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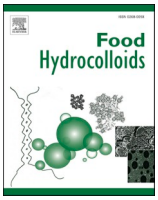

Tomato (*Solanum lycopersicum*) leaf juice as enzyme source: A study on the impact of endogenous proteases on plant proteins

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ABSTRACT

Tomato leaves are by-products of tomato production and can be considered as source of enzymes due to its endogenous proteolytic activity. The endogenous proteases can alter the functionality of other proteins. In this study, we investigated the effect of tomato leaf juice on the properties of a selected number of plant proteins, with focus on the endogenous proteases. The effect was analyzed at molecular scale using SDS-PAGE and HPSEC and at colloidal scale in terms of protein dispersibility, particle size distribution and viscosity. Lastly, the effects on the gelation behavior and rheological properties were characterized. We observed partial hydrolysis of all plant proteins under the tested conditions. The hydrolysis was specific for each protein and incomplete. The partial hydrolysis resulted in significant changes in protein dispersibility and particle size. For plant proteins with low protein dispersibility, dispersibility increased due to size reduction of the swollen particles. For plant proteins with high protein dispersibility, dispersibility decreased due to the formation of larger aggregates. The partial hydrolysis also resulted in small increase of viscosity, with exception of soy protein isolate, of which the viscosity decreased. Finally, the partial hydrolysis resulted in reduction of the least gelling concentration for all plant proteins, while some of the formed gels exhibiting higher gel strength than the corresponding water gels. This suggests that partial hydrolysis led to formation of more interactions, but these interactions were mostly weak. To conclude, our findings provided a comprehensive overview of the effects of tomato leaf juice proteases on plant proteins.

1. Introduction

Tomato leaves are by-products of the tomato production and take up to about 40 % of the plant mass (Taylor & Fraser, 2011). Based on the global tomato production in the last 10 years (FAOSTAT), it is estimated that at least 120 million tons of tomato leaves are produced annually worldwide. Despite of its large availability, tomato leaves are currently considered as waste since they are mostly discarded, or in some cases composted (Fernández-Gómez et al., 2013). In tomato leaves, up to 28 % of dry matter is protein (Abo Bakr et al., 1982; Yu et al., 2022), making tomato leaves a potential protein source for food and feed industry.

Among all leaf proteins, RuBisCo (ribulose bisphosphate carboxylase oxygenase), as a key enzyme for photosynthesis (Fiorentini & Galoppini, 1983; Santamaría-Fernández & Lübeck, 2020), is considered as the main target for food industry, due to its good nutritional values and functionality (Martin et al., 2014; Nieuwland et al., 2021). However, leaf proteins are very heterogenous. For example, part of the leaf proteins are endogenous proteases. These proteases have important functions for the plants, such as plant defense, stress response and protein turnover (Messdaghi & Dietz, 2000). Additionally, the presence of these endogenous proteases leads to protein degradation during leaf processing (Scalet et al., 1984; Wang et al., 2004) and therefore comprises the quantity and quality of extracted proteins from leaves. Nevertheless, our previous research showed that (part of) the endogenous proteases in tomato leaf juice resulted in a selective and limited hydrolysis of whey proteins, which subsequently led to an improved gelation behavior (Yu et al., 2024). Hence, it was concluded that tomato leaves can also be considered as a source of enzymes.

Enzymatic modification is considered as a green method to improve the functionality of proteins, including their gelling properties (Nielsen, 2009). Moreover, the utilization of enzymes from natural biological sources has gained attention due to the increasing awareness of

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sustainability. Enzymatic modification was applied to many different types of proteins such as dairy proteins, plant proteins and animal proteins. For example, much less material (3 % w/w, at neutral pH) was required for whey protein isolates to form gel with proteins hydrolyzed by *Bacillus lichenifonnis* as compared to the corresponding gel formed with intact proteins (15 % w/w) (Ju et al., 1995). In another study, shorter gelation time and higher gel strength were reported for whey protein isolates when hydrolyzed by the same enzyme (Tarhan et al., 2016). Similar findings were reported for plant proteins such as pea protein isolate (Chen & Campanella, 2022), quinoa protein isolate (Wang et al., 2022) and peanut protein isolate (Zhao et al., 2011). The key to this application is to control the hydrolysis degree since proteins will lose their integrity and functionality upon complete hydrolysis. At low degree of hydrolysis, hydrophobic groups can be exposed, which then leads to the formation of aggregates between hydrolyzed peptides and intact proteins (Creusot & Gruppen, 2007). Such aggregates are promoted upon secondary heating and lead to gel formation (Chen & Campanella, 2022). In some cases, aggregation and gelation take place even without the secondary heating step. In this case, a secondary hydrolysis step is usually involved where the hydrolyzed products from the first hydrolysis step are exposed to the same or different enzymes (Gong et al., 2015). The obtained (thixotropic) products through such enzymatic hydrolysis and without the secondary heating step is defined as plastein (Gong et al., 2015). Until today, the production of plastein been demonstrated for many protein sources, such as casein (Sun & Zhao, 2012), soy, zein (Gong et al., 2015), squid (Ono et al., 2004) and fish silage (Raghunath & McCurdy, 1991).

The aim of this study is therefore to investigate the effect of endogenous proteases present in tomato leaf juice on a selected number of plant proteins. Commercial and in-house made protein isolates and concentrates from soy, yellow pea, mung bean, lupin and faba bean are included. The enzymatic effects on the plant proteins are firstly investigated at molecular scale (using SDS-PAGE and HPSEC). Then, the effect on protein dispersibility, particle size distribution and viscosity of plant proteins is demonstrated. Finally, the effects on gelation behavior (least gelling concentration) and rheological properties of plant proteins are also characterized. With this study, we hope to provide insights on the utilization of tomato leaves as enzyme source.

2. Material and methods

2.1. Plant material and chemicals

Tomato plants (*Solanum lycopersicum*, cultivar: Moneymaker) were grown from December 2022 to April 2023 in the greenhouse (Wageningen University, the Netherlands). Detailed information of growing conditions of the plants and harvesting of the leaves was described in Yu et al. (2024). In short, leaves were harvested in batches from approximately 2.5 m tall tomato plants with mature fruits to minimize the time between harvesting and processing. In each batch, leaves from 3 to 4 plants were harvested. The middle stem was removed from every plant and therefore the harvested leaves consisted of leaflets, petioles, rachises and petiolules (Altartouri et al., 2015). All leaves harvested within one batch were manually mixed and juiced within 1 h after the harvest. The juice was stored in a −20 °C freezer (Section 2.2) until October 2023 when the experiments for this study were conducted.

Soy protein isolate (SPI, trade name SUPRO 500E IP) and mung bean protein isolate (MPI, trade name UNIMUNG M70) were purchased from Barentz (Hoofddorp, the Netherlands). Soy protein concentrate (SPC, trade name ALPHA 8 IP) was purchased from Solae LLC (St. Louis, Missouri, United States). Pea protein isolate (PPI, trade name NUTRALYS F85M) was purchased from Roquette Frères (Lestrem, France). Lupin protein isolate (LPI) was purchased from Prolupin GmbH (Grimmen, Germany). Faba bean protein concentrate (FPC, trade name VITESSENCE CT3602) was purchased from Ingredion (Westchester, Illinois, USA). Pea protein concentrate (PPC), mung bean protein concentrate (MPC), lupin protein concentrate (LPC) and faba bean protein concentrate 2/fine fraction (FPF) were produced in-house, using air classification method according to Bühler et al. (2022), Lie-Piang et al. (2023) and Schlangen et al. (2022). Sodium carbonate anhydrous was purchased from VWR International (Radnor, Pennsylvania, United States). L-aspartic acid, 2-mercaptoethanol, sodium hydroxide, hydrochloric acid, thyroglobulin, bovine serum albumin, β-lactoglobulin, α-lactalbumin, aprotinin, bacitracin, phenylalanine, L-tyrosine, Folin-Ciocalteu phenol reagent, trichloroacetic acid, potassium phosphate monobasic and dibasic were purchased from Sigma-Aldrich (Darmstadt, Germany). Sodium metabisulfite and trifluoroacetic acid were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, United States). Acetonitrile (Ultra LC-MS grade) was purchased from Actu-All Chemicals b. v. (Oss, the Netherlands). 2x concentrated Laemmli sample buffer (65.8 mM Tris-HCl, pH 6.8, 2.1 % SDS, 26.3 % w/v glycerol, 0.01 % bromophenol blue), running buffer, Bio-Rad Precision Plus Protein™ Dual Xtra Prestained Protein Standards, Bio-safe Coomassie Staining buffer and 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels for SDS-PAGE were purchased from Bio-Rad Laboratories (Hercules, California, United States). Ultrapure water (MilliQ water) was purified by using a Milli-Q IQ 7000 Ultrapure Lab Water System (Merck KGaA, Darmstadt, Germany).

2.2. Juice production

Tomato leaf juice was produced in the same way as described in Yu et al. (2024). In short, tomato leaves were juiced with an Angel juicer II 7500 (Angel Juicers, Queensland, Australia) into green juice and pulp. The green juice was mixed with 10 % (w/w) sodium metabisulfite to a final concentration of 0.2 % (w/w) and centrifuged (15,000g for 1 h at 18 °C). Supernatant was separated from the pellet by carefully pouring, after which the supernatant was filtered through a double folded cheesecloth. The filtered supernatant was immediately frozen at −20 °C until further analysis. The filtered supernatant was referred to as TLJ in this study.

2.3. Compositional analysis

The dry matter content of all plant proteins were measured by leaving samples in an hot air oven at 105 °C overnight. The dry matter content of TLJ was defined as the weight after freeze drying (Epsilon 2-10D LSCplus, Martin Christ, Osterode, Germany). Each sample was measured in triplicates.

The protein content of all plant proteins, their oven dried pellets (Section 2.6) and TLJ was measured using the Dumas nitrogen combustion method, with a rapid N exceed® analyzer (Elementar, Langenselbold, Germany). Around 250 mg of sample was weighed in a tin foil sheet and wrapped tightly without headspace. Subsequently samples were combusted at 900 °C with oxygen. L-aspartic acid was used as standard sample and each sample was measured in triplicates. Different N conversion factors were used for the plant proteins, their oven dried pellets and TLJ. The results are summarized with references in Table 1, together with the corresponding dry matter content and protein content on dry basis. Besides proteins, the rest of dry matter in TLJ was assumed to be soluble carbohydrates, given the fact that they are the most dominant component in leaves (Tamayo Tenorio et al., 2018).

2.4. Specific protease activity measurement

The specific protease activity was measured according to Arbita et al. (2020), with β-lactoglobulin as substrate. 500 μL of 1 % (w/v) β-lactoglobulin solution in 0.1 M potassium phosphate buffer (pH 7.5) was mixed with 100 μL TLJ. The mixture was then incubated at 37 °C for 10 min. Subsequently, 500 μL of 5 % (w/v) trichloroacetic acid was added to the mixture. The resulting mixture was incubated at 37 °C for 30 min to stop the reaction. After that, the mixture was centrifuged at 10,000 g

Table 1
The dry matter content (%), protein content on dry basis (%) and N conversion factor with references for each plant protein and TLJ.

