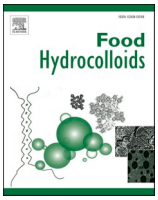
Food Hydrocolloids 151 (2024) 109812

Contents lists available at ScienceDirect

Food Hydrocolloids

journal homepage: www.elsevier.com/locate/foodhyd



Tomato (*Solanum lycopersicum*) leaf juice induced whey protein gelling: Unveiling the potential of endogenous proteases in novel applications

Yafei Yu [a], Marietheres Kleuter [b], Antoine H.P. America [c], Luisa M. Trindade [b], Atze Jan van der Goot [a,*]

[a] Laboratory of Food Process Engineering, Wageningen University, PO Box 17, 6700, AA, Wageningen, the Netherlands
[b] Plant Breeding, Wageningen University, Droevendaalsesteeg 1, 6708, PB, Wageningen, the Netherlands
[c] BU Bioscience, Wageningen Plant Research, Wageningen, 6700, AP, the Netherlands

ARTICLE INFO

Keywords:
Green biomass
Agricultural by-products
Tomato leaves
Plant proteins
Enzymes
Inhibition

ABSTRACT

Tomato leaves are by-products of tomato production and a potential source of proteins. Part of these proteins are proteases that carry out important functions for the plants. These endogenous proteases can bring challenges in downstream protein extraction, and can alter protein functionality. The aim of this study was to characterize the endogenous proteases in tomato leaf juice and to investigate the hydrolysis by these proteases on both endogenous leaf proteins and WPI. In addition, we characterized the gelation behavior, rheological properties and structural and thermal properties change of WPI by the juice proteases. 38 different proteases were identified in the juice, along with 4 protease inhibitors. The proteases hydrolyzed both endogenous leaf proteins and WPI, with predominantly an endopeptidase manner for the latter. The protein hydrolysis was positively affected by the incubation time and temperature. The protease activity in juice could not be fully eliminated by heat deactivation or the addition of protease inhibitors. The protein hydrolysis however remained limited (max. 43 %) under all conditions below 60 °C. This hydrolysis resulted in small changes in both secondary structure and thermal properties of WPI. Limited hydrolysis led to WPI gelation at lower protein concentration. Gel formed with WPI after limited hydrolysis had higher gel strength and more brittle structure than the gel with intact proteins. To conclude, the complex and active nature of proteases in tomato leaves poses challenge for downstream protein extraction. Alternatively, juice from tomato leaves could be used as natural gelling agent for WPI.

1. Introduction

Tomato plants are widely cultivated for their fruits, both on open fields and in greenhouses. On a global scale, there are around two million hectares of greenhouses (Pardossi et al., 2004). Among all greenhouse plants, tomato is the most commonly grown vegetable plant (Alkoaik & Ghaly, 2006). Tomato fruits however only represent about 60 % of the total plant mass (Taylor & Fraser, 2011). The production of tomato fruit in the greenhouses therefore generates a lot of by-products, being estimated up to about 49 ton/ha/year, which mainly consists of leaves and stems. This number is almost two times higher than that of the by-products (28.5 ton/ha/year) produced by other plants in greenhouses (Fernández-Gómez et al., 2013). Tomato leaves contain 70 % of organic matter (Alkoaik & Ghaly, 2006) and particularly up to 30 % of proteins (Abo Bakr et al., 1982; Yu et al., 2022). After the harvest of fruits, tomato leaves are usually discarded (Fernández-Gómez et al., 2013), resulting in a loss of valuable components present in the leaves.

To extract proteins from tomato leaves, several challenges need to be addressed. The first challenge is that there are distinctive differences between the type of proteins present in leaves and those present in other seed crops, such as soy, pea and other legumes. As described by Tamayo Tenorio et al., (2018), proteins present in the seed crops are mainly storge proteins, in the form of protein bodies, which are fairly uniform and stable (Fiorentini & Galoppini, 1983). The proteins in leaves are however heterogenous, as they are mostly functional proteins that carry different roles for the plants, including photosynthesis, enzymatic activity, structural support, defense mechanism, transport and signaling (Fiorentini & Galoppini, 1983). Additionally, leaf proteins are changing upon aging. For instance, previous research demonstrated a reduction of net protein content in tomato leaves as a function of plant age and leaf

