Protein polymerization, similar to aggregation, is an exothermic process and may lead to a lowering in enthalpy values (*37*).

FTIR Spectral Characteristics. Figure 4 shows the effect of TG and heat treatments on the FTIR spectral characteristics of oat globulin. Because heat treatments were shown to induce changes in FTIR spectral characteristics of oat globulin (*7*), the protein−enzyme mixture was dialyzed and freeze-dried after incubation without heat inactivation. As FTIR experiments were conducted at room temperature, residual TG activity during spectral measurements would be minimal. The control sample (**Figure 4A**) shows several major bands in the amide I region. From the locations and relative intensities of the infrared bands, it was evident that α-helix (1652 cm^{-1}) and random coils (1643 cm^{-1}) are the major secondary structures in oat globulin, followed by β-sheet (1634 and 1680 cm^{-1}) and β-turns (1660 and 1668 cm^{-1}). According to circular dichroism (CD) data (*39*), oat globulin, similar to most plant proteins, has a low α-helix content and a large quantity of β-sheet and random coil structures. However, discrepancies between CD and FTIR spectroscopy in quantitative estimation of protein secondary structures have been reported (*25*, *40*). TG treatment caused no marked changes in intensity and position of the major bands associated with the secondary structure composition (**Figure 4B**), suggesting that TG did not lead to significant conformational changes in oat globulin. The most dramatic changes were marked increases in the intensity of the 1626 cm^{-1} (shifted to 1624 cm^{-1}) and 1682 cm^{-1} bands. These two bands are attributed to antiparallel β-sheets and strands and are associated with the aggregation process (*24*). Heat-induced aggregates were prepared by heating a 1% (w/v) dispersion of oat globulin at 100 °C according to a procedure previously described (*5*), and the FTIR spectrum of the heated sample (**Figure 4C**) also showed pronounced increases in intensity of the aggregation peaks at 1682 and 1626 (shifted to 1623) cm^{-1}. There were no marked changes in the intensity of the other major bands, but increases in band intensity and shift in band position were observed in two minor peaks, including the side-chain vibration band at 1612 cm^{-1} and β-type band at 1691 cm^{-1} (**Figure 4C**). The results indicate that both TG-induced polymerization and heat-induced coagulation led to similar changes in secondary structures as measured by FTIR, although DSC data suggest more extensive protein denaturation in the heat aggregation process (*4*).

Table 2. Effect of Transglutaminase Treatment on the Power Law Constants, Apparent Viscosity, and Casson Yield Stress of Oat Globulin Dispersions[a]

incubation time (min)	flow behavior index	consistency coefficient (Pa s^n)	apparent viscosity[b] (mPa s)	Casson yield stress (Pa)
0	0.96	0.04	3.96	0
30	0.90	0.07	4.00	0.060
60	0.63	0.66	7.52	0.53
90	0.54	0.84	9.02	1.20
120	0.29	3.6	12.8	2.30
180	0.26	4.9	13.5	2.60
240	0.18	7.8	15.6	5.80

[a] Averages of three determinations. [b] Apparent viscosity was measured at 100 s^{-1}.

Flow Properties. Table 2 shows the steady flow rheometric data of oat globulin incubated with TG at various time periods. With increase in incubation time, there was a progressive decrease in flow behavior index (n). The dimensionless index, described as a reflection of the closeness to Newtonian flow (*41*), was close to unity initially, suggesting a near-Newtonian flow. The progressive decrease in n value indicates increasing tendency toward pseudoplastic behavior. There were progressive increases in consistency coefficient (m), an index of viscosity, apparent viscosity, and yield stress value (**Table 2**). All of these changes in flow properties are consistent with the formation of polymerized protein molecules, similar to that observed when oat globulin was heat-coagulated (*15*), although heating at 100 °C for 30 min led to much greater changes in flow properties (except in flow behavior index) than TG treatment.

Conclusions. In the present study, a microbial transglutaminase was used to polymerize oat globulin. The acidic polypeptides were more reactive than the basic polypeptides, but the difference was not as dramatic as that observed in legume 11S globulins. The relatively low solubility of oat globulin may contribute to lower enzyme reactivity when compared to other protein substrates. The polymerization led to marked changes in physicochemical and structural properties of the protein, and the changes were similar to those observed in heat-treated oat globulin. The polymerized protein has low solubility near neutral pH (data not shown), and the poor solubility may affect its utilization as a functional food ingredient. The solubility and other functional properties of polymerized oat globulin have been determined, and the results will be presented in a separate paper.

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