Plant proteins	Dry matter content (%)	N conversion factor	Protein content on d.b (%)	References for N conversion factor
SPI	96.9 ± 0.1	5.7	79.0 ± 0.0	Krul (2019)
SPC	93.9 ± 0.1	5.71	59.5 ± 0.1	Krul (2019) and Mariotti et al. (2008)
PPI	95.3 ± 0.1	5.5	69.7 ± 0.0	Holt and Sosulski (1979)
PPC	94.1 ± 0.1	5.5	46.7 ± 0.1	Schlangen et al. (2022)
MPI	94.7 ± 0.2	5.7	65.1 ± 0.1	Schlangen et al. (2022)
MPC	93.4 ± 0.1	5.7	52.8 ± 0.1	Schlangen et al. (2022)
LPI	97.5 ± 0.2	5.7	84.0 ± 0.3	Berghout et al. (2014)
LPC	93.4 ± 0.2	5.7	46.9 ± 0.6	Berghout et al. (2014)
FPC	94.7 ± 0.1	5.71	53.3 ± 0.2	Bühler et al. (2020) and Mariotti et al. (2008)
FPF	93.0 ± 0.1	5.71	53.0 ± 0.3	Bühler et al. (2020) and Mariotti et al. (2008)
TLJ	5.5 ± 0.4	4.4	25.3 ± 0.1	Kiskini et al. (2016) and Milton and Dintzis (1981)

for 5 min. An aliquot of 400 μL supernatant was taken and mixed with 1 mL 0.5 M Na_2CO_3 solution and 200 μL 0.5 N Folin-Ciocalteu phenol reagent. The new mixture was incubated at 37 °C for 30 min and followed by centrifuge at 10,000 g for 5 min. Finally, the absorbance of 1 mL supernatant was measured at 660 nm using a DR6000 UV VIS Spectrophotometer (Hach, Colorado, USA). TLJ was measured three times. One unit of protease activity was defined as the amount of enzyme that hydrolyzes β-lactoglobulin to produce color equivalent to 1 μmol (181 μg) of tyrosine per minute at the tested conditions. Therefore, series of tyrosine solutions with different concentrations (0, 27.5, 55, 110 and 220 μmol/L in 0.1 M potassium phosphate buffer) were used to make standard curve. The specific protease activity was calculated with the following equation (Anson, 1938; Arbita et al., 2020):

$$\textit{Specific protease activity}\ (\textit{units / mg proteins}) = \frac{TE * v_A}{v_E * t * v_C * c_{pt}} \quad \text{Eq. 1}$$

Where *TE* represents the tyrosine equivalent derived from the standard curve (μmol); v_A, v_E and v_c represent the total volume of assay (mL), volume of used enzyme (mL) and volume used in colorimetric measurement (mL), respectively; t represents the reaction time (min) and c_{pt} represents the concentration of proteins in TLJ (mg proteins/mL TLJ, derived from Section 2.3).

2.5. Protein hydrolysis analysis

2.5.1. Analysis of hydrolysis over time

The hydrolysis of plant proteins by proteases in TLJ over time was analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition. The sample preparation method was adapted from Liese et al. (2023). A reducing sample buffer was prepared by mixing the Laemmli sample buffer with 2-mercaptoethanol at ratio of 19:1 (v/v). Frozen TLJ was thawed at room conditions and the pH was adjusted to 7 with 2 M sodium hydroxide. Afterwards, each plant protein was dispersed in TLJ at concentration of 3 % (w/v) on dry basis. The mixture was thoroughly mixed using a vortex and the pH of the mixture was re-adjusted to 7 with 2 M sodium hydroxide or 2 M hydrochloric acid. Control samples were made in the same way by dispersing plant proteins in MilliQ water at the same concentration. The mixtures were then incubated at room temperature on an rotator at 40 rpm. During incubation, samples were taken at 1 h, 2 h and 18 h and immediately mixed with reducing sample buffer at ratio of 1:1 (v/v). The new mixtures were thoroughly mixed using a vortex, then heated at 95 °C for 10 min and subsequently frozen at −20 °C. On the next day, these mixtures were thawed at room temperature and heated again at 95 °C for 10 min. Then, these samples were diluted with a MilliQ water-reducing buffer mixture (1:1, v/v) to a protein concentration in dispersion of 2 mg/mL. The dispersion was subsequently centrifuged at 10,000g for 5 min 15 μL of supernatants and 10 μL of protein standard marker were loaded into different lanes on the gel. The electrophoresis was carried out with 200 V for about 1 h. Afterwards, the gels were washed three times with MilliQ water, stained with Bio-safe Coomassie stain and detained with MilliQ water. The gels were scanned using GS-900 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, USA).

2.5.2. (Soluble) protein molecular weight distribution

The molecular weight distribution of (soluble) proteins before and after hydrolysis were analyzed using High Pressure Size Exclusion Chromatography (HPSEC) method using an Ultimate 3000 HPLC (Thermo Fisher, Waltham, USA) (Möller et al., 2022). Two columns of TSKGel G4000SWXL 5 μm 300 × 7.8 mm and TSKGel G3000SWXL 5 μm 300 × 7.8 mm were used. A 10 μL of each supernatant (Section 2.6) was injected each time with a flow rate of 1.5 mL/min at 30 °C, and with eluent of 30% acetonitrile in MilliQ containing 0.1% trifluoroacetic acid. The run time for each sample was approximately 35 min. UV detection was at 214 nm. Data analysis was performed in Chromeleon 7.2 CDS software (Thermo Fisher, Waltham, USA). The HPLC peaks were divided into 6 ranges based on molecular weights: >550 kD, 260–550 kD, 160–260 kD, 56–160 kD, 9–56 kD and 1–9 kD. A calibration curve of the molecular weight on a logarithmic scale against elution time was plotted for thyroglobulin (670 kDa), bovine serum albumin (66.5 kDa), β-lactoglobulin (36 kDa), α-lactalbumin (14.5 kDa), aprotinin (6.51 kDa), bacitracin (1.42 kDa) and phenylalanine (165 Da). The relative area was calculated as the peak area integrated from each peak divided by the total of all peak areas in one sample. The relative areas were used as a measure for the relative abundance of proteinaceous components that were in each molecular weight range in one sample. Each sample was made twice and analyzed in duplicates.

2.6. Protein dispersibility

2.6.1. Sample preparation

Plant proteins dispersed in TLJ and MilliQ water at concentration of 3 % (w/v) on dry basis were prepared at pH 7. The mixtures were incubated at room temperature on an rotator at 40 rpm for 1 h. One hour incubation time was chosen due to the fact that hydrolysis degree was already evident at 1 h and did not increase significantly with time (Fig. 1).

2.6.2. Protein dispersibility measurement

After incubation, samples were centrifuged at 17,217 g for 20 min, after which supernatants were separated from the wet pellets by carefully pouring. The supernatants were analyzed for soluble protein molecular weight distribution (Section 2.5.2) and the wet pellets were weighed and dried in an hot air oven for overnight. The oven dried pellets were weighed and the protein content of these pellets was determined using the Dumas nitrogen combustion method (Section 2.3). Each sample was made twice and analyzed in duplicates. The protein dispersibility were calculated with the following equation:

$$\textit{Protein dispersibility}\ (\%) = \frac{\left(M_{pt.plant\ protein} - M_{pt.oven\ dried\ pellet}\right)}{M_{pt.plant\ protein}} \quad \text{Eq. 2}$$

Where $M_{pt.plant\ protein}$, $M_{pt.oven\ dried\ pellet}$ and $M_{pt.plant\ protein}$ represent the mass

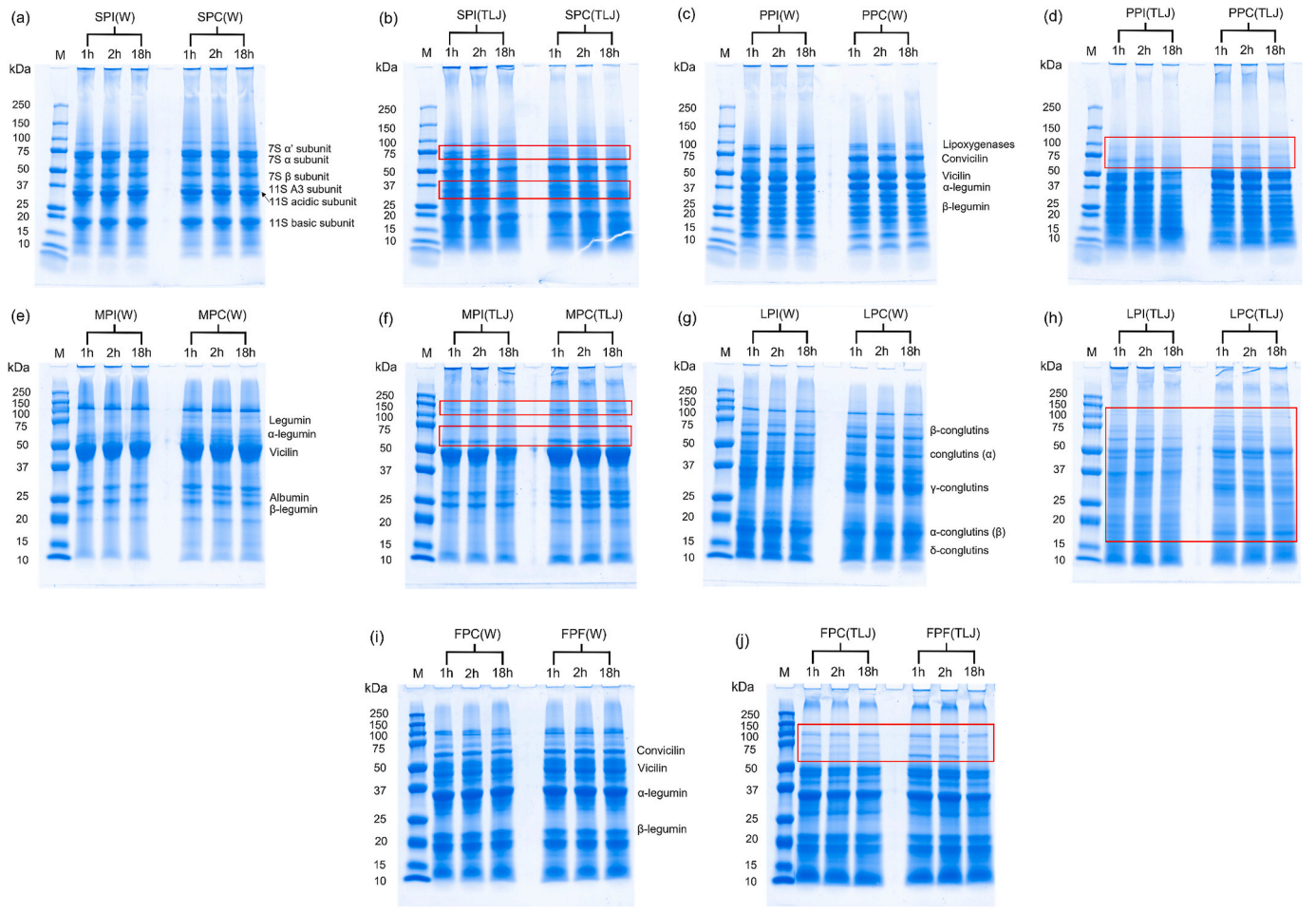

Fig. 1. SDS-PAGE of plant proteins (a–j) dispersed in water (W) and tomato leaf juice (TLJ) after incubation (3 % w/v, room temperature, 40 rpm) for different time (1 h, 2 h and 18 h). M represents the protein standard marker. The red line frames indicated the area where major protein hydrolysis took place.

of total proteins in wet pellet, oven dried pellet and plant protein sample, respectively.

