

How fractionation procedure of mung bean protein affects transglutaminase crosslinking

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ARTICLE INFO

Keywords:

Transglutaminase
Mung bean
Protein fractionation
Rheological properties
Microstructure

ABSTRACT

Transglutaminase is often used to improve functional properties of plant proteins. Here, we report on the effects of the fractionation procedure on transglutaminase susceptibility of mung bean proteins at three length scales: molecular, colloidal and bulk. Dispersions of 4 wt% mung bean protein fractions were treated with transglutaminase. The four fractions were obtained through (1) dry fractionation (fine fraction); (2) dry fractionation with additional heating; (3) lab-scale wet fractionation; and (4) a commercial protein isolate. Analysis of rheological properties (at 9.5 wt% dry matter) revealed that transglutaminase crosslinking formed tougher materials when using the fine fraction, the heated fine fraction, and the wet fractionated isolate, while the rheological properties of the protein isolate were hardly changed. Proteins in all fractions were crosslinked by transglutaminase at molecular scale, as confirmed by an increase in large molecular weight bands in gel electrophoresis, but the extent of crosslinking depended on the fraction used. The molecular changes also resulted in altered physical chemical properties for some fractions, as revealed by protein dispersibility, confocal laser scanning microscopy, and particle size distribution. At the colloidal scale, transglutaminase crosslinking led to an increased particle size in the fine fraction and heated fine fraction, while particle sizes of the wet fractionated isolate and commercial protein isolate were mostly unaffected. The differences between the fractions were explained by three mechanistic crosslinking routes. To conclude, protein fractionation procedure greatly affected susceptibility to transglutaminase.

1. Introduction

In the past decade, plant proteins have become an important ingredient in foods, amongst other with the aim to replace meat and dairy products. Especially, soy and yellow pea protein are used in plant based foods, because of their favorable functional properties (Kyriakopoulou, Dekkers, & van der Goot, 2019; Vatansever, Tulbek, & Riaz, 2020). Another legume that is gaining attention as a potential food ingredient is mung bean (*Vigna radiata*). Mung beans are inexpensive to cultivate in comparison to soy and are drought resistant (Brishti et al., 2021). Mung beans contain a high amount of protein (± 25 g protein/100 g dry matter) that is rich in essential amino acids (Du et al., 2018; Mubarak, 2005; Yi-Shen, Shuai, & Fitzgerald, 2018). So far, the starch fraction of mung bean has been widely studied, while mung bean protein is still underexplored. Protein extracted from mung bean showed great

potential to form structures in food, and in particular, in meat analogue applications (Brishti et al., 2017, 2021; Schlangen, Dinani, Schutyser, & van der Goot, 2022). However, mung bean protein, when combined with wheat gluten, generally gives weaker structures upon heating compared with soy protein when combined with wheat gluten, suggesting that more protein interactions occur in the soy based material compared with mung bean (Schlangen et al., 2023; Schreuders et al., 2019). The introduction of additional protein crosslinks in mung bean, for example through, heating, chemical treatment or enzymatic treatment, could be a route to obtain higher viscosity and stronger gels, which enhances its potential as a value added ingredient in plant-based meat and dairy alternatives (Buchert et al., 2010; Singh, 1991).

Transglutaminase (TGase) is an enzyme that is widely studied for crosslinking different plant proteins, such as soy, pea, and Bambara groundnut protein (Djoullah, Djemaoune, Husson, & Saurel, 2015; Liu

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<https://doi.org/10.1016/j.foodhyd.2023.109067>

Received 31 March 2023; Received in revised form 30 June 2023; Accepted 10 July 2023

Available online 15 July 2023

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et al., 2021; Mattice & Marangoni, 2021; Nivala, Nordlund, Kruus, & Ercili-Cura, 2021; Ruzengwe, Amosou, & Kudanga, 2020; Shand, Ya, Pietrasik, & Wanasundara, 2008; Sun & Arntfield, 2011; Zhang et al., 2021). TGase catalyses intra- and inter-molecular crosslinking reactions between lysine (acyl acceptor) ϵ -amino groups and glutamine (acyl donor) γ -carboxamide groups and, thus, forms covalent bonds (Gaspar & De Góes-Favoni, 2015). The crosslinking of proteins by TGase can lead to high molecular weight proteins that can give stronger gels and increased water holding capacity (Gaspar & De Góes-Favoni, 2015; Shand, Ya, Pietrasik, & Wanasundara, 2008). Recently, it was shown that TGase improved the mechanical and rheological properties of gels produced from pea protein isolate, while gels from mung bean protein isolate were relatively unaffected (Schlangen et al., 2023). Then, it was hypothesized that the lysine and glutamine amino groups in mung bean protein isolate were less available or accessible for TGase to crosslink.

The availability of amino groups is partly determined by the structure of the protein, which in turn is dependent on the protein fractionation procedure. Conventional wet fractionation based on alkaline extraction and isoelectric precipitation, yields ingredients with a protein purity of 80 wt% at least (Assatory, Vitelli, Rajabzadeh, & Legge, 2019; Rivera, Siliveru, & Li, 2022), but at the cost of protein structural changes and, thus, often a loss of native protein characteristics (Schutyser, Pelgrom, van der Goot, & Boom, 2015). An alternative to wet fractionation is dry fractionation. With dry fractionation, plant proteins are enriched in a fine fraction obtained via milling and air classification (Pelgrom, Boom, & Schutyser, 2015; Schlangen et al., 2022). An advantage of dry fractionation is that the protein native structure is preserved. The fraction enriched in proteins after dry fractionation often has a different technological and nutritional functionality compared with that of the commercial protein isolate counterparts (Opazo-Navarrete, Schutyser, Boom, & Janssen, 2018; Vogelsang-O'Dwyer et al., 2020). This is because of the mild process conditions used during dry fractionation and the fact that the fractions obtained contain many other components next to protein. Therefore, the susceptibility of proteins to TGase is expected to be different depending on processing routes used to make the protein-rich fractions and molecular architecture, with a lower susceptibility in more aggregated protein structures. Indeed, the accessibility of TGase is determined by the steric availability of the lysine and glutamine residues.

Pelgrom, Boom, & Schutyser (2015) and Schutyser et al. (2015) showed that TGase can be used to crosslink mildly fractionated pea proteins and found that this resulted in a stronger heat-induced gel compared with un-crosslinked pea proteins. However, to date, there are no other studies that report on the use of TGase to crosslink plant proteins in fine fractions obtained through dry fractionation. Furthermore, there are no prior studies on the effect of fractionation procedure on the susceptibility of proteins to TGase crosslinking activity. The current work aims to fill the above research gaps.

Thus, the aim of this study was to investigate the effect of mung bean protein fractionation procedures on the susceptibility of those proteins to TGase crosslinking. A fine fraction, a heated fine fraction, a pH precipitated wet fraction and a commercial protein isolate were compared on their susceptibility to TGase crosslinking activity. We hypothesize that the dispersibility of the protein will affect the susceptibility to TGase. Susceptibility to TGase was studied at three different length scales: (1) molecular scale (gel electrophoresis), (2) colloidal scale (particle size, protein dispersibility, and microstructure), and (3) bulk scale (rheology). Here, colloidal scale refers to the nanometre to micrometre range and thus referring to protein present in particles that are solvated, but not in solution.

2. Materials & methods

2.1. Materials

Dehulled mung beans (*Vigna radiata*) were obtained from Vladex

(Middelharnis, the Netherlands). The average dry matter content was assumed to be the same as for flour from dehulled mung beans and was 91.5 wt%. Mung bean protein isolate (UNIMUNG M70) was obtained from Barentz (Hoofddorp, the Netherlands). The protein content and dry matter content of the protein isolate were 68.9 wt% (N x 5.7) on dry basis and 94.3 wt%, respectively. The supplier of the protein isolate reported a maximum fat content of 6 wt%.

Transglutaminase (ACTIVA wm) was obtained from Ajinomoto Co. (Ajinomoto, Tokyo, Japan). The composition of the enzyme preparation is 1% transglutaminase and 99% maltodextrin, and the activity was reported by the suppliers as 100 U/g. We use the term transglutaminase (TGase) in this study to refer to the enzyme preparation that includes both transglutaminase and maltodextrin. *N*-Ethylmaleimide (NEM) and sodium chloride were obtained from Sigma-Aldrich (Missouri, USA).