* Corresponding author. PO Box 17, 6700, AA, Wageningen, the Netherlands.
E-mail addresses: yafei.yu-woudstra@wur.nl (Y. Yu), marietheres.kleuter@wur.nl (M. Kleuter), twan.america@wur.nl (A.H.P. America), luisa.trindade@wur.nl (L.M. Trindade), atzejan.vandergoot@wur.nl (A.J. van der Goot).

https://doi.org/10.1016/j.foodhyd.2024.109812
Received 22 September 2023; Received in revised form 16 January 2024; Accepted 23 January 2024
Available online 24 January 2024

position, which was suspected to be linked with leaf senescence (Yu et al., 2022). Leaf senescence is a process where predominantly intact proteins are degraded to peptides and free amino acids in aging leaves. These degraded products are then transferred to provide other organs such as fruits and young vegetative tissue with additional nutrients (Havé et al., 2017). This process compromises protein yield and leads to co-extraction of degraded products such as peptides and amino acids (Yu et al., 2022, 2023). Proteases play a crucial role in leaf senescence as they break down proteins to facilitate the recycling of amino acids. Furthermore, proteases in plants are also involved in other functions, such as plant defense mechanism, stress response, protein turnover and more (Messdaghi & Dietz, 2000; Pinedo et al., 2000).

The presence of proteases leads to another challenge for protein extraction from leaves, which is postharvest protein degradation (Wang et al., 2004). Such protein degradation, catalyzed by a group of active proteases (Scalet et al., 1984), happens during storage or processing, and results in loss of protein quality and lower protein recovery (Zhang et al., 2015). Several attempts have been made to inhibit the postharvest protein degradation by steam blanching and addition of inhibitors, acid and formaldehyde (Guo et al., 2007; Hadidi et al., 2019). So far, most of inhibition methods are limited by logistics and processing time (Guo et al., 2007). In addition, the use of inhibitors increases processing costs.

Nevertheless, the enzymatic hydrolysis by proteases, if controlled to be limited, has been shown to improve protein functionalities, including gelling capacity (Mookerjee & Tanaka, 2023). For example, limited hydrolysis of proteins resulted in gelation with less material or at lower temperature for whey proteins (Ju et al., 1995) and plant proteins such as pea proteins (Chen & Campanella, 2022), sunflower proteins (Sanchez & Burgos, 1997) and peanut proteins (Zhang et al., 2021). Gels formed with limited protein hydrolysis exhibited a higher storage modulus as compared to the unhydrolyzed proteins (Ju et al., 1995; Tarhan et al., 2016). Additionally, “milk-clotting” proteases from other biological material such as medicinal plant *Calotropis procera* (Kumar Dubey & Jagannadham, 2003), ginger (Gagaoua et al., 2015), green fig fruits (Afsharnezhad et al., 2019), red seaweed (Arbita et al., 2020), non-edible parts of fennel (Bey et al., 2018) and lettuce leaves (Lo Piero et al., 2002) have also been identified, demonstrated by their effects on caseins and potential as natural gelling agents for the cheese making industry.

To better understand the relationship between the presence of proteases and protein extraction from tomato leaves, we investigated the composition of proteases in tomato leaf juice and hydrolysis of both endogenous leaf proteins and WPI by these proteases. We further characterized the effect of juice proteases on the gelation behavior, rheological properties and structural and thermal properties change of WPI.

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). Similar growing conditions were applied as described by Yu et al. (2022 and 2023). Temperature and humidity in the greenhouse, the use of nutrition, pesticides and insecticides during the plant growth were summarized in Supplementary material Figure S1, Table S1 and S2, respectively. Leaves were harvested on 17th and 18th April 2023. The plants were approx. 2.5 m tall and contained red and green fruits. To minimize the time between leaf harvesting and processing, three to four plants were harvested in one batch. The plants were cut at the bottom of the stem, and the stem was removed from the rest of the leaves. The leaves therefore consisted of leaflets, petioles, rachises and petiolules (Altartouri et al., 2015). All leaves harvested in one batch was manually mixed and immediately transferred to labs for further processing. In total, 27 plants were harvested in 7 batches.