2.7. Particle size distribution

2.7.1. Sample preparation

The particle size distribution of plant proteins dispersions was analyzed using static light scattering. Plant proteins dispersed in TLJ and MilliQ water at concentration of 3 % (w/v) on dry basis at pH 7 were prepared and incubated at room temperature on an rotator at 40 rpm for 1 h.

2.7.2. Particle size distribution measurement

After incubation, samples were analyzed with a Mastersizer 3000 (Malvern Instruments Ltd., UK) at 20 °C. A volume-based model (Mie theory for non-spherical particles) was used because of the multi-component nature of the plant proteins. A refractive index of 1.46 was used with an absorption index of 0.1. Each sample was measured in triplicates, and the averaged value (without standard deviation) of the triplicates is presented in this study. A blank TLJ sample was incubated (room temperature, 1 h, 40 rpm) and added to the Mastersizer. However, the concentration of particles was not high enough to allow measurement.

2.8. Least gelling concentration analysis

2.8.1. Sample preparation

The least gelling concentration method was adapted from Coffmann and Garciaj (1977) and Schlangen et al. (2022). Plant proteins were dispersed in TLJ at concentrations of 3, 6, 9, 12 and 15 % (w/v) on dry basis at pH 7. Due to the fact that lower concentrations did not lead to gel formation, two more concentrations (16 and 17 % (w/v)) were tested for PPI and LPC. Similarly, seven more concentrations (16, 17, 18, 19, 20, 21 and 22 % (w/v)) were tested for LPI. All samples were then incubated at room temperature on an rotator at 40 rpm for 1 h.

2.8.2. Least gelling concentration measurement

After incubation, all samples were immediately heated at 95 °C in a water bath for 30 min and subsequently cooled down to room temperature. The samples were then inverted and the least gelling concentration was determined at the concentration where the sample did not slip or fall down after the inversion. All samples were made and evaluated three times.

2.9. Rheological properties

2.9.1. Sample preparation

The rheological properties of samples were analyzed by small angle oscillatory shear (SAOS) and large amplitude oscillatory shear (LAOS) measurements, with methods adapted from Kornet et al. (2021) and Nieuwland et al. (2021). All samples were made and analyzed three times. Plant proteins dispersed in TLJ and MilliQ water at concentration of 15 % (w/v) on dry basis at pH 7 were prepared and incubated at room temperature on an rotator at 40 rpm for 1 h.

2.9.2. SAOS (viscosity and temperature sweep) measurement

After incubation, approximately 6 mL of dispersion was added into MCR702 rheometer combined with a concentric cylinder CC-17 geometry (Anton Paar, Graz, Austria). The geometry was covered with a moisture trap to prevent water evaporation. Dispersions were equilibrated at 20 °C for 2 min and the flow properties were measured with shear rate ranging from 1 to 100 s^{-1} at 20 °C. Dispersions with low viscosities (MPI and LPC in TLJ and blank TLJ and MilliQ water samples) were measured using a double-gap DG 26.7 geometry (Anton Paar, Graz, Austria), at shear rate ranging from 10 to 100 s^{-1} at 20 °C. The viscosity curve of blank water and TLJ samples can be found in supplementary material Figure S.1. In addition, the shear-thinning behavior was analyzed by normalizing viscosity in relation to the first measured viscosity point of each sample, the result is presented in supplementary material Figure S.2.

After the viscosity measurement, dispersions were allowed to recover at 20 °C for 5 min. Subsequently, a temperature sweep was conducted by increasing the temperature from 20 to 95 °C at a rate of 1.8 °C/min. The temperature was kept at 95 °C for 10 min and subsequently lowered to 20 °C at a rate of 1.8 °C/min, after which the dispersion was kept at 20 °C for 10 min. During the temperature sweep, the frequency and shear strain were kept constant at 1 Hz and 1 % and the storage modulus (G') and loss modulus (G'') were recorded. The individual G' value reported in this study corresponded to the last measure point of the temperature sweep. The loss factor (tan δ) was calculated with the following equation:

$$tan\,\delta = \frac{G''}{G'} \quad \text{Eq. 3}$$

Where G'' and G' represent the loss modulus and storage modulus of the last measurement point of the temperature sweep, respectively.

2.9.3. LAOS (shear strain sweep) measurement

Right after the temperature sweep, a shear strain sweep was conducted with shear strain ranging from 0.1 to 1000 %, while the frequency was kept constant at 1 Hz. During the shear strain sweep, the G' and G'' were recorded. The end of the linear viscoelastic regime was defined as the shear strain where the first G' modulus data point deviated 5 % (Schlangen et al., 2022) and was expressed as the critical strain (γ_c). The crossover strain ($\gamma_{G'=G''}$) was determined as the last shear strain point where G' was higher than G''. A summary of all the data derived from the LAOS analysis is presented in Supplementary material Table S1.

2.10. Statistical analysis

The statistical analysis was carried out by using IBM SPSS statistics, version 28.0.1.1 (IBM, Armonk, US). Significant differences between all tested samples were analyzed with one-way ANOVA using a multivariate general linear model and with a post hoc Duncan test. Differences were considered significant when $P \leq 0.05$ and were shown as small upper letters.

3. Results and discussion

3.1. Partial protein hydrolysis evident on molecular level for all plant proteins

The specific protease activity of TLJ was 0.04 ± 0.002 units/mg proteins, which was at similar level as the specific protease activities reported for other natural sources, such as red seaweed (0.06 units/mg protein) (Arbita et al., 2020) and kiwi fruit (0.09 units/mg protein) (Mazorra-Manzano et al., 2013). The effect of TLJ proteases on plant proteins was firstly investigated at molecular scale, using SDS-PAGE and HPSEC methods. The results of SDS-PAGE are presented in Fig. 1. Here we discussed the effect of TLJ proteases on proteins from different plants separately.

For unhydrolyzed soy proteins (Fig. 1a), major bands like 7S α′ and 7S α subunits (75 kD), 7S β subunit (50 kD), 11S A3 subunit (37 kD) and 11S acidic and basic subunits (35 and 20 kD, respectively) (Iwabuchi & Yamauchi, 1987) were identified under reducing conditions. These bands represent the two major storage proteins namely glycinin (11S globulin) and β-conglycinin (7S globulin) in soybean (Nguyen et al., 2016). No differences were observed in SPI and SPC (Fig. 1a). When dispersed in TLJ, the band intensities of 7S α' and 7S α subunits and 11S acidic subunit decreased significantly, suggesting protein hydrolysis as a result of the proteolytic activity in TLJ. Such decrease of band intensity was already evident after 1 h incubation time and it continued when incubation time reached 18 h. In addition, an overall smearing of bands occurred from 10 to 75 kD, which also suggests the formation of hydrolyzed products with various sizes (Fig. 1b). It is important to note that the intensities of other major bands like 7S β subunit, 11S A3 subunit and 11S basic subunits did not change, indicating selective hydrolysis of proteins. This finding differs from our previous finding on WPI where all proteins were hydrolyzed by TLJ proteases, albeit to different extent (DH: BSA 40 %, β-lg 33 % and α-lg 10%, respectively) (Yu et al., 2024). It is suspected that enzyme specificity is responsible for the difference in hydrolyzation degree for the various proteins.

Similar results as with soy were observed for pea proteins (Fig. 1c and d), mung bean proteins (Fig. 1e and f) and faba bean proteins (Fig. 1i and j). In the case of pea proteins, several major bands were identified such as lipoxygenases (~90 kD) (Crévieu et al., 1997), convicilin (~70 kD), vicilin (48–52 kD) and α-legumin and β-legumin (38–40 kD and 19–22 kD, respectively) (Rubio et al., 2014) on unhydrolyzed protein gels (Fig. 1c). Upon (partial) hydrolysis, the band intensities of lipoxygenases and convicilin significantly decreased for both PPI and PPC, indicating strong specificity of proteases in TLJ on these proteins (Fig. 1d). In addition, the vicilin subunit in PPI decreased its intensity. In the meanwhile, the band intensity at the same size increased for PPC, which might be a result of the formation of hydrolyzed products with similar size as vicilin. In the case of mung bean proteins, major bands such as legumin (~75 kD), α-legumin and β-legumin (60 kD and 25 kD, respectively), vicilin (50 kD) and albumin (~26 kD) (Schlangen et al., 2023) were identified on unhydrolyzed protein gel (Fig. 1e). TLJ proteases specifically hydrolyzed α-legumin in both MPI and MPC, leaving a new distinctive band with size slightly larger than 50 kD. In addition, the band corresponding to size from 100 to 150 kD also disappeared upon hydrolyzation in both cases (Fig. 1f). Finally, in the case of faba bean proteins, major bands such as convicilin (60 kDa), vicilin (46–55 kDa), α-legumin and β-legumin (38–40 kDa and 23 kDa, respectively) were identified on unhydrolyzed protein gel (Fig. 1i) (Warsame et al., 2018). The TLJ proteases again specifically hydrolyzed subunits with large size in between 50 and 150 kD including convicilin, leaving the rest unhydrolyzed (Fig. 1i). LPI showed bands such as α-conglutin, β-conglutin, γ-conglutin, and δ-conglutin (Fig. 1g) (Grasberger et al., 2023). Furthermore, LPC contained more γ-conglutin subunit as demonstrated by its higher band intensity than that in LPI. Upon hydrolysis by TLJ proteases, the intensities of all abovementioned major bands decreased, but did not completely disappear. As compared to the rest of tested plant proteins, the hydrolysis of lupin proteins by TLJ proteases seemed to be less specific, but the hydrolysis remained overall limited (partial) (Fig. 1h). This phenomenon is suspected to be caused by several factors. Firstly, the protease activity in TLJ is limited by endogenous protease inhibitors, which was also identified in TLJ (Yu et al., 2024). Secondly, under normal circumstances, proteases from different cellular components in leaves do not come in contact with each other. However, when leaf tissue is lysed to make TLJ, these proteases can interact and result in self-cleavage or cleavage of other proteases (Gill & Parks, 2008). Thirdly, Creusot and Gruppen (2008) reported that the aggregating peptides as the products of partial hydrolysis prevent further digestion by proteases due to their large size and net increase in hydrophobicity (Kuipers & Gruppen, 2008). In conclusion, we observed partial

hydrolysis of all tested plant proteins by proteases in TLJ. The partial hydrolysis included the hydrolysis of only part of the proteins and incomplete hydrolysis of the proteins that could be hydrolyzed. The former is probably due to specificity of proteases in TLJ, while the latter is probably due to regulated enzyme activity and formation of aggregating peptides.