2.2. Methods

2.2.1. Preparation of the fractions

Four different mung bean protein fractions were used in this study. An overview of their fractionation pathways is visualized in Fig. 1. Milling and air classification parameters used were based on previous research results (Schlangen et al., 2022). For preparation of the fine fraction, the legumes were first pre-milled into grits with a pin mill (LV 15 M, Condux-Werk, Wolfgang bei Hanau, Germany). Next, the grits were milled into a flour with a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany). The ZPS50 mill speed used was 8000 rpm, the classifier wheel speed was set to 4000 rpm, the air flow was 52 m³/h, and the feed rate was ~500 g/h. A batch size of 3 kg mung beans was used. The obtained flour was separated into a fine (protein-rich) and coarse (starch-rich) fraction by air classification with a ATP50 classifier (Hosokawa-Alpine, Augsburg, Germany). Here, a classifier wheel speed of 10,000 rpm, an air flow of 47 m³/h, and a feed rate of ~250 g/h were used. The heated fine fraction was prepared by mixing the fine fraction (either 9.5 wt% dry matter for rheology, or 4 wt% protein for other experiments) with MilliQ water on a vortex for 1 min, followed by heating this dispersion at 90 °C for 30 min. Subsequently, the dispersion was rapidly cooled with cold tap water.

The wet fractionation process was based on an extraction process developed by Kornet et al. (2021). The fine fraction was dispersed under mild agitation in demineralized water and the pH was adjusted to 8 with a solution of 1.7 M NaOH. After 2 h, the dispersion was centrifuged at 10,000×g for 30 min. The supernatant was taken for further purification by adjusting the pH to 4.5 with a solution of 1 M HCl. Then the dispersion was centrifuged again at 10,000×g for 30 min. The obtained pellet was separated from the supernatant and redispersed at pH 7. The resulting dispersion was agitated for at least 2 h. The dispersion was frozen and lyophilized with a Christ Alpha 1-2-LD freeze-dryer (Salm en Kipp, Breukelen, the Netherlands) at -48 °C and 0.75–0.92 mbar. The wet fractionated isolate was used in dry powder form for all analyses. The fine fraction, heated fine fraction, and wet fractionated isolate were compared with a commercial protein isolate. The fractionation procedure of the commercial protein isolate is unknown.

Protein contents of the fractions were determined using a Rapid N Exceed Dumas (Elementar, Langensfeld, Germany) in duplicate. Based on previous research, a nitrogen conversion factor of 5.7 was used for mung bean protein (Schlangen et al., 2022). The dry matter content of the fractions was determined by drying around 1 g of sample overnight in an oven (Binder GmbH, Tuttlingen, Germany) at 105 °C. The ash content of the fractions was determined by drying around 500 mg of sample in a Nabertherm ash oven (Nabertherm GmbH, Lilienthal, Germany) at 500 °C for 3 h. An overview of the compositions of the fractions is shown in Table 1.

2.2.2. Rheological properties of the dispersions

The rheological properties of the samples with and without TGase were analysed by small angle oscillatory shear (SAOS) measurements

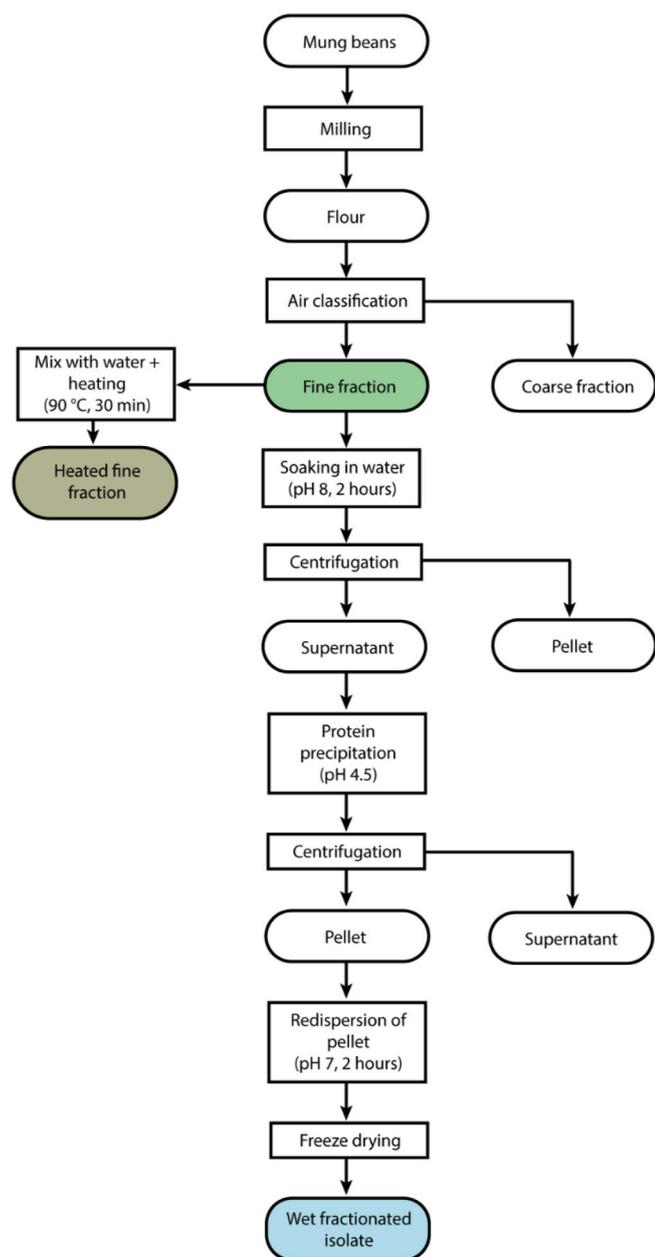


Fig. 1. Fractionation scheme of fine fraction, heated fine fraction, and wet fractionated isolate prepared from mung beans.

Table 1

Composition of mung bean fractions and pH of mung bean fractions dispersed in MilliQ at 9.5 wt% dry matter.

	Dry matter content (g/100 g)	Ash content (g/100 g dry matter)	Protein content (g/100 g dry matter)	pH of dispersions (–)
Fine fraction	92.71 ± 0.05	7.06 ± 0.05	58.00 ± 0.23	6.05
Heated fine fraction	92.71 ± 0.05	7.06 ± 0.05	58.00 ± 0.23	5.89
Wet fractionated isolate	96.88 ± 0.25	4.72 ± 0.01	82.62 ± 4.73	6.75
Protein isolate	94.20 ± 0.12	3.68 ± 0.00	69.34 ± 0.11	6.87

and a strain sweep. The fractions were dissolved in demineralized water at a concentration of 9.5 wt% dry matter with 0 U/g protein (without) or 7.6 U/g protein (with) TGase. The samples were mixed vigorously with a vortex. The rheological properties of the samples were measured with a MCR301 rheometer (Anton Paar, Graz, Austria) combined with a CC-17 concentric cylinder geometry. Two SAOS measurements were performed: a temperature sweep and a frequency sweep. The temperature sweep was performed by subjecting the samples to an incubation step of 50 °C for 30 min (based on optimal conditions found in previous research by Schlangen et al. (2023) and controlled by a water bath) at a frequency of 1 Hz and a strain amplitude of 1%. Subsequently, the samples were cooled down to 20 °C at a rate of 3 °C/min and kept at 20 °C for 5 min to equilibrate. Next, the incubated samples were subjected to a frequency sweep from 0.01 to 10 Hz (at a strain of 1%). Straight after the SAOS measurements, a strain sweep was performed. The strain was varied from 0.1 to 1000% in a logarithmic manner (6 points per decade, at a frequency of 1 Hz). The storage modulus (G') and loss modulus (G'') were recorded during all measurements. The end of the linear viscoelastic regime (LVR) was calculated for all curves and was defined as the maximum strain before a 5% G' deviation from linear. The strain at the end of the LVR in the strain sweeps was expressed as the critical strain (γ_c). The critical strain and corresponding stress values were plotted in a texture map, as previously described by Schreuders et al. (2021). Rheological measurements were performed in triplicate, except for the wet fractionated isolate with and without TGase, which was analysed in duplicate, due to limited availability of material.

2.2.3. Crosslinking of diluted dispersions with transglutaminase

To study the susceptibility of the proteins to TGase under diluted conditions, the fractions were dissolved in MilliQ at 4 wt% protein with a NaCl concentration of 0.005 M. The diluted dispersions were treated with 0 or 7.6 U/g protein TGase and mixed vigorously with a vortex. The samples were then incubated at 50 °C for 30 min in a ThermoMixer F2.0 (Eppendorf, Hamburg, Germany). The enzyme concentration and incubation conditions were based on conditions used previously (Schlangen et al., 2023). Subsequently, 8 mM *N*-ethylmaleimide (NEM) was added to all samples to stop the enzymatic reaction.