A commercial WPI (trade name BiPro) was purchased from Agropur Cooperative (Saint-Hubert, Longueil, Canada). 2-mercaptoethanol, sodium hydroxide, Pepstatin A, bovine serum albumin (BSA) (66.5 kDa), beta-lactoglobulin (β-lg) (36 kDa), alpha-lactalbumin (α-lg) (14 kDa), aprotinin (6.51 kDa), bacitracin (1.42 kDa), phenylalanine (165 Da), paraffin oil and cOmplete™ Protease Inhibitor Cocktail with and without EDTA were purchased from Sigma-Aldrich (Darmstadt, Germany). Sodium metabisulfite, formic acid, phosphoric acid, SDS, TCEP (tri-chloro-ethyl-phosphine), TEAB (tri-ethyl ammonium bicarbonate) and MS-grade trypsin and trifluoroacetic acid were purchased from Thermo Fisher Scientific (Ochten, the Netherlands). Acetonitrile (Ultra LC-MS grade) and methanol (HPLC grade) were 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 (Lunteren, The Netherlands). 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

The harvested leaves were juiced using an Angel Juicer II 7500 (Angel Juicers, Queensland, Australia) into a dark green juice and pulp (Tamayo Tenorio et al., 2016), the pulp was discarded. Afterwards, 10 % (w/w) sodium metabisulfite stock solution was added to the green juice to make a final concentration of 0.2 % (w/w). The mixture was then centrifuged at 15,000 g for 1 h at 18 °C. Supernatant was separated from the green pellet by pouring. Subsequently, the supernatant was filtered through a double-folded cheesecloth and divided into 50, 125 and 250 mL plastic tubes. The tubes were immediately stored at −20 °C until further analysis. The filtered supernatant was further referred to as juice that contained proteases in this study.

2.3. Compositional analysis

The protein content of WPI was measured with Dumas nitrogen combustion method (Yu et al., 2022) using a rapid N exceed® analyzer (Elementar, Langenselbold, Germany). About 150 mg of sample was weighed in a tin foil sheet and closed tightly without headspace. The sample was subsequently combusted at 900 °C in the presence of oxygen. L-aspartic acid was used as the standard. Each sample was measured in triplicates. The WPI had protein content of 91.46 ± 0.37 % with a N conversion factor of 6.25.

The dry matter content of juice was measured by the moisture loss after freeze drying with Epsilon 2-10D LSCplus (Martin Christ, Germany). The dry matter content of juice was 5.52 ± 0.4 %.

2.4. Protease composition

The protease composition in the juice was analyzed using Liquid Chromatography-Mass Spectrometry (LC-MS/MS). The frozen juice was thawed and well mixed at room temperature before the sample preparation. Two aliquots of 20 and 80 μL were taken, respectively. The aliquots were mixed with 10 % (w/v) SDS solution at ratio of 1: 1 (v/v). The addition of SDS was to maximize the protein solubility. The mixture was incubated in the dark at room temperature for 30 min with addition of 10 mM TCEP and 20 mM iodo-acetamide in 50 mM TEAB (pH 7.5). Subsequently, phosphoric acid was added to the mixture to make a final SDS concentration of 1.2 % (w/v). Afterwards, the proteins in the mixture were precipitated with 80 % methanol in 50 mM TEAB on a micro S-trap filter device (Protifi, Fairport NY, USA). Addition of phosphoric acid and methanol ensures protein denaturation and precipitation, which inhibits undesired enzymatic activities. Samples were

further processed according to manufacturer's instruction. In short, the precipitated proteins were digested with 1 μg MS grade trypsin during overnight incubation at 37 °C in an incubator. The peptide digest was eluted using 50 % acetonitrile in 0.1 % formic acid, dried using a SpeedVac vacuum concentrator (Thermo Fisher Scientific, Ochten, the Netherlands) and finally re-dissolved in 0.1 % formic acid. The redissolved peptides were analyzed using a Dionex Ultimate 3000 UPLC connected to a QexactivePlus (ThermoFisher, SanJose, USA). Sample was injected and separated on an analytical C18 RP column (15 cm*2.1 mm, HSS T3 Waters, Milford USA) and on-line sprayed using the ESI electrospray ion-source into the entrance of the MS. Peptides were separated in analytic mode with a gradient of 2 %–40 % acetonitrile in 0.1 % formic acid during 40 min. The column was rinsed with 80 % acetonitrile and re-equilibrated to 2 % acetonitrile in 0.1 % formic acid at a flow rate of 200 μL/min at 55 °C column temperature. Data were acquired in a DDA (data-dependent analysis) MSMS selection mode using a top 10 precursor selection method. The juice was analyzed in duplicates.