We further characterized the hydrolysis of plant proteins by analyzing the (relative) distribution of soluble proteins with HPSEC method. The result is presented in Fig. 2. Here we quantified the relative changes of (soluble) proteins using the peak area with different molecular weight divided by the sum peak area of all detected proteinaceous components in each sample. It is important to note that the HPSEC analyses were performed under only denaturing conditions while the SDS-PAGE analysis was performed under both denaturing and reducing conditions. The molecular weight of proteinaceous component therefore differs in these two techniques because (not all) disulfide bonds are broken under only denaturing conditions, resulting in potentially larger size in HPSEC analysis. As shown in Fig. 2, the relative size distribution of proteinaceous components in all plant proteins changed significantly after treatment with TLJ. It is consistently observed that the relative amount of large proteins (with molecular weights of >550 kD, 260–550 kD and 160–260 kD) reduced, while that of smaller proteins and peptides (with molecular weights of 56–160 kD, 9–56 kD and 1–9 kD) increased for all samples after hydrolysis. Such changes confirmed TLJ protease activity, as shown in Fig. 1. Also here, it was found that the hydrolysis was not complete since there were still considerable amounts (minimum 3.2 % of detected proteinaceous components) of large proteins (with molecular weight of >550 kD) left after 1 h incubation with TLJ. This result aligned with the SDS-PAGE result.

3.2. Significant change in protein dispersibility associated with the original protein dispersibility

The next step was to investigate the effect of TLJ proteases on plant proteins at colloidal scale. As a first analysis, the protein dispersibility was quantified and the results are presented in Fig. 3. The term protein dispersibility (Schlangen et al., 2023) was used here instead of protein solubility (Möller et al., 2022; Yu et al., 2023), due to the fact that some plant proteins (in particular MPC) contained stable (non-soluble) aggregates that remained suspended in the supernatant after high speed centrifugation (picture not shown).

As shown in Fig. 3, SPI, SPC, PPI and MPI had low protein dispersibility when dispersed in water (25.8 %, 20.5 %, 18.2 % and 15.7 %, respectively). Low protein dispersibility suggested that these dispersions mostly contained dense particles and (partly) swollen particles that end up in the pellet after high speed centrifugation. These particles can be a result of protein denaturation and aggregation induced during the processing (Li et al., 2007; Peng, Kyriakopoulou, Ndiaye, et al., 2021a). However, when dispersed in TLJ, the corresponding dispersibilities of SPI, SPC, PPI and MPI significantly increased to 41.6 %, 40.2 %, 41.4 % and 45.4 %, respectively (Fig. 1a–f). Such increase of dispersibility after (partial) protein hydrolysis was also reported for peanut proteins (~5–80 %, DH 5.4 %, pH 6) (Zhao et al., 2011) and chickpea proteins (~40–60 %, DH 4 %, pH 6 and ~10–80 %, DH 2.9 %, pH 6, respectively) (Mokni Ghribi et al., 2015; Yust et al., 2010). Protein hydrolysis resulted in products with smaller molecular sizes (Fig. 2) and thus newly exposed ionizable amino and carboxyl groups, which alters protein hydrophilicity and dispersibility. In the meanwhile, LPI and FPC showed a high protein dispersibility (around 50 % in both cases) when dispersed in water. The corresponding protein dispersibility did not significantly change, despite of the fact that partial protein hydrolysis was evident at the molecular level for these dispersions (Fig. 1g–j). PPC, MPC, LPC and FPF also showed high protein dispersibilities in water (76.8 %, 77.0 %, 84.9 % and 80.8 %, respectively), similar to LPI and FPC,. However, when dispersed in TLJ, the corresponding dispersibilities significantly decreased to 68.6 %, 64.5 %, 67.8 % and 62.5 %, respectively. Dent et al. (2023) summarized the effect of partial protein hydrolysis on protein

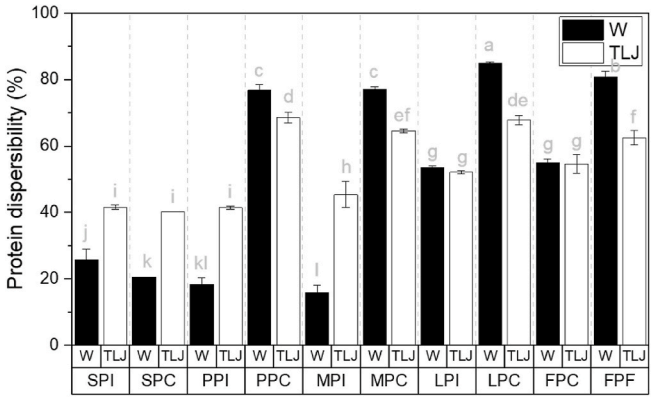

Fig. 3. Protein dispersibility of plant proteins dispersed in water (W) and tomato leaf juice (TLJ) after incubation (3 % w/v, room temperature, 1 h, 40 rm). Significant differences between all tested samples at $P \leq 0.05$ are indicated as small upper letters.

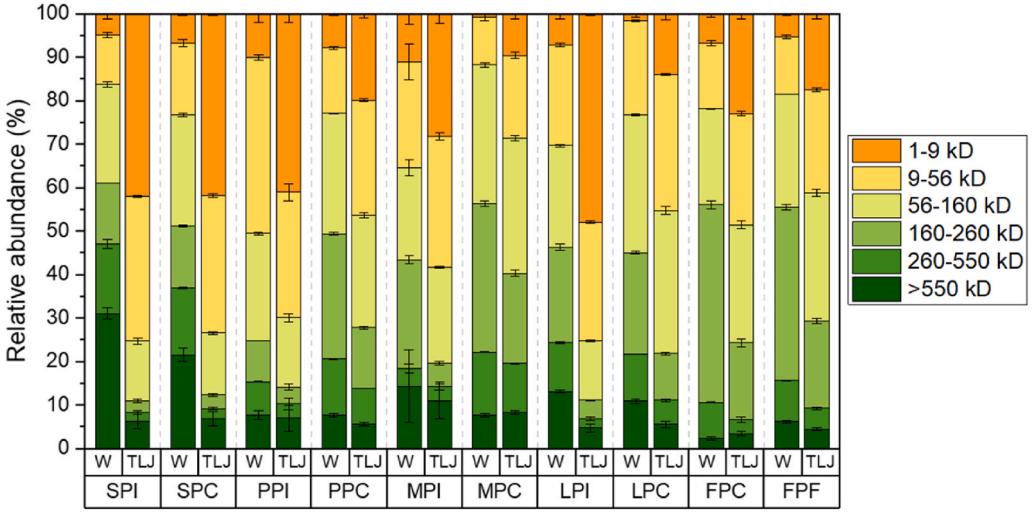

Fig. 2. Relative abundance of soluble proteins with different molecular weights (1–9 kD, 9–56 kD, 56–160 kD, 160–260 kD, 260–550 kD and >550 kD) from plant proteins dispersed in water (W) and tomato leaf juice (TLJ) after incubation (3 % w/v, room temperature, 1 h, 40 rpm).

dispersibility. When dispersibility starts high, proteins are likely to be native, hydrolysis then leads to formation of aggregates (Creusot & Gruppen, 2007) and loss of dispersibility (Kuipers & Gruppen, 2008). When dispersibility starts low, part of the proteins are already aggregated or denatured, hence hydrolysis leads to free (and smaller) peptides from the aggregates, resulting in increased dispersibility. Our findings in this study (Fig. 3) are in-line with this theory.

3.3. Significant change in particle size associated with corresponding change in protein dispersibility

The second analysis of the effect of TLJ proteases on plant proteins at colloidal scale is particle size distribution. The results are presented in Fig. 4. Here we expect that mainly dense (inert) and swollen particles (upon hydration) were measured by the Mastersizer. This instrument measures light scattering in a dynamic dispersed system. In such system, small soluble proteins scatter light much less intensely than the large dense and swollen particles, making them more difficult to detect. In general, we observed significant changes in particle size distribution in all plant proteins as a result of partial hydrolysis. For the explanation of these results, we distinguish between dispersions with low and high protein dispersibility (in water).

Unhydrolyzed SPI and SPC had a low protein dispersibility and contained particles with predominant size of 1000 μm. Upon partial hydrolysis, the peaks shifted and resulted in particles with size of around 100 μm (Fig. 4a and b). As discussed in Section 3.2, we expect that dispersions like SPI and SPC contained mostly (at least 80 %) dense and swollen particles that ended up in the pellet after high speed centrifugation, resulting in a general low protein dispersibility. The dense particles are likely to be inert and therefore not accessible to enzymes in dispersant. The size of these particles would therefore not change upon partial hydrolysis. In the meanwhile, the swollen particles are accessible to enzymes. Upon (partial) hydrolysis, part of the swollen particles dissolved, which then resulted in smaller (swollen) particles (Fig. 4a and b). Subsequently, the release of the hydrolyzed products (e.g., peptides) caused a net increase in protein dispersibility (Fig. 3). Such decrease in particle size was also observed for PPI and MPI (Fig. 4c and e). This was in line with expectations given the low protein dispersibility due to the presence of dense and swollen particles. The decrease in particle size in these dispersions was however somewhat less than SPI and SPC.

Dispersions with high protein dispersibility in water were unhydrolyzed PPC, MPC, LPC and FPF. These proteins dispersions contained particles that have predominant size of 10 μm. Upon partial hydrolysis, the majority of particles in the dispersions had size of around 100 μm (Fig. 4d–f, h and j). In these dispersions, the majority of proteins (at least 76 %) were soluble and dispersible (Fig. 3). Upon hydrolysis, the products (e.g., peptides) could form larger aggregates (Creusot & Gruppen, 2007) (Fig. 4d–f, h and j), which then resulted in a decrease in protein dispersibility (Fig. 3). For LPI and FPC, no significant differences were observed in protein dispersibility upon partial hydrolysis (Fig. 3). Also no significant change in their particle size was observed. Despite the fact that the peaks had different shapes, the majority of particles retained a similar size (around 100 μm) before and after hydrolysis in both cases (Fig. 4g and i). In conclusion, for dispersions with mostly dense and swollen particles, smaller particles were formed due to the increase of the amount of (newly dissolved) soluble proteins upon partial hydrolysis. For dispersions that have mostly soluble (and dispersible) proteins, larger aggregates were formed upon partial hydrolysis,