Another set of samples was produced to study the effect of TGase on solely the protein fraction in the supernatants obtained after centrifugation. Here, the fractions were dissolved in MilliQ at 4 wt% protein (NaCl concentration of 0.005 M) and centrifuged at 5000×g at 21 °C for 10 min. Next, the supernatant was treated with TGase as described before. Subsequently, 8 mM *N*-ethylmaleimide (NEM) was added to all samples to stop the enzymatic reaction. We refer to this set of samples with post-centrifugation treatment.

2.2.4. Gel electrophoresis

Gel electrophoresis under reducing conditions was performed to obtain insights on the crosslinking of different protein subunits, using an Invitrogen™ system according to manufacturer's instructions (ThermoFisher Scientific, Waltham, MA, USA). The TGase treated dispersions were centrifuged (5000×g, 21 °C, 10 min), and the supernatants were analysed to investigate effects on the proteins induced by TGase. We refer to these samples as pre-centrifugation treatment. Additionally, the post-centrifugation treated samples were analysed as explained in section 2.2.3. The pre- and post-centrifugation treated samples, before and after TGase crosslinking, were first diluted to 2 mg protein/mL with MilliQ. Subsequently, 13 µL of the sample was mixed with 5 µL of NuPAGE™ LDS sample buffer and 2 µL NuPAGE™ reducing agent. The mixtures were heated at 95 °C for 5 min in a thermoshaker (IKA-Werke GmbH, Staufen, Germany) and rapidly cooled in an ice bath afterwards. Aliquots of 7 µL were injected into a NuPAGE™ precast gradient gel (4–12% polyacrylamide). The first and last well of the gel were injected with 10 µL of Precision Plus Protein™ Marker (Bio Rad Laboratories). The gel was run at 200 V for 35 min using an XCell SureLock™ Mini-Cell

filled with NuPAGE™ MES SDS running buffer. The gel was stained with SimplyBlue™ SafeStain solution for 4.5 h, rinsed in MilliQ overnight and subsequently digitalised with a ChemiDoc XRS (Bio Rad Laboratories, Inc., Hercules, CA, USA).

2.2.5. Protein dispersibility

The protein dispersibilities of the dispersed fraction from section 2.2.3 with and without TGase were analysed. The term protein dispersibility is used instead of solubility, because it is possible that part of the protein is present in the supernatant as colloidal aggregates rather than fully dissolved. The dispersions were centrifuged at 5000×g at 21 °C for 10 min. The protein contents of the dispersions and the supernatants were analysed using Dumas (Dumatherm, Gerhardt GmbH & Co. KG, Königswinter, Germany) with a protein conversion factor of 5.7. The protein dispersibility was calculated using Eq. (1).

$$\text{Protein dispersibility} = \frac{\text{Protein content in supernatant}}{\text{Protein added}} \times 100\% \quad (1)$$

2.2.6. Microstructure characterization

The microstructures of the dispersions without and with TGase were characterized using confocal laser scanning microscopy (CLSM) (Nikon C2, Nikon Instrument Inc., Tokyo, Japan). Rhodamine B (Sigma Aldrich, St. Louis, MO) was dissolved in MilliQ until a final concentration of 1 mg/mL. Approximately 60 μl of each dispersion and 5 μl of the Rhodamine B staining solution were loaded onto glass slides and mixed with a pipette tip. The stained samples were analysed using CLSM with a 20x objective and 60x objective (data not shown). A laser line of 561 nm

was used for excitation to induce fluorescence emission. Images were made using two samples taken from two separate dispersions. Representative images are shown.

2.2.7. Particle size analysis

The particle size distributions of the dispersions (pre-centrifugation) with and without TGase were measured using static light scattering with a Mastersizer 2000 (Malvern Instruments Ltd., UK). A volume-based mode was used because of the multi-component nature of the protein fractions. The particle size distribution of the post-centrifugation treated samples with and without TGase was measured using dynamic light scattering with a Zetasizer Lab (Malvern Panalytical Ltd., Malvern, UK). The post-centrifugation treated samples were diluted to 1:8 (v/v) in MilliQ before analysis. The measurements of the diluted samples were carried out at 25 °C after 120 s equilibration time. A refractive index of 1.45 was used for the measurements with the Mastersizer as well as the Zetasizer. Particle size analyses were performed in triplicate.

2.2.8. Statistical analysis

All measurements were performed in duplicate unless stated otherwise. The mean values and standard errors were calculated and used as a measure of error. Significant differences of the measured dispersibility values were analysed by ANOVA with a post hoc Duncan test. Significant differences of the measured rheological properties were determined by an independent *t*-test. Equality of variances was analysed with Levene's test, unequal variances were assumed when $P > 0.05$. Significance was defined as $P < 0.05$.

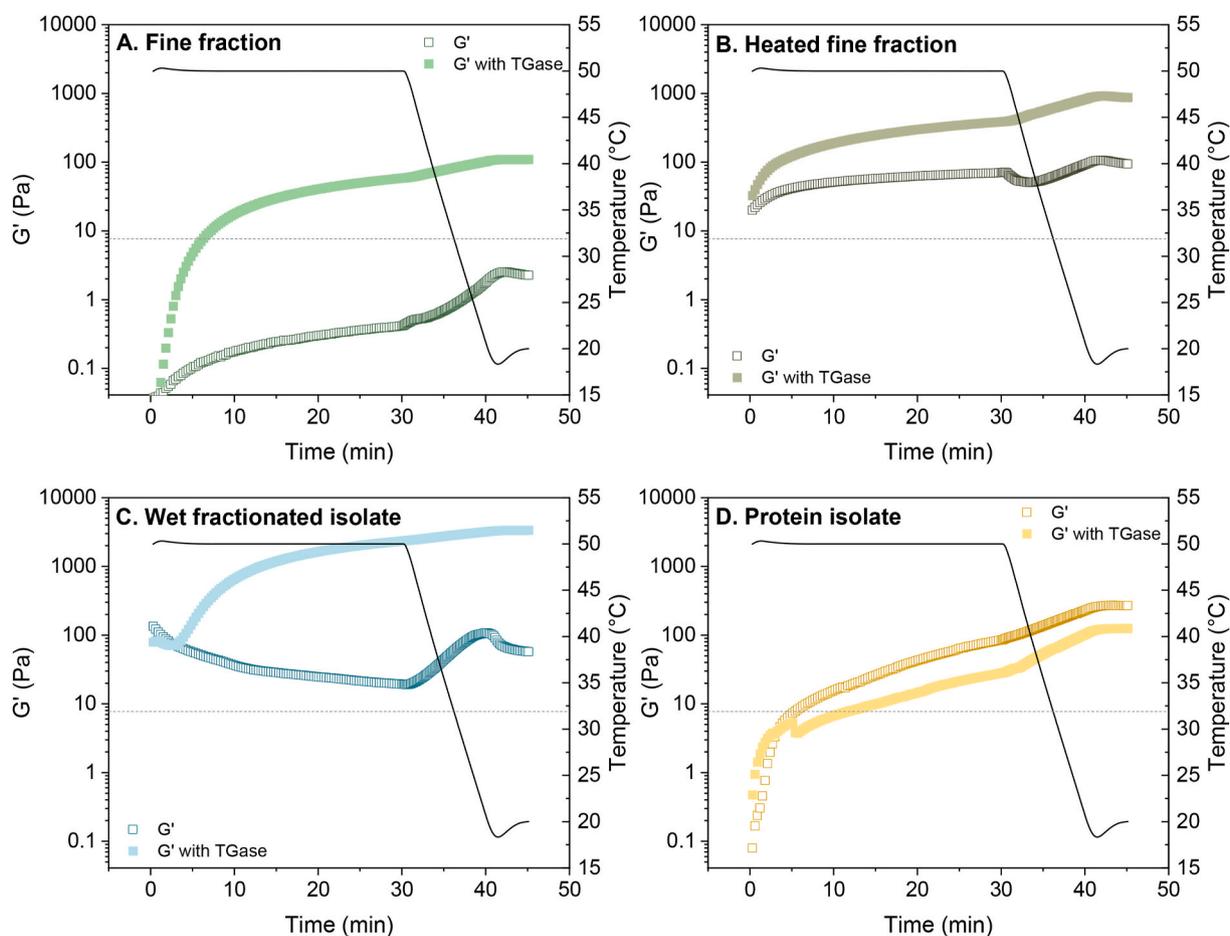


Fig. 2. Representative temperature sweep of A: fine fraction, B: heated fine fraction, C: wet fractionated isolate, and D: protein isolate without (dark, open symbols) and with (light, closed symbols) TGase. Black lines indicate incubation temperature profile. Horizontal dashed line indicates minimum torque limit, which was taken as a factor 10 higher than reported by the supplier.