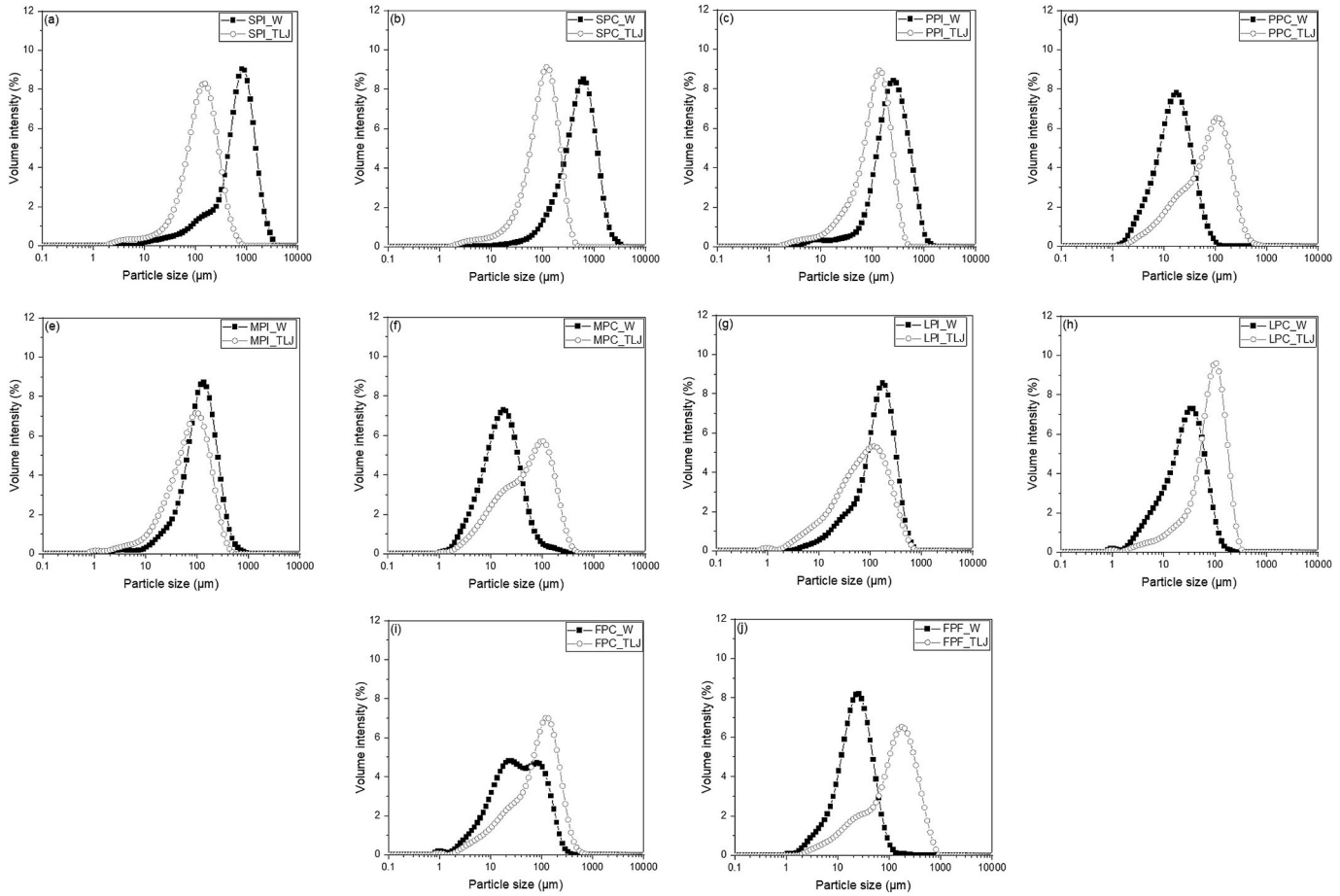

Fig. 4. Particle size distribution of swollen and dense particles in plant proteins (a–j) dispersed in water (W) and tomato leaf juice (TLJ) after incubation (3 % w/v, room temperature, 1 h, 40 rpm).

leading to a decrease in protein dispersibility.

3.4. Small change in viscosity

The last analysis of the effect of TLJ proteases on plant proteins at colloidal scale is viscosity measurement and the results are presented in Fig. 5. We discussed the result of viscosity in combination with the results of protein dispersibility and particle size, because viscosity is influenced by protein dispersibility and particle size. In general, we observed an increase of viscosity for (almost) all samples upon hydrolysis, with exceptions of SPI and PPI. A decrease of viscosity was observed for SPI while the viscosity hardly changed for PPI. We therefore discussed the result of SPI and the rest of the samples separately. SPI is known for its exceptional water holding capacity (9.2 g water/g SPI, 2 % w/v dispersion in water) (Peng et al., 2020c) as compared to other proteins such as SPC (7.2 g water/g SPC, 2 % w/v dispersion in water) (Peng, Kyriakopoulou, Ndiaye, et al., 2021a). Such high water holding capacity was also reflected in viscosity since SPI had the highest viscosity (35 Pa s at shear rate of 1 s^{-1}) among all test proteins (Fig. 5a). At room temperature, SPI dispersion in water exhibited a cold-set gel like behavior, which was unique among all test dispersions (data not shown). This gel (highly likely formed through swollen protein aggregates forming a network) was weakened upon protein hydrolysis, leading to fewer swollen aggregates (Fig. 4a) and subsequently lower viscosity. Similar results were reported where the viscosity of soy protein decreased significantly at DH of 3 % and remained stable at higher DH (Nielsen, 2009).

The viscosities of the rest of the plant proteins (except SPI and PPI) increased (to somewhat different extent) upon partial hydrolysis (Fig. 5 b and d – j). A higher viscosity originates from more interactions and the formation of a network. Similar results were reported for whey proteins where its viscosity continuously increased from DH of 4 %–6 % (Nielsen, 2009). Plant proteins however consist not only of soluble proteins (like whey proteins), but also of insoluble proteins in the form of dense or swollen particles. Interestingly, although we observed different effects for different plant proteins on their protein dispersibility and particle size upon hydrolysis, the net effect on viscosity seemed to be consistent (for all proteins except SPI and PPI). When dispersed in both water and TLJ, all plant protein dispersions showed (to somewhat different extent) shear-thinning behavior as function of shear rate (Supplementary material Figure S2), indicating that the interaction and network was broken at higher shear rate.

3.5. Less material required for gelation with (some) gels exhibiting higher gel strength

As the final step, the effect of TLJ proteases on plant proteins was investigated in terms of least gelling concentration and rheological properties, since these properties are influenced by changes at both molecular and colloidal scale. The least gelling concentration results are presented in Fig. 6. When dispersed in water, high concentrations of material was required to form self-standing gels that did not slip or break upon inversion at pH 7. For example, 12 % (w/v) of SPI was required to form such gel, which was the lowest gelling concentration among all plant proteins. Notably, an reduction in the least gelling concentration was consistently observed for all plant proteins when dispersed in TLJ (Fig. 6). For example, the lowest gelling concentration among all plant proteins was achieved at 6 % (w/v) for PPC and MPC, as compared to the corresponding control samples dispersed in water (15 % (w/v) in both cases). This finding is in-line with our previous study in which we found

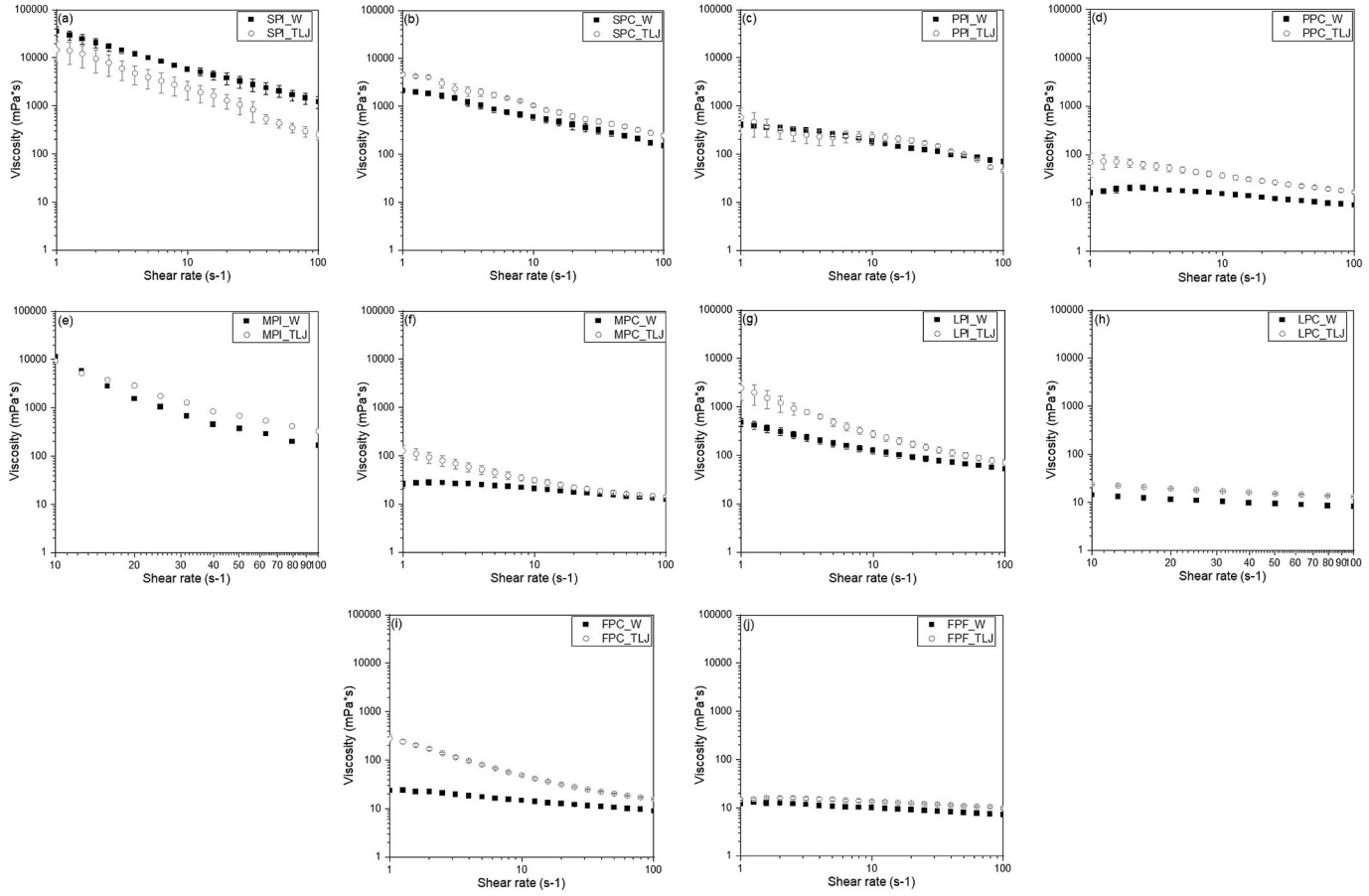

Fig. 5. Viscosity as a function of shear rate (at 20 °C) of plant proteins (a–j) dispersed in water (W) and tomato leaf juice (TLJ) after incubation (15 % w/v, room temperature, 1 h, 40 rpm).

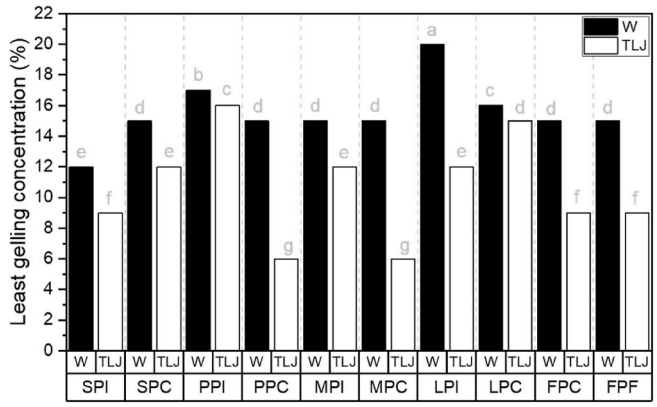

Fig. 6. Least gelling concentration of plant proteins dispersed in water (W) and tomato leaf juice (TLJ) after incubation (room temperature, 1 h, 40 rpm) and subsequent heating (95 °C, 30 min). Significant differences between all tested samples at $P \leq 0.05$ are indicated as small upper letters.

that less material was required for whey protein isolates to form a self-standing gel when dissolved in TLJ (3 % w/v, pH 7) as compared to water (15 % w/v, pH 7) (Yu et al., 2024). It is believed that partial hydrolysis of proteins leads to exposure of hydrophobic groups, which leads to additional interaction points between hydrolyzed products (e.g., peptides) and intact proteins (Creusot & Gruppen, 2007). These interactions then lead to the formation of aggregates (Kuipers & Gruppen, 2008; Otte et al., 1997), which can be further promoted upon heating and therefore results in gel formation (Chen & Campanella, 2022). It is interesting to point out that the extent of this effect varied among different proteins despite of the general reduction in the least gelling concentration. For example, 3 % and 1 % reduction in the least gelling concentration was observed for SPI and PPI, while 8 % was observed for LPI. Such difference could be attributed to different enzyme specificity to different plant proteins (Creusot et al., 2006; Ju et al., 1995) (Fig. 1).

The rheological properties of plant proteins dispersed in water and TLJ are presented in Fig. 7 and Supplementary material Table S1. Upon heating (20–95 °C), a consistent increase of G' was observed for almost all the plant proteins (except for SPI in water), regardless of the dispersant. Such increase of G' was likely attributed to protein denaturation and aggregation (Renkema & Van Vliet, 2002). The exceptional decrease in G' for SPI in water (Fig. 7a) was probably due to the fact that SPI was already mostly denatured before heating, leading to the lack of ability to form additional interactions and network upon heating (Li et al., 2007; Peng, Kyriakopoulou, Rahmani, et al., 2021b). Remarkably, an increase of G' was observed for SPI dispersed in TLJ, suggesting that the hydrolyzed products could form network upon heating, despite of the lower viscosity (Fig. 5a). On an overall level, higher G' values during the initial heating stage were observed for almost all plant proteins in TLJ compared to in water (Fig. 7a–c and f – j). Two exceptions were PPC and MPI, where the G' values of gel formed with TLJ were similar to that with water. The overall higher G' values indicated formation of additional interactions as a result of partial hydrolysis.

During the temperature sweep, both protein gels formed with water and TLJ showed solid-like behavior, as evidenced by the tan δ value, which is defined as the ratio of G'' and G' at the end of temperature sweep (equation (3)). When tan δ is less than 1, material exhibits solid-like behavior (Schurz, 1967). Protein gels formed with water and TLJ had tan δ values in the range of 0.17–0.64, confirming their solid-like texture (Supplementary material Table S1). When focusing on the last G' value at the end of the temperature sweep, a significant higher G' value was observed for SPC, PPI and MPC gels made with TLJ (2.8 kPa, 2.1 kPa and 4.6 kPa, respectively) than that with water (0.7 kPa, 0.2 kPa and 0.3 kPa, respectively) (Fig. 7b, c and f). A higher G' value suggests a higher gel strength. This finding aligns with other research where partial hydrolysis resulted in (to somewhat different extent) improved gel strength for different proteins, such as pea protein isolate (100 Pa vs. 4 Pa) (Chen & Campanella, 2022), quinoa protein isolate (100 Pa vs. 5 Pa) (Wang et al., 2022) and peanut protein isolate (374 Pa vs. 3.5 Pa, DH 2.1 %) (Zhao et al., 2011). It is interesting to note that despite less material was required for all plant proteins to make a self-standing gel with TLJ, not all TLJ gels showed higher gel strength than the corresponding water gels, even for proteins from the same crop. This suggests that indeed more interactions were formed upon partial hydrolysis, leading to a net effect on the reduction of the least gelling concentration. However, the interactions might be weak since at the same gelling concentration not all plant proteins showed an improved gel strength. To improve the functionality of proteins by enzymatic hydrolysis, the key is to control the degree of hydrolysis. For different plant proteins, the required degree of hydrolysis for improved functionality might differ (Mookerjee & Tanaka, 2023). Therefore, it is interesting to further investigate the optimal hydrolysis conditions (e.g., time, temperature and enzyme-to-substrate ratio) to achieve better gelling properties (i.e., lower gelling concentration and higher gel strength) for each plant protein tested.

After the temperature sweep, we performed a shear strain sweep where the rheological stability of a gel against large oscillatory deformation at a constant frequency and temperature was determined. From such test, we derived two parameters being the critical strain (γ_c) and the crossover strain ($\gamma_{G' = G''}$). The former was defined as the shear strain at the end of linear viscoelastic regime where the measured G′ value deviated 5 % from the first G′ value (Schlangen et al., 2022). After this point, the initial gel structure is interrupted. The latter was defined as the last shear strain point where the measured G' value was higher than the G'' value. These two parameters together indicate how well a gel material can withstand deformation. When focusing on the γ_c values, only SPI gel with TLJ (0.14 %) showed a significant lower value as compared to the corresponding water gel (6.37 %), while no significant differences were observed for other proteins (Supplementary material Table S1). A lower γ_c value indicated that the SPI gel with TLJ was easier to disrupt. When focusing on the $\gamma_{G' = G''}$ values, significant lower values were observed for SPI, SPC, PPI, LPI and FPF gels made with TLJ than the corresponding water gels (Supplementary material Table S1). In combination with the γ_c results, one can interpret that materials with lower γ_c and $\gamma_{G' = G''}$ values had more brittle texture that yielded sooner. Similar result was also found in WPI gel made with TLJ and water (γ_c: 1.47 % vs. 5.04 % and $\gamma_{G' = G''}$: 88 % vs. 344 %, respectively) (Yu et al., 2024).

3.6. Reflection on the utilization of tomato leaves as enzyme source

Our research demonstrated the proteolytic activity in TLJ on a selected number of plant proteins. These proteins were dispersed in TLJ and after 1 h incubation at room temperature, the hydrolysis was evident on molecular scale for all tested proteins. Additionally, the hydrolysis was selective and not complete (partial) for each dispersion. The partial hydrolysis led to significant changes at colloidal scale and improved gelation behavior (lower gelling concentration). Some of the formed gels with hydrolyzed proteins also exhibited higher gel strength than the gels formed with intact proteins. The current research is a continuation of our previous research on the effect of the endogenous proteases in TLJ on the functionality of whey protein isolates (Yu et al., 2024). The findings of these two studies demonstrated the potential of utilizing tomato leaves as enzyme source, in particular as a source of proteases. The advantage of using TLJ as enzyme source is that the enzymes remain stable and active under frozen conditions. The diversity of proteases in the juice ensures its applicability to various types of dairy and plant proteins. Additionally, it allows the utilization of TLJ as a whole product, instead of only fractionating a small part of the juice. In comparison to the commercial enzyme source, the use of enzymes from TLJ might have less environmental impact since no purification and

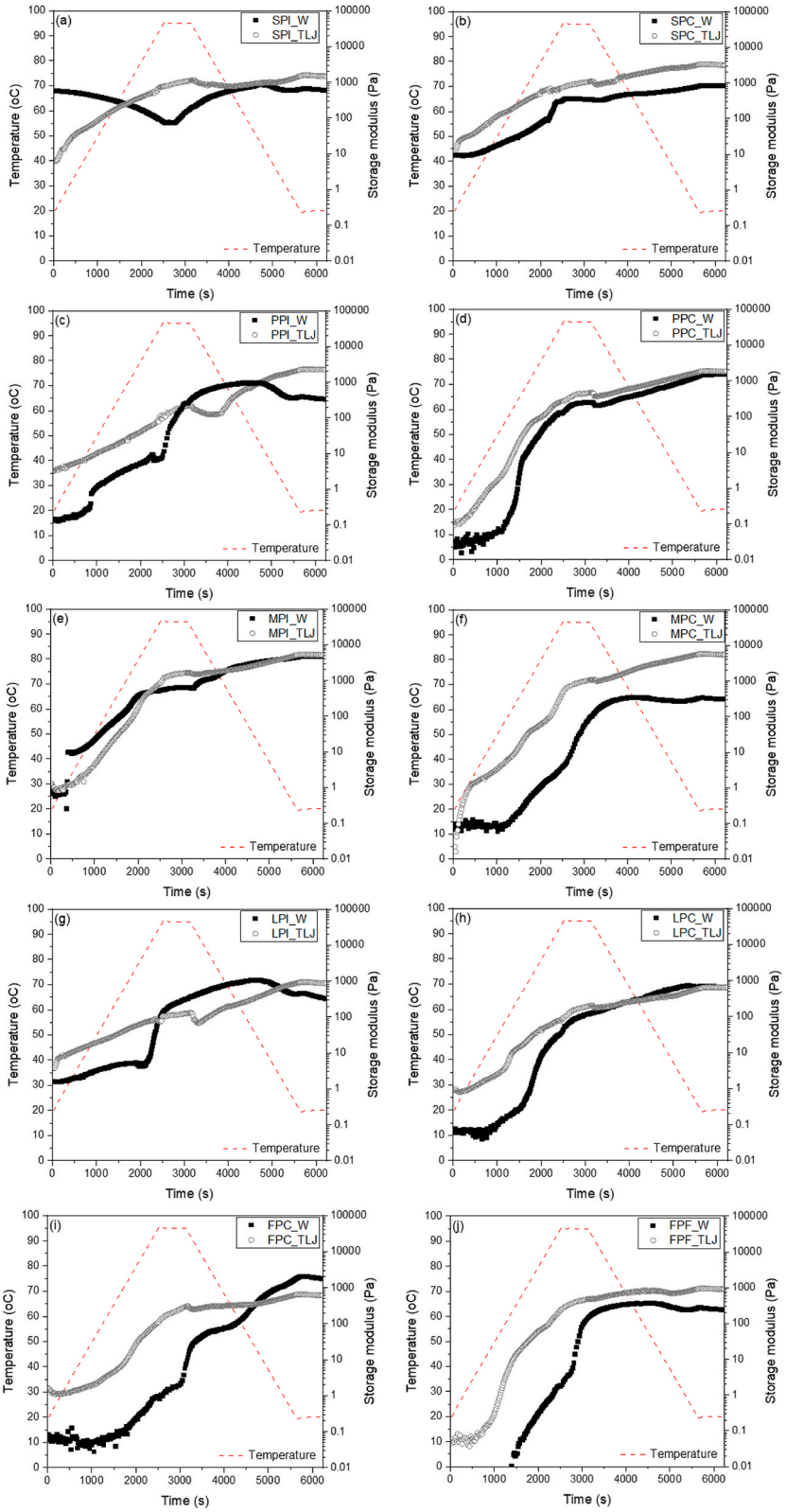

Fig. 7. Storage modulus (G′) during temperature sweep (20–95 °C, f = 1 Hz, •γ = 1%) of plant proteins (a–j) dispersed in water (W) and tomato leaf juice (TLJ) after incubation (15 % w/v, room temperature, 1 h, 40 rpm).

concentration (e.g., drying) are applied in the process. Further research can be conducted to investigate the optimal hydrolysis conditions (e.g., time, temperature and enzyme-to-substrate ratio) to achieve optimal functionalities for different proteins.