3. Results & discussion

3.1. Effect of crosslinking at bulk scale

Bulk changes were studied with rheology at a standardized dry matter content of 9.5 wt%. The compositions of all fractions are presented in Table 1, which shows that the fine fractions contain most non-proteinaceous components. The development of the elastic modulus (G') with time is shown in Fig. 2, where the dispersions were incubated for 30 min at 50 °C with and without TGase. A frequency sweep was performed after incubation showing that $G' > G''$ over the entire frequency range for all samples, indicating solid-like behaviour (Supplementary Material: Fig. S1). During incubation, there were clear differences in the rheological properties of the samples depending on the composition and fractionation procedure of the fractions. Without TGase, we observed an increase in G' over time in the fine fraction, heated fine fraction, and protein isolate (Fig. 2A, B, and D). Thus, heating at 50 °C alone changed the rheological properties in these fractions. We believe that the increase in G' was due to hydration and solvent inclusion of the protein aggregates at 50 °C and consequently the increase in particle-particle interactions. Comparing the G' profiles of the fine fraction and heated fine fraction without TGase, we noted that the heated fine fraction had higher G' values overall (Fig. 2A and B). This suggests that the heated fine fraction forms a network-like structure at the start of the measurement already, which can be explained by the additional heating step in the fractionation procedure. The wet fractionated isolate without TGase showed a decrease in G' during the heating period, and an increase in G' started upon cooling. The decrease in G' during the heating period may be due to a temperature-dependent decrease in viscosity. A decrease in viscosity normally occurs in materials, including gels, in case no additional interactions or crosslinks are created upon heating. The wet fractionated isolate had a relatively high G' value (~100 Pa) at the start of the measurement, and might therefore already be in a certain gelled state. It is important to note that the fine fraction and heated fine fraction are multi-component ingredients, as they still contain other components, such as fibre. Fibre could increase G' during incubation by holding water, but it might also interfere with protein network formation.

The application of TGase resulted in distinct rheological effects amongst the various fractions. TGase crosslinking increased the G' of the fine fraction, heated fine fraction, and wet fractionated isolate (Fig. 2A, B, and C), indicating a stiffening of the materials. On the other hand, TGase crosslinking decreased the G' of the protein isolate (Fig. 2D), indicating a softer material. In the fine fraction, we believe that TGase crosslinking within individual protein particles resulted in an enhanced G' value. The same explanation may hold for the heated fine fraction. However, here the effect is smaller, because an initial network may already have been formed due to the processing applied to make this fraction. The highest G' values with TGase were found for the wet fractionated isolate (Fig. 2C). The decreased G' in the protein isolate with TGase does not necessarily indicate a reduced susceptibility to TGase, as there is still a difference with the moduli without TGase. The limited effects of TGase on the protein isolate aligns with previous studies by Shand et al. (2008) and Pelgrom, Boom, & Schutyser (2015). They also demonstrated a limited effect of TGase on rheological properties in commercial pea protein isolate compared with native pea protein. The results were explained by considering partial or complete denaturation of the proteins in the isolate, which led to a decrease in solubility.

The texture map depicts the properties of all fractions without and with TGase (Fig. 3). The addition of TGase to the fine fraction, heated fine fraction, and wet fractionated isolate led to an increase in the critical strain and the corresponding stress values, and thus resulted in tougher materials compared with those without TGase. The strongest and most stretchable material was obtained by addition of TGase to the wet fractionated isolate. Previous research by Shand et al. (2008) also

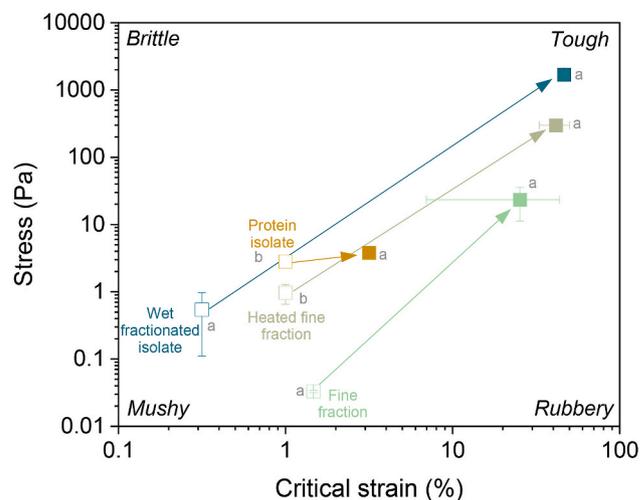


Fig. 3. Texture map at the end of the linear viscoelastic regime for fine fraction, heated fine fraction, wet fractionated isolate, and protein isolate without (open symbols) and with (closed symbols) TGase. Different letters indicate significant differences ($P < 0.05$) in stress between TGase treatment and the control within each fraction. Significance for critical strain (%) could not be determined, because at least one group had 0 variance. Arrows indicate the changes in behaviour when TGase is added and are solely provided to guide the eye.

showed that addition of TGase to pea protein isolate and soy protein isolate led to tougher materials. The addition of TGase to mung bean protein isolate in this study induced a more subtle change towards higher critical strain values. The small effect of TGase on rheological properties of the protein isolate is therefore considered to be a result of the fractionation procedure.

One effect of the fractionation procedure is that it can alter the amino acid availability. However, we previously reported that mung bean protein isolate has a higher level of lysine and glutamine (70.3 mg lysine/g protein and 181.3 mg glutamine including glutamic acid/g protein) (Schlengen et al., 2023), compared with the mung bean fine fraction (64.0 mg lysine/g protein and 162.3 mg glutamine (including glutamic acid)/g protein) (Schlengen et al., 2022). It can thus be concluded that the differences in rheological properties between TGase treated fine fractions and protein isolates cannot be explained by amino acid availability.

3.2. Effect of crosslinking on molecular length scale

The formation of crosslinked protein molecules through addition of TGase was studied using SDS-PAGE (Fig. 4). Here, we analysed both the entire dispersion (pre-centrifugation) as well as only the supernatant fractions after centrifugation (post-centrifugation) to gain a better understanding of the difference of TGase treatment between all proteins compared to solely soluble proteins. The suspensions without TGase were also studied under non-reducing conditions to obtain insight on their natural molecular aggregation states.

Clear differences were visible in molecular composition between the different fractions without TGase (Fig. 4) (Brishti et al., 2021; Rahma, Dudek, Mothes, Görnitz, & Schwenke, 2000; Zhong & Xiong, 2020). Here, the band patterns of the pre-centrifugation treatment (full dispersion) without TGase (lane 2) and the treatment without TGase on the corresponding supernatant (post-centrifugation) (lane 4) were similar, as anticipated, given that both fractions have a similar history. The fine fraction, heated fine fraction, and wet fractionated isolate had a high intensity band at 50 kDa, indicating presence of vicilin (lane 2 in Fig. 4A and B, C). Furthermore, the fine fraction and heated fine fraction contained albumin, indicated by the bands at ~26 kDa (lane 2 in Fig. 4A and B). Theoretically, the soluble albumins should have been removed in the fractionation process of the wet fractionated isolate and protein