There are a few aspects to consider when using tomato leaves as a source of proteases for future industrial application. Firstly, tomato leaves are not a common food source. One of the reasons explaining this is the presence of toxic glycoalkaloids, specifically tomatine and dehydrotomatine (Kozukue et al., 2004), which are more concentrated in tomato leaves than fruits (Friedman, 2004). During the processing of

tomato leaves, these toxins distributed in all obtained fractions (Liese et al., 2023) and hence they will be present in TLJ. It is therefore important to quantify the level of these toxins in future applications containing TLJ and evaluate the risk upon human consumption. So far, EFSA has not published human risk assessment for tomato glycoalkaloids due to lack of data (Schrenk et al., 2020). Another approach is to test the proteolytic activity in juices obtained from edible leaves like spinach and lettuce. The second aspect to consider is how to apply TLJ. The most similar food application is high protein drinks that contain 15 % dry matter. Here, extensive hydrolysis could lower the viscosity. Subtle changes in viscosity and gel strength might lead to a novel texture and mouthfeel. In addition, proteases from other natural sources such as red seaweed (Arbita et al., 2020) and kiwi fruit (Mazorra-Manzano et al., 2013) have been used to coagulate milk proteins, hence demonstrating their ability to replace calf rennet. Given the fact that TLJ proteases had similar level of activity as these enzymes (Section 3.1), TLJ proteases can be thus potentially used as cheese coagulant as well.

CRediT authorship contribution statement

Yafei Yu: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marietheres Kleuter:** Writing – review & editing, Resources. **Luisa M. Trindade:** Writing – review & editing, Resources. **Atze Jan van der Goot:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2024.110245.

References

Abo Bakr, T. M., Mohamed, M. S., & Moustafa, E. K. (1982). Leaf protein isolates from some Egyptian crops. *Food Chemistry, 9*(4), 295–301. https://doi.org/10.1016/0308-8146(82)90081-4

Altartouri, H., Abu Maizer, A., Idais, A., Tamimi, H., & Arafeh, R. (2015). Computerized extraction of morphological and geometrical features for plants with compound leaves. *Journal of Theoretical and Applied Information Technology, 81*(3), 474–480. https://www.researchgate.net/publication/282002103.

Anson, M. L. (1938). The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *Journal of General Physiology, 22*(1), 79–89. https://doi.org/10.1085/jgp.22.1.79

Arbita, A. A., Paul, N. A., Cox, J., & Zhao, J. (2020). Extraction, partial purification and characterization of proteases from the red seaweed Gracilaria edulis with similar cleavage sites on κ-casein as calf rennet. *Food Chemistry, 330*, Article 127324. https://doi.org/10.1016/j.foodchem.2020.127324

Berghout, J. A. M., Boom, R. M., & Van Der Goot, A. J. (2014). The potential of aqueous fractionation of lupin seeds for high-protein foods. *Food Chemistry, 159*, 64–70. https://doi.org/10.1016/j.foodchem.2014.02.166

Bühler, J. M., Dekkers, B. L., Bruins, M. E., & Van Der Goot, A. J. (2020). Modifying faba bean protein concentrate using dry heat to increase water holding capacity. *Foods, 9*(8), 1077–1093. https://doi.org/10.3390/foods9081077

Bühler, J. M., van der Goot, A. J., & Bruins, M. E. (2022). Quantifying water distribution between starch and protein in doughs and gels from mildly refined faba bean fractions. *Current Research in Food Science, 5*, 735–742. https://doi.org/10.1016/j.crfs.2022.03.013

Chen, D., & Campanella, O. H. (2022). Limited enzymatic hydrolysis induced pea protein gelation at low protein concentration with less heat requirement. *Food Hydrocolloids, 128*, Article 107547. https://doi.org/10.1016/J.FOODHYD.2022.107547

Coffmann, C. W., & Garciaj, V. V. (1977). Functional properties and amino acid content of a protein isolate from mung bean flour. *International Journal of Food Science and Technology, 12*(5), 473–484. https://doi.org/10.1111/j.1365-2621.1977.tb00132.x

Creusot, N., & Gruppen, H. (2007). Hydrolysis of whey protein isolate with Bacillus licheniformis protease: Fractionation and identification of aggregating peptides. *Journal of Agricultural and Food Chemistry, 55*(22), 9241–9250. https://doi.org/10.1021/jf071584s

Creusot, N., & Gruppen, H. (2008). Hydrolysis of whey protein isolate with Bacillus licheniformis protease: Aggregating capacities of peptide fractions. *Journal of Agricultural and Food Chemistry, 56*(21), 10332–10339. https://doi.org/10.1021/jf801422j

Creusot, N., Gruppen, H., van Koningsveld, G. A., de Kruif, C. G., & Voragen, A. G. J. (2006). Peptide-peptide and protein-peptide interactions in mixtures of whey protein isolate and whey protein isolate hydrolysates. *International Dairy Journal, 16*(8), 840–849. https://doi.org/10.1016/j.idairyj.2005.06.010

Crévieu, I., Carré, B., Chagneau, A. M., Quillien, L., Guéguen, J., & Bérot, S. (1997). Identification of resistant pea (pisum sativum L.) proteins in the digestive tract of chickens. *Journal of Agricultural and Food Chemistry, 45*(4), 1295–1300. https://doi.org/10.1021/jf960806b

Dent, T., Campanella, O., & Maleky, F. (2023). Enzymatic hydrolysis of soy and chickpea protein with Alcalase and Flavourzyme and formation of hydrogen bond mediated insoluble aggregates. *Current Research in Food Science, 6*, Article 100487. https://doi.org/10.1016/j.crfs.2023.100487

FAOSTAT. (n.d.). Retrieved September 14, 2023, from https://www.fao.org/faostat/en/#data/QCL.

Fernández-Gómez, M. J., Díaz-Raviña, M., Romero, E., & Nogales, R. (2013). Recycling of environmentally problematic plant wastes generated from greenhouse tomato crops through vermicomposting. *International journal of Environmental Science and Technology, 10*(4), 697–708. https://doi.org/10.1007/S13762-013-0239-7

Fiorentini, R., & Galoppini, C. (1983). The proteins from leaves. *Qualitas Plantarum Plant Foods for Human Nutrition, 32*(3–4), 335–350. https://doi.org/10.1007/BF01091193

Friedman, M. (2004). Analysis of biologically active compounds in potatoes (Solanum tuberosum), tomatoes (Lycopersicon esculentum), and jimson weed (Datura stramonium) seeds. *Journal of Chromatography A, 1054*(1–2), 143–155. https://doi.org/10.1016/j.chroma.2004.04.049

Gill, S. E., & Parks, W. C. (2008). Metalloproteinases and their inhibitors: Regulators of wound healing. *The International Journal of Biochemistry & Cell Biology, 40*(6–7), 1334–1347. https://doi.org/10.1016/J.BIOCEL.2007.10.024

Gong, M., Mohan, A., Gibson, A., & Udenigwe, C. C. (2015). Mechanisms of plastein formation, and prospective food and nutraceutical applications of the peptide aggregates. *Biotechnology Reports, 5*(1), 63–69. https://doi.org/10.1016/j.btre.2014.12.003

Grasberger, K., Hammershøj, M., & Corredig, M. (2023). Stability and viscoelastic properties of mixed lupin-whey protein at oil-water interfaces depend on mixing sequence. *Food Hydrocolloids, 138*, Article 108485. https://doi.org/10.1016/j.foodhyd.2023.108485

Holt, N. W., & Sosulski, F. W. (1979). Amino acid composition and protein quality of field peas. *Canadian Journal of Plant Science, 59*(3), 653–660. https://doi.org/10.4141/cjps79-103

Iwabuchi, S., & Yamauchi, F. (1987). Electrophoretic analysis of whey proteins present in soybean globulin fractions. *Journal of Agricultural and Food Chemistry, 35*(2), 205–209. https://doi.org/10.1021/jf00074a010

Ju, Z. Y., Otte, J., Madsen, J. S., & Qvist, K. B. (1995). Effects of limited proteolysis on gelation and gel properties of whey protein isolate. *Journal of Dairy Science, 78*(10), 2119–2128. https://doi.org/10.3168/JDS.S0022-0302(95)76839-4

Kiskini, A., Vissers, A., Vincken, J. P., Gruppen, H., & Wierenga, P. A. (2016). Effect of plant age on the quantity and quality of proteins extracted from sugar beet (beta vulgaris L.) leaves. *Journal of Agricultural and Food Chemistry, 64*(44), 8305–8314. https://doi.org/10.1021/acs.jafc.6b03095

Kornet, R., Veenemans, J., Venema, P., van der Goot, A. J., Meinders, M., Sagis, L., & van der Linden, E. (2021). Less is more: Limited fractionation yields stronger gels for pea proteins. *Food Hydrocolloids, 112*, Article 106285. https://doi.org/10.1016/j.foodhyd.2020.106285

Kozukue, N., Han, J. S., Lee, K. R., & Friedman, M. (2004). Dehydrotomatine and α-tomatine content in tomato fruits and vegetative plant tissues. *Journal of Agricultural and Food Chemistry, 52*(7), 2079–2083. https://doi.org/10.1021/jf0306845

Krul, E. S. (2019). Calculation of nitrogen-to-protein conversion factors: A review with a focus on soy protein. *JAOCS, Journal of the American Oil Chemists' Society, 96*(4), 339–364. https://doi.org/10.1002/aocs.12196

Kuipers, B. J. H., & Gruppen, H. (2008). Identification of strong aggregating regions in soy glycinin upon enzymatic hydrolysis. *Journal of Agricultural and Food Chemistry, 56*(10), 3818–3827. https://doi.org/10.1021/jf703781j

Li, J. Y., Yeh, A. I., & Fan, K. L. (2007). Gelation characteristics and morphology of corn starch/soy protein concentrate composites during heating. *Journal of Food Engineering, 78*(4), 1240–1247. https://doi.org/10.1016/j.jfoodeng.2005.12.043

Lie-Piang, A., Hageman, J., Vreenegoor, I., van der Kolk, K., de Leeuw, S., van der Padt, A., & Boom, R. (2023). Quantifying techno-functional properties of ingredients from multiple crops using machine learning. *Current Research in Food Science, 7*, Article 100601. https://doi.org/10.1016/j.crfs.2023.100601

Liese, H. W., Valkenburg, T. A. A., America, A. H. P., Scholten, E., & Bruins, M. E. (2023). Toxin removal during protein extraction from tomato leaves. *Innovative Food Science and Emerging Technologies, 88*, Article 103454. https://doi.org/10.1016/j.ifset.2023.103454

Mariotti, F., Tomé, D., & Mirand, P. P. (2008). Converting nitrogen into protein - beyond 6.25 and Jones' factors. *Critical Reviews in Food Science and Nutrition, 48*(2), 177–184. https://doi.org/10.1080/10408390701279749

Martin, A. H., Nieuwland, M., & De Jong, G. A. H. (2014). Characterization of heat-set gels from RuBisCO in comparison to those from other proteins. *Journal of Agricultural and Food Chemistry, 62*(44), 10783–10791. https://doi.org/10.1021/jf502905g