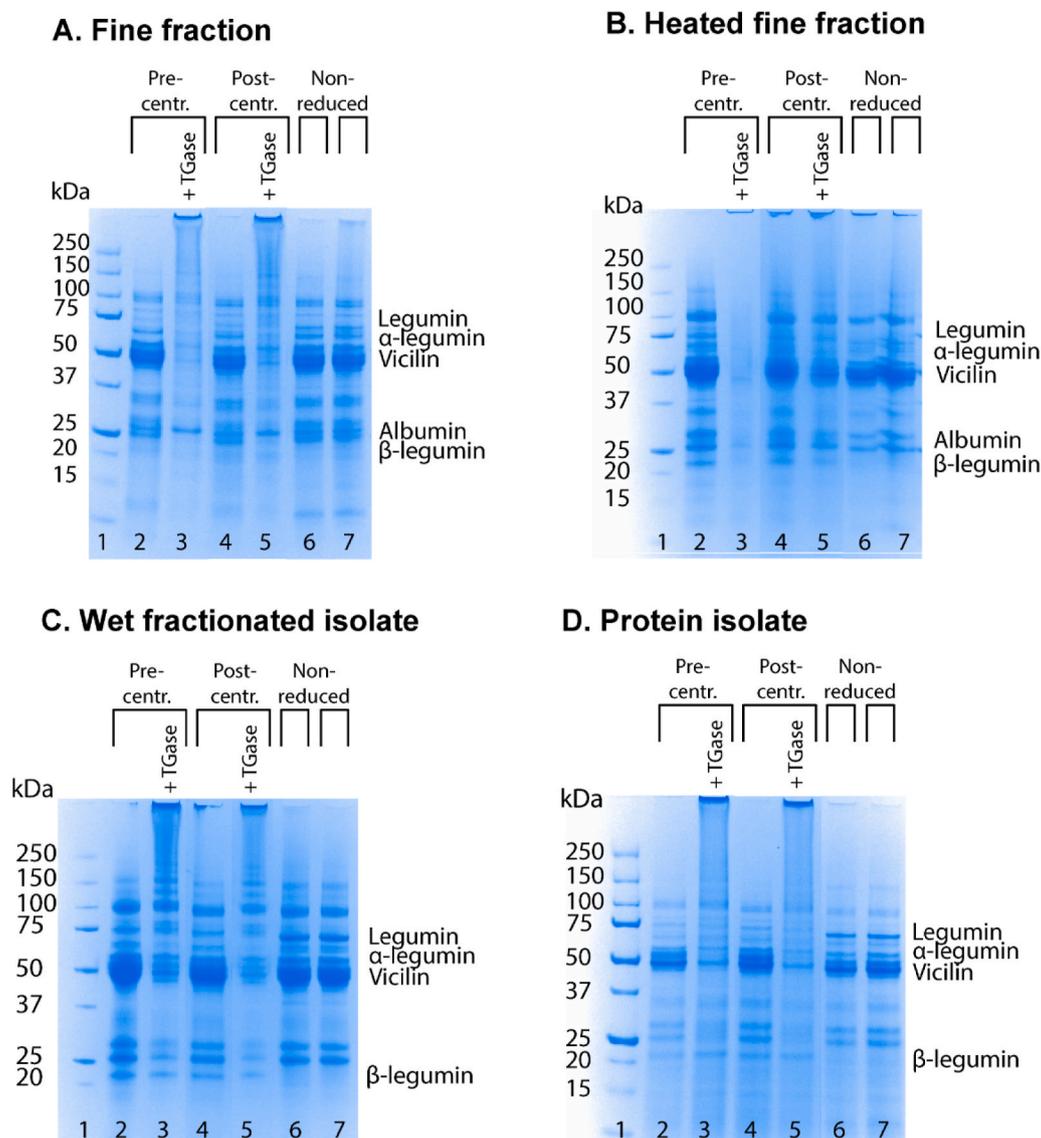


Fig. 4. SDS-PAGE of the pre-(lane 2–3) and post-centrifugation (lane 4–5) treated fractions of A) fine fraction, B) heated fine fraction, C) wet fractionated isolate, D) protein isolate without and with TGase under reducing conditions. Non-reduced represents the pre- (lane 6) and post-centrifugation treated (lane 7) fractions without TGase in non-reducing conditions.

isolate, and should thus not be present in those fractions. The band patterns of the fine fraction, heated fine fraction, and wet fractionated isolate without TGase were relatively similar, but there was a clear difference compared with the composition of the protein isolate. The protein isolate consisted of mainly vicilin and α -legumin, as indicated by the clear bands at ~ 50 kDa and ~ 60 kDa (lane 2 in Fig. 4D). The differences in molecular composition between the protein isolate and the other fractions can be related to the isolation and precipitation of the protein isolate (Fig. S2).

Crosslinking with TGase led to clear changes in the electrophoretic migration in all pre- and post-centrifugation treated samples (Fig. 4). All samples had crosslinked proteins, which shows that TGase was able to crosslink all fractions. With TGase addition, the band intensities of the low molecular weight polypeptides decreased and new high molecular weight bands appeared, indicating crosslinking of proteins. This is in accordance with previous research on Bambara groundnut protein isolate, faba bean protein, and pea globulins, for which it was also described that addition of TGase led to the disappearance of most low molecular weight polypeptide bands (Djoullah et al., 2015; Nivala, Mäkinen, Kruus, Nordlund, & Ercili-Cura, 2017; Ruzengwe et al., 2020).

Most samples with TGase also showed a high intensity band retained at the bottom of the well. These bands indicate the presence of insoluble, high molecular weight compounds, induced by TGase, that are not able to migrate through the gel (for example lane 3 in Fig. 4A). As the SDS-PAGE profiles of the pre-centrifugation and post-centrifugation treatments were relatively similar, it may be suggested that crosslinking is predominantly taking place between soluble protein subunits (Fig. 4).

Some protein subunits were found to be more susceptible to TGase crosslinking than others. More specifically, all low molecular weight bands in the fine fraction decreased in intensity upon addition of TGase except for the band at ~ 26 kDa (lane 2–5 in Fig. 4A). This result confirmed previous reports that the major albumin subunit at ~ 26 kDa is less available or less accessible for TGase to crosslink (Djoullah et al., 2015). This effect was less evident for the heated fine fraction, suggesting that heating increased the availability of this subunit for TGase crosslinking, by for example facilitating exposure of its amino acid residues (Fig. 4B). Earlier reports showed an increased reaction yield for pea albumin in denatured state due to improved accessibility to the lysine and glutamine groups (Djoullah, Husson, & Saurel, 2018). Thus, it

could be concluded that the heated fine fraction had a higher total amount of proteins available for TGase crosslinking compared to the fine fraction. In the wet fractionated isolate, TGase addition mostly reduced the intensity of the band at ~50 kDa (Fig. 4C), suggesting that crosslinking mostly occurred to the vicilin-like storage protein (Rahma et al., 2000). Vicilin was also prone to TGase crosslinking in the fine fraction (Fig. 4A lane 2 versus 3). Previously, Nivala et al. (2017) showed that vicilin was highly prone to TGase crosslinking in faba bean protein. They argued that vicilin is more soluble than legumin, which explained their increased availability for TGase crosslinking (Nivala et al., 2017). It is also worth noting that the less reactive albumins are lost in the wet fractionated isolate, due to the extraction process (Kornet et al., 2021). Therefore, the total amount of reactive protein subunits in the wet fractionated isolate may be higher than in the fine fraction and heated fine fraction.

SDS-PAGE analyses proved that TGase crosslinking occurred at molecular length scale in all protein fractions independent of fractionation procedure applied. However, SDS-PAGE is a qualitative measurement and only measures the soluble protein fraction. Therefore, protein dispersibility, microstructure, and particle size analyses were performed at the colloidal length scale to better understand the different effects of TGase on the change in rheological properties of mung bean protein.

3.3. Protein dispersibility changes upon TGase addition

The next step to explain the effects above is to analyse the dispersibility of the protein fractions. It can be expected that dispersible protein has a higher susceptibility to TGase than protein that is not dispersible. Here, we use the term protein dispersibility rather than protein solubility, as the mung bean proteins may form colloiddally stable aggregates that remain suspended after low speed centrifugation.

There were clear differences in protein dispersibility values between the different fractions before TGase treatment (Fig. 5). The protein isolate had the lowest dispersibility, while the wet fractionated isolate had the overall highest dispersibility. The latter is presumably due to the removal of water-insoluble compounds during protein extraction in the wet fractionated isolate. However, the prevention of agglomeration during freeze drying instead of spray drying may also explain the high dispersibility of the wet fractionated isolate. The greater availability of dispersible protein in the wet fractionated isolate may have resulted in a large increase in the G' upon TGase addition as shown in section 3.1. Further, it might be that crosslinking the low quantity of dispersible protein present in the protein isolate was not enough to induce a change

in G' and form a strong gel (Fig. 2). Heating of the fine fraction increased its protein dispersibility compared to the non-heated fine fraction. This was probably due to the loosening of the native structures with temperature leading to increased hydration. Aggregation is less likely to have taken place in the heated fine fraction, because this would have led to a decrease in protein dispersibility. These results demonstrated that fractionated method changes protein dispersibility due to changes in colloidal interactions. Based on this information, it is thus plausible that denaturation and aggregation during the protein isolation process had reduced the protein dispersibility, thereby limiting the accessibility of the protein isolate reactive groups to TGase.

To determine the possible changes with addition of TGase, we studied the amount of protein that was dispersible after the TGase treatment as well (Fig. 5). Clear differences in protein dispersibility were observed when TGase was added to the protein fractions. We found that even though TGase crosslinking led to changes in molecular sizes (Fig. 4), it did not always change the dispersibility of the samples (Fig. 5). The addition of TGase decreased the protein dispersibility of the fine fraction, heated fine fraction (though not significant), and the protein isolate, probably due to covalent TGase crosslinking. This aligns with the results of the gel electrophoresis, where TGase crosslinking resulted in an increase in molecular weight of the protein subunits (Fig. 4). Previous research also showed a decrease in protein solubility of faba bean protein upon TGase treatment (Nivala et al., 2017). Contradictory, the protein dispersibility of the wet fractionated isolate was not affected by TGase addition. Thus, the crosslinked proteins of the wet fractionated isolate remained dispersible according to this measurement.