Mazorra-Manzano, M. A., Perea-Gutiérrez, T. C., Lugo-Sánchez, M. E., Ramirez-Suarez, J. C., Torres-Llanez, M. J., González-Córdova, A. F., & Vallejo-Cordoba, B. (2013). Comparison of the milk-clotting properties of three plant extracts. *Food Chemistry, 141*(3), 1902–1907. https://doi.org/10.1016/j.foodchem.2013.05.042

Messdaghi, D., & Dietz, K. J. (2000). Characterization of an extracellular chymostatin-sensitive serine protease preferentially expressed in young plant tissues. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 1480*(1–2), 107–116. https://doi.org/10.1016/S0167-4838(00)00092-3

Milton, K., & Dintzis, F. R. (1981). Nitrogen-to-Protein conversion factors for tropical plant samples. *Association for Tropical Biology and Conservation, 13*(3), 177–181. https://doi.org/10.2307/2388122

Mokni Ghribi, A., Maklouf Gafsi, I., Sila, A., Blecker, C., Danthine, S., Attia, H., Bougatef, A., & Besbes, S. (2015). Effects of enzymatic hydrolysis on conformational and functional properties of chickpea protein isolate. *Food Chemistry, 187*, 322–330. https://doi.org/10.1016/j.foodchem.2015.04.109

Möller, A. C., Li, J., van der Goot, A. J., & van der Padt, A. (2022). A water-only process to fractionate yellow peas into its constituents. *Innovative Food Science & Emerging Technologies, 75*, Article 102894. https://doi.org/10.1016/J.IFSET.2021.102894

Mookerjee, A., & Tanaka, T. (2023). Influence of enzymatic treatments on legume proteins for improved functional and nutritional properties: Expansion of legume protein utilization as food ingredients. *Current Opinion in Food Science, 49*, Article 100974. https://doi.org/10.1016/J.COFS.2022.100974

Nguyen, Q., Hettiarachchy, N., Rayaprolu, S., Jayanthi, S., Thallapuranam, S., & Chen, P. (2016). Physicochemical properties and ACE-I inhibitory activity of protein hydrolysates from a non-genetically modified soy cultivar. *JAOCS, Journal of the American Oil Chemists' Society, 93*(4), 595–606. https://doi.org/10.1007/s11746-016-2801-1

Nielsen, P. M. (2009). Enzymes in protein modification. In *Enzymes in food technology* (2nd ed.). https://doi.org/10.1002/9781444309935.ch13

Nieuwland, M., Geerdink, P., Engelen-Smit, N. P. E., van der Meer, I. M., America, A. H. P., Mes, J. J., Kootstra, A. M. J., Henket, J. T. M. M., & Mulder, W. J. (2021). Isolation and gelling properties of duckweed protein concentrate. *ACS Food Science and Technology, 1*(5), 908–916. https://doi.org/10.1021/acsfoodscitech.1c00009

Ono, S., Kasai, D., Sugano, T., Ohba, K., & Takahashi, K. (2004). Production of water soluble antioxidative plastein from squid hepatopancreas. *Journal of Oleo Science, 53*(5), 267–273. https://doi.org/10.5650/jos.53.267

Otte, J., Lomholt, S. B., Ipsen, R., Stapelfeldt, H., Bukrinsky, J. T., & Qvist, K. B. (1997). Aggregate Formation during hydrolysis of β-lactoglobulin with a glu and asp specific protease from Bacillus licheniformis. *Journal of Agricultural and Food Chemistry, 45*(12), 4889–4896. https://doi.org/10.1021/jf9704571

Peng, Y., Kersten, N., Kyriakopoulou, K., & van der Goot, A. J. (2020c). Functional properties of mildly fractionated soy protein as influenced by the processing pH. *Journal of Food Engineering, 275*, Article 109875. https://doi.org/10.1016/J.JFOODENG.2019.109875

Peng, Y., Kyriakopoulou, K., Ndiaye, M., Bianeis, M., Keppler, J. K., & van der Goot, A. J. (2021a). Characteristics of soy protein prepared using an aqueous ethanol washing process. *Foods, 10*(9), 2222. https://doi.org/10.3390/foods10092222

Peng, Y., Kyriakopoulou, K., Rahmani, A., Venema, P., & van der Goot, A. J. (2021b). Isochoric moisture heating as a tool to control the functionality of soy protein. *Lebensmittel-Wissenschaft & Technologie, 150*, Article 111979. https://doi.org/10.1016/j.lwt.2021.111979

Raghunath, M. R., & McCurdy, A. R. (1991). Synthesis of plasteins from fish silage. *Journal of the Science of Food and Agriculture, 54*(4), 655–658. https://doi.org/10.1002/jsfa.2740540417

Renkema, J. M. S., & Van Vliet, T. (2002). Heat-induced gel formation by soy proteins at neutral pH. *Journal of Agricultural and Food Chemistry, 50*(6), 1569–1573. https://doi.org/10.1021/jf010763l

Rubio, L. A., Pérez, A., Ruiz, R., Guzmán, M.Á., Aranda-Olmedo, I., & Clemente, A. (2014). Characterization of pea (Pisum sativum) seed protein fractions. *Journal of the Science of Food and Agriculture, 94*(2), 280–287. https://doi.org/10.1002/jsfa.6250

Santamaría-Fernández, M., & Lübeck, M. (2020). Production of leaf protein concentrates in green biorefineries as alternative feed for monogastric animals. *Animal Feed Science and Technology, 268*, Article 114605. https://doi.org/10.1016/j.anifeedsci.2020.114605

Scalet, M., Alpi, A., & Picciarelli, P. (1984). Proteolytic activities in alfalfa (medicago sativa L.) leaves. *Journal of Plant Physiology, 116*(2), 133–145. https://doi.org/10.1016/S0176-1617(84)80070-X

Schlangen, M., Raak, N., Dinani, S. T., Corredig, M., & van der Goot, A. J. (2023). How fractionation procedure of mung bean protein affects transglutaminase crosslinking. *Food Hydrocolloids, 145*, Article 109067. https://doi.org/10.1016/j.foodhyd.2023.109067

Schlangen, M., Taghian Dinani, S., Schutyser, M. A. I., & van der Goot, A. J. (2022). Dry fractionation to produce functional fractions from mung bean, yellow pea and cowpea flour. *Innovative Food Science and Emerging Technologies, 78*, Article 103018. https://doi.org/10.1016/j.ifset.2022.103018

Schrenk, D., Bignami, M., Bodin, L., Chipman, J. K., del Mazo, J., Hogstrand, C., Hoogenboom, L., Leblanc, J. C., Nebbia, C. S., Nielsen, E., Ntzani, E., Petersen, A., Sand, S., Schwerdtle, T., Vleminckx, C., Wallace, H., Brimer, L., Cottrill, B., Dusemund, B., … Grasl-Kraupp, B. (2020). Risk assessment of glycoalkaloids in feed and food, in particular in potatoes and potato-derived products. *EFSA Journal, 18*(8), 6222. https://doi.org/10.2903/j.efsa.2020.6222

Schurz, J. (1967). Rheological methods. In *Polymer fractionation* (pp. 317–339). Academic Press. https://doi.org/10.1016/B978-1-4832-3245-4.50016-9.

Sun, H., & Zhao, X.-H. (2012). Angiotensin I converting enzyme inhibition and enzymatic resistance in vitro of casein hydrolysate treated by plastein reaction and fractionated with ethanol/water or methanol/water. *International Dairy Journal, 24*(1), 27–32. https://doi.org/10.1016/j.idairyj.2011.11.012

Tamayo Tenorio, A., Kyriakopoulou, K. E., Suarez-Garcia, E., van den Berg, C., & van der Goot, A. J. (2018). Understanding differences in protein fractionation from conventional crops, and herbaceous and aquatic biomass - consequences for industrial use. *Trends in Food Science and Technology, 71*, 235–245. https://doi.org/10.1016/j.tifs.2017.11.010

Tarhan, O., Spotti, M. J., Schaffter, S., Corvalan, C. M., & Campanella, O. H. (2016). Rheological and structural characterization of whey protein gelation induced by enzymatic hydrolysis. *Food Hydrocolloids, 61*, 211–220. https://doi.org/10.1016/j.foodhyd.2016.04.042

Taylor, M. A., & Fraser, P. D. (2011). Solanesol: Added value from solanaceous waste. *Phytochemistry, 72*(11–12), 1323–1327. https://doi.org/10.1016/j.phytochem.2011.03.015

Wang, X., Cheng, L., Wang, H., & Yang, Z. (2022). Limited Alcalase hydrolysis improves the thermally-induced gelation of quinoa protein isolate (QPI) dispersions. *Current Research in Food Science, 5*, 2061–2069. https://doi.org/10.1016/J.CRFS.2022.10.027

Wang, Y. T., Yang, C. Y., Chen, Y. T., Lin, Y., & Shaw, J. F. (2004). Characterization of senescence-associated proteases in postharvest broccoli florets. *Plant Physiology and Biochemistry, 42*(7–8), 663–670. https://doi.org/10.1016/j.plaphy.2004.06.003

Warsame, A. O., O'Sullivan, D. M., & Tosi, P. (2018). Seed storage proteins of faba bean (Vicia faba L): Current status and prospects for genetic improvement. *Journal of Agricultural and Food Chemistry, 66*(48), 12617–12626. https://doi.org/10.1021/acs.jafc.8b04992

Yu, Y., Kleuter, M., America, A. H. P., Trindade, L. M., & van der Goot, A. J. (2024). Tomato (Solanum lycopersicum) leaf juice induced whey protein gelling: Unveiling the potential of endogenous proteases in novel applications. *Food Hydrocolloids, 151*, Article 109812. https://doi.org/10.1016/j.foodhyd.2024.109812

Yu, Y., Kleuter, M., de Ruijter, N. C. A., Dinani, S. T., Trindade, L. M., & van der Goot, A. J. (2023). An in-depth analysis of initial processing steps in the extraction of proteins from tomato (Solanum lycopersicum) leaves. *Innovative Food Science & Emerging Technologies, 87*, Article 103424. https://doi.org/10.1016/J.IFSET.2023.103424

Yu, Y., Kleuter, M., Taghian Dinani, S., Trindade, L. M., & Jan van der Goot, A. (2022). The role of plant age and leaf position on protein extraction and phenolic compounds removal from tomato (Solanum lycopersicum) leaves using food-grade solvents. *Food Chemistry, 406*, Article 135072. https://doi.org/10.1016/J.FOODCHEM.2022.135072

Yust, M. del M., Pedroche, J., Millán-Linares, M. del C., Alcaide-Hidalgo, J. M., & Millán, F. (2010). Improvement of functional properties of chickpea proteins by hydrolysis with immobilised Alcalase. *Food Chemistry, 122*(4), 1212–1217. https://doi.org/10.1016/j.foodchem.2010.03.121

Zhao, G., Liu, Y., Zhao, M., Ren, J., & Yang, B. (2011). Enzymatic hydrolysis and their effects on conformational and functional properties of peanut protein isolate. *Food Chemistry, 127*(4), 1438–1443. https://doi.org/10.1016/j.foodchem.2011.01.046