3.4. Microstructure changes upon TGase addition

CLSM was used to determine possible changes in the microstructure of the protein aggregates without and with TGase. Without TGase, we observed clear differences in microstructure between the different protein fractions (Fig. 6). The fine fraction and heated fine fraction had similar irregularly shaped particles, while the particles present in the wet fractionated isolate were slightly larger (Fig. 6A, B, and C). The microstructure of the protein isolate revealed distinct wrinkled surface particles at all conditions tested, which is typical for spray dried proteins (Brishti et al., 2020; Lan, Xu, Ohm, Chen, & Rao, 2019) (Fig. 6D). To further understand protein-enzyme interactions, dispersions were prepared with 1 M NaCl, treated with or without TGase and their microstructure was studied with CLSM. The high concentration of NaCl can

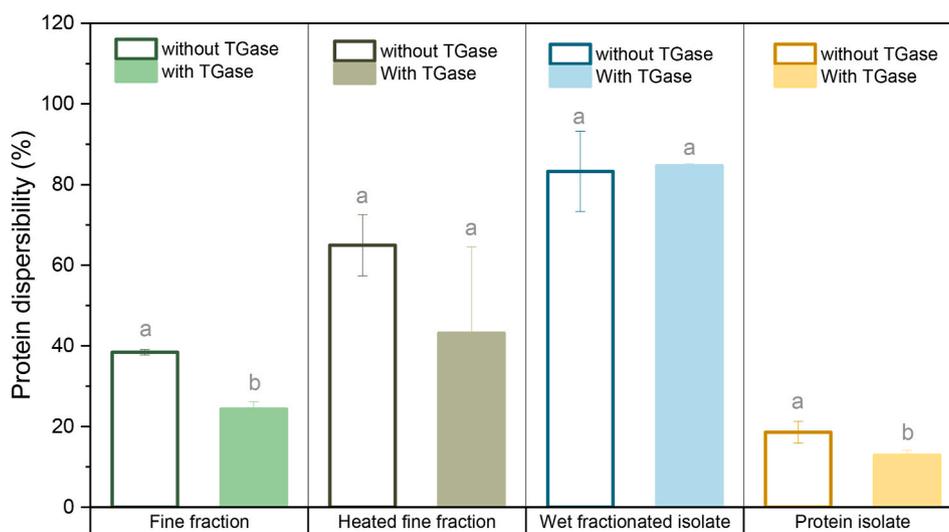


Fig. 5. Protein dispersibility at 4 wt% protein, without (0 U/g protein) and with (7.6 U/g protein) TGase of fine fraction, heated fine fraction, wet fractionated isolate, and protein isolate. Different letters indicate significant differences between treatments of the same fraction ($P < 0.05$).

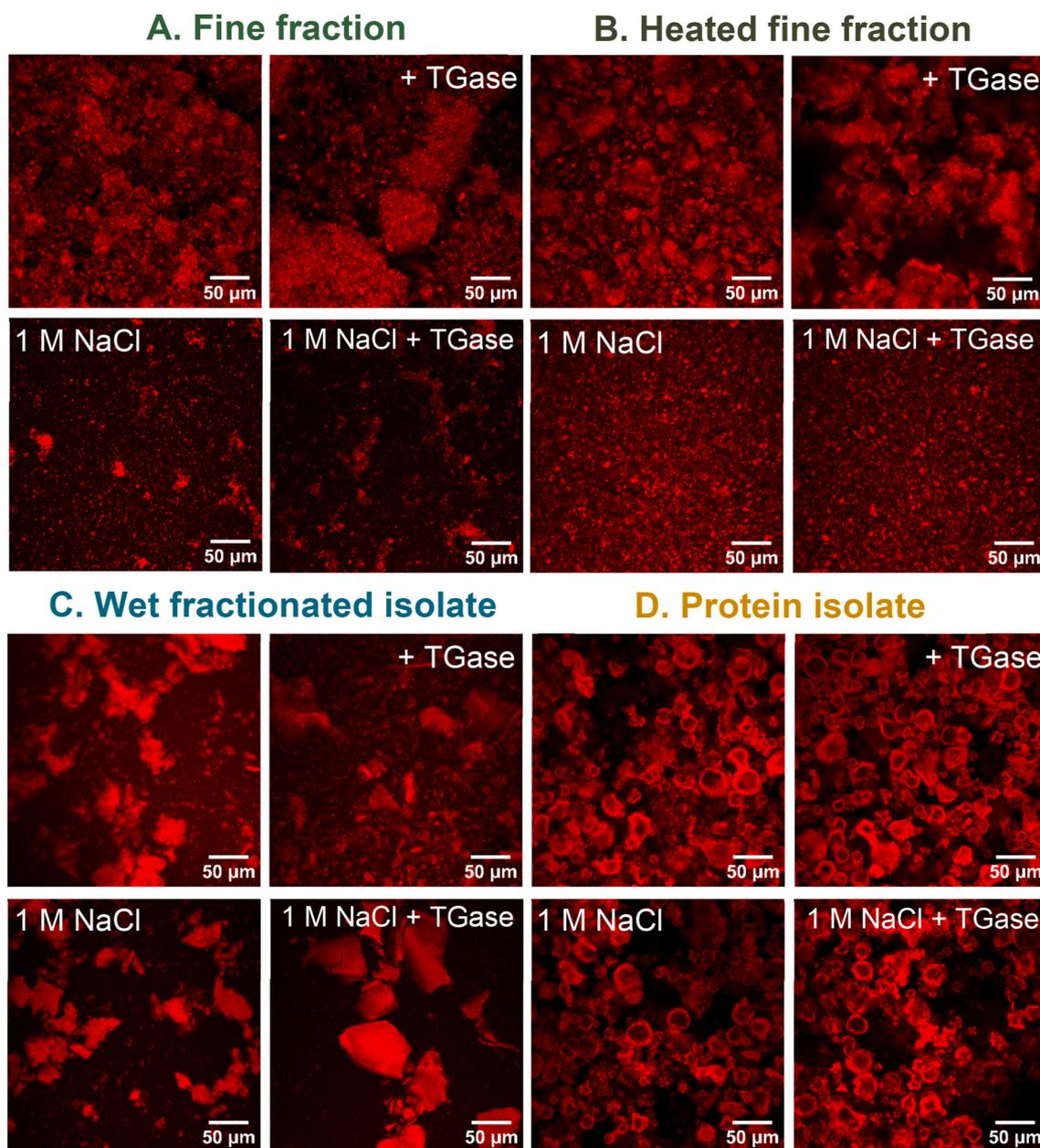


Fig. 6. The microstructure of dispersions of A) fine fraction, B) heated fine fraction, C) wet fractionated isolate, and D) protein isolate at low (0.005 M) NaCl concentration and high (1 M) NaCl concentration without TGase and with TGase. Protein is stained in red with Rhodamine B. Magnification 20x, scale bar represents 50 μm.

change protein dispersibility of the fractions, possibly exposing different amino acid groups and thus changing protein-enzyme interactions. Without TGase, the addition of NaCl to the fine fraction and the heated fine fraction decreased the size of the visible structures compared to the microstructure without NaCl (Fig. 6A and B). Thus, salt limited the swelling of the particles. The microstructures of the wet fractionated isolate and protein isolate before TGase treatment remained unchanged upon an increase in NaCl concentration (Fig. 6). We conclude that those particles were denser and did not have the ability to swell and adsorb water.

Treatment with TGase did not change the microstructure of all dispersions. The addition of TGase in the fine fraction and heated fine fraction induced aggregation, as visualized by larger particles, as well as larger voids in the CLSM images (Fig. 6A and B). This is in agreement with the increase in G' (Fig. 2A and B) and the decrease in protein dispersibility (Fig. 5) upon addition of TGase to those fractions. No clear

differences were evident between dispersions with and without TGase for the wet fractionated isolate and the protein isolate (Fig. 6C and D). Previously, Nivala et al. (2021) also showed that addition of TGase to faba bean protein gels did not change the microstructure. They suggested that inter-molecular covalent crosslinks formed by TGase reinforced the particle structures without causing massive rearrangements in spatial distribution (Nivala et al., 2021). TGase crosslinks mostly soluble proteins, whereas mostly proteins captured in insoluble particles are visible as bright parts in the CLSM picture. This could explain the lack of discernible differences in the microstructure upon the addition of TGase to the wet fractionated isolate and protein isolate. At 1 M NaCl, only the microstructure of the fine fraction was affected by TGase treatment, (Fig. 6A). The microstructures of the heated fine fraction, wet fractionated isolate, and protein isolate at 1 M NaCl remained unchanged upon TGase treatment (Fig. 6B, C, and D). Due to changes in colloidal state at high NaCl concentration, it seems that the proteins

don't swell and become less accessible for TGase.

3.5. Changes in particle size distribution upon TGase addition

To better determine potential enzyme-induced aggregation of protein particles, the dispersions before and after TGase crosslinking were tested for changes in their particle size distribution. Particle size distributions of the full dispersions with and without TGase are shown in Fig. 7. Without TGase, the protein isolate contained larger size particles than the fine fraction and heated fine fraction, further supporting the differences in microstructure (Fig. 6). A profound effect of TGase addition on particle size distribution was observed: the particle size distribution of the fine fraction and heated fine fraction shifted to larger particle sizes (Fig. 7A and B). This suggests that TGase is either forming inter-aggregate crosslinks, or it is making individual particles more prone to aggregation. These results are in agreement with the decrease in protein dispersibility, the observed change in microstructure and the increase in protein subunit size as described previously (Figs. 4, 5 and 6). The particle size distribution of the protein isolate was unaffected by TGase addition. This implies that no changes in size occurred at the colloidal scale in the protein isolate, suggesting that these particles are mostly inert. It is important to acknowledge that crosslinking of protein subunits was observed at the molecular scale in the protein isolate (Fig. 4). Consequently, even though crosslinking may have occurred in the limited number of dispersible particles present in the protein isolate,

they would not have grown to a size that would have been detectable by the Mastersizer. This can be partly a result from the fact that the Mastersizer analysis favours the detection of larger particles.

As previously suggested, the limited effect of TGase in the protein isolate could be due to its relatively low dispersibility (Fig. 5). To test this hypothesis, the post-centrifugation treated samples (obtained as a supernatant by centrifugation) were treated with TGase and their particle size distribution were analysed by dynamic light scattering (Fig. 8). Here, without TGase, the post-centrifugation treated fine fraction had a slightly smaller particle size than the other post-centrifugation treated fractions. TGase crosslinking affected the various post-centrifugation treated fractions differently. The particle size distribution of the post-centrifugation treated fine fraction and protein isolate did not change upon addition of TGase (Fig. 8A, D). The reason for this could be that the soluble proteins are diluted and dispersible, lowering the chance of particle-particle crosslinking. On the other hand, the post-centrifugation treated proteins of the heated fine fraction were susceptible to TGase treatment (Fig. 8B). By addition of TGase, the main peak in the particle size distribution broadened towards larger particle sizes in the post-centrifugation treated heated fine fraction. Here, TGase might induce inter-aggregate crosslinking between soluble proteins, increasing the overall particle size. Previous research showed that the particle size of TGase treated proteins will increase when inter-aggregate crosslinks are formed, while it may decrease when intra-aggregate crosslinks are formed (Djoullah, Krechiche, Husson, & Saurel, 2016). Therefore, the

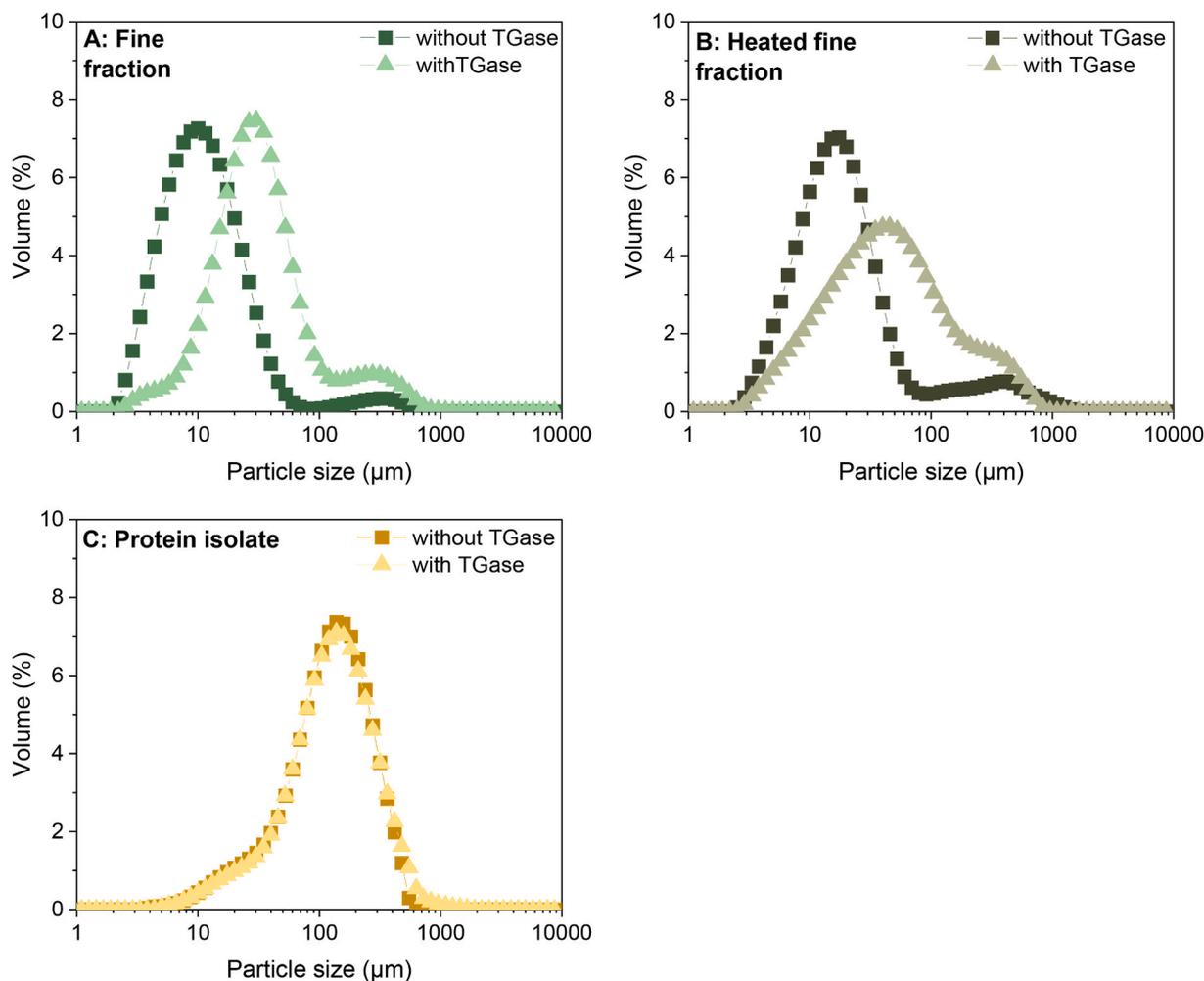


Fig. 7. Representative particle size distributions of dispersions of A) fine fraction, B) heated fine fraction, and C) protein isolate, without TGase (squares) and with TGase (triangles). Results for the wet fractionated isolate are missing, as these could not be measured due to their small particle size (<2 μm) and low obscuration levels.

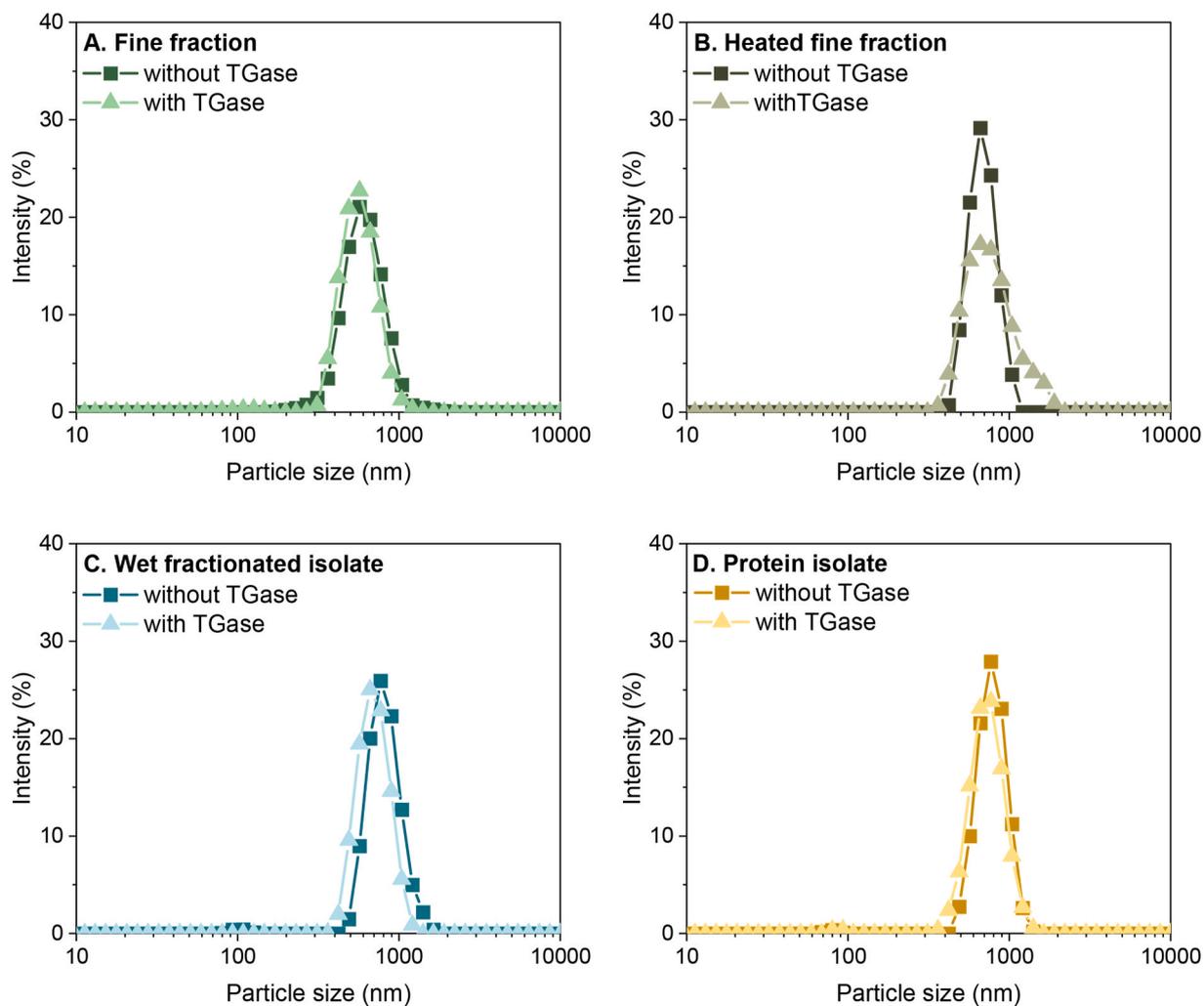


Fig. 8. Particle size distributions of the post-centrifugation treated fractions of A) fine fraction, B) heated fine fraction, C) wet fractionated isolate, and D) protein isolate, without TGase (squares & dark colour) and with TGase (triangles & light colour).

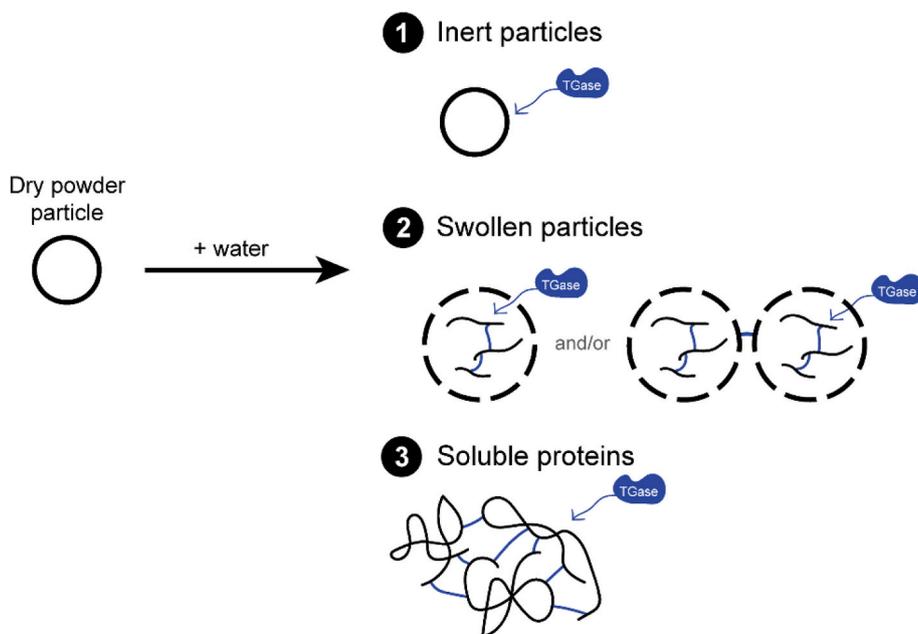


Fig. 9. Schematic illustration of proposed mechanisms of TGase crosslinking.

slight decrease in particle size in the post-centrifugation treated wet fractionated isolate by TGase addition is of interest (Fig. 8C). When combined with the results of the protein dispersibility and microstructure, this observation may imply that the majority of crosslinks formed in the soluble wet fractionated isolate are intra-aggregate crosslinks.

3.6. Proposed crosslinking mechanism

The protein isolate has a higher number of glutamine and lysine groups relative to the fine fraction, as previously reported (Schlangen et al., 2022, 2023). Therefore, this cannot account for the limited TGase crosslinking observed in the protein isolate as compared with the fine fraction. To explain the susceptibility of proteins to TGase, we hypothesize that the mung bean protein is present in three states, each having a different susceptibility to TGase (Fig. 9). The first state is a dense inert particle that does not swell in contact with water. This could be the result of intensive heat treatment and lack of hydration during when mixing the powder in water (for example in the case of the protein isolate). The second state is related to dispersed colloidal particles that have absorbed water, leading to swollen particles. The third state is that of the proteins that are soluble. Protein in dense inert particles are hypothesized not to react with TGase (mechanism 1). Protein present in swollen particles can react with TGase, and their crosslinking may lead to an increase in the density of the particles (Flory & Rehner, 1943) and/or potentially crosslinking of particles (mechanism 2). Soluble protein can most easily react with TGase, forming protein aggregates that might remain in solution or form particles (mechanism 3). The specific mechanism(s) of crosslinking depend on the fractionation procedure of the fractions and their composition. Approximately 80% of the protein isolate is not dispersible and particles appeared dense (Figs. 5 and 6), implying that protein is mostly present as the dense inert particles that hardly react as explained by mechanism 1. However, the roughly 20% of dispersible protein in the protein isolate will be crosslinked by TGase through mechanism 3, explaining the increase in molecular size (Fig. 4D). Furthermore, the subtle decrease in rheological properties may be due to densifying of the 20% dispersible protein, which then contributes less to the overall network upon crosslinking by TGase (Fig. 2D). The wet fractionated isolate is highly dispersible (Fig. 5), suggesting that mechanism 3 is primarily responsible for TGase crosslinking. The fine fraction and heated fine fraction contain both dispersible and non-dispersible protein (Fig. 5). TGase crosslinking of the dispersible protein mainly follows mechanism 3. For the non-dispersible protein, we hypothesize that the proteins will be present in swollen particles after hydration that allow for the possibility for TGase to crosslink proteins inside the particles. The possibility that TGase would also crosslink the swollen particles with each other cannot be excluded, which would explain the increase in particle size upon TGase addition in the fine fraction and heated fine fraction (Fig. 7). The hypothesis of swollen particles is supported by the effect of salt addition. Salt reduced the swelling of particles as shown in section 3.4, confirming that without salt, water and thus also the TGase can penetrate the particles.

4. Conclusions

This study describes the effects of mung bean protein fractionation on susceptibility to crosslinking by TGase and the consequences for its products at three different length scales. We showed that TGase crosslinking was independent of fractionation procedure at the molecular scale, but was greatly dependent on fractionation procedure at colloidal and bulk scale. To explain the different crosslinking effects of TGase on the various mung bean protein fractions, we hypothesized that mung bean protein can be present in different states. The first state is a dense protein particle, which does not swell upon hydration and is rather inert. The second state is a swollen particles in which TGase can diffuse, leading to both intra- and interparticle crosslinks. The last state is that of

soluble proteins. These proteins can be readily crosslinked leading to still soluble aggregates or swollen particles. We concluded that proteins in the commercial protein isolate are mostly present as dense protein particles, which explains the limited effect on the rheological properties, despite some detected molecular crosslinks of the small soluble protein fractions present in this isolate. The fine fraction and heated fine fraction possess mostly swollen particles, which explains observed changes on all length scales. The relatively high dispersibility and high amount of highly TGase reactive globulins increased susceptibility to TGase in the wet fractionated isolate, explaining the large effect on the rheological properties, but limited effect on colloidal scale. We can conclude that changes in microstructure and protein dispersibility, as a result of fractionation procedure, affect susceptibility of the proteins to TGase.

Author contributions

MS: Conceptualization, Investigation, Writing – Original draft, Writing – review & editing, Funding acquisition. NR: Conceptualization, Writing – review & editing, Supervision. STD: Conceptualization, Writing – review & editing, Supervision. MC: Conceptualization, Writing – review & editing, Supervision. AJvdG: Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

Acknowledgements

The first author would like to thank Katherine Findlay Grasberger for the warm welcome at AU food and introduction to lab equipment. This study was financially supported by the Good Food Institute and by VLAG Graduate School.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodhyd.2023.109067>.

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