The collected spectra that were processed using FragPipe version 20.0 with MSFragger version 3.8, IonQuant version 1.9.8 and Philosopher version 5.0 (da Veiga Leprevost et al., 2020; Kong et al., 2017; Teo et al., 2021; Yu et al., 2021). The MS/MS data were compared to the complete tomato proteome sequence from Uniprot (Tomato_UP000004994_4081.fasta), with an enzyme specific search (trypsin, max 2 missed cleavages). Such specific search identified and quantified trypsin-cleaved proteins, where the peptide sequences end with arginine or lysine. Fixed modification of cysteine with carbamidomethyl, and variable modification of methionine oxidation were used as potential peptide modifications. Identification results of both juice aliquots were combined into a single result file. The identified protein table (Supplementary material proteomics file) was filtered for "peptidase" and "prote(in)ase" to generate a protease table (Table 1).

2.5. Enzymatic hydrolysis of WPI

2.5.1. Effect of incubation time

The enzymatic hydrolysis by proteases in the juice was determined by using High Pressure Size Exclusion Chromatography (HPSEC) method and with WPI as substrate. The frozen juice was thawed at room temperature and the pH of the juice was adjusted to 7 by adding 6 M sodium hydroxide. pH 7 was chosen to avoid acid coagulation of WPI since the natural pH of the juice was 5.21 ± 0.08. Subsequently, 1.5 g of WPI was dissolved in 50 mL juice to make a WPI concentration of 30 mg/mL. A control sample was made by dissolving 1.5 g WPI in 50 mL MilliQ water, the pH of the control sample was 6.73. To investigate the effect of incubation time, all samples were incubated at 20 °C in a thermomixer with 300 rpm. Samples were taken at 5, 15, 30, 45, 60, 120, 240 and 420 min and immediately mixed with MilliQ water at ratio of 1: 5 (v/v) to make a final WPI concentration of 5 mg/mL. The mixtures were centrifuged at 10,000 g for 5 min and the supernatant was analyzed by HPSEC.

2.5.2. Effect of heat deactivation

To investigate the effect of heat deactivation, aliquots of juice (at pH 7, without WPI) was pipetted into 5 mL pre-heated (at 98 °C) Eppendorf tubes and heated at 98 °C in a thermomixer for 10, 20, 30 and 60 min, respectively. After cooling down at room conditions for 10 min, WPI was dissolved in the juice at concentration of 30 mg/mL. The mixtures were incubated at 20 °C for 1 h with 300 rpm and diluted with MilliQ water to a WPI concentration of 5 mg/mL. The new mixtures were centrifuged at 10,000 g for 5 min and the supernatant was analyzed by HPSEC method.

2.5.3. Effect of incubation temperature and protease inhibitors

To investigate the effect of incubation temperature and the addition of protease inhibitors, aliquots of juice (at natural pH, without WPI) were firstly mixed with different protease inhibitors, namely cOmplete™ Protease Inhibitor Cocktail with EDTA at one or two times dosage as suggested by the manufacturer (+EDTA and 2x + EDTA), cOmplete™ Protease Inhibitor Cocktail without EDTA at one or two times dosage as suggested by the manufacturer (-EDTA and 2x-EDTA) and cOmplete™ Protease Inhibitor Cocktail with and without EDTA with additional pepstatin (+EDTA + pep and -EDTA + pep), respectively. To make a 1M pepstatin stock solution, 6.9 mg pepstatin was dissolved in 10 mL methanol. The stock solution was added at 1: 1000 ratio (v/v) to the corresponding samples (+EDTA + pep and -EDTA + pep). cOmplete™ protease inhibitors target at broad range of serine and cysteine proteases (COmplete Protease Inhibitor Cocktail Tablets Roche, 2023). The addition of EDTA and pepstatin target at metalloproteases (Hazra et al., 2012) and aspartic proteases, respectively. The pH of the juice samples containing proteases inhibitors were adjusted to 7 with 6 M sodium hydroxide. WPI was then dissolved in these samples at 30 mg/mL. The mixtures were incubated for 1 h with 300 rpm at 4, 20, 40 and 60 °C, respectively. After incubation, samples were diluted with MilliQ water to a WPI concentration of 5 mg/mL. The new mixtures were centrifuged at 10,000 g for 5 min and the supernatant was analyzed by HPSEC method. All samples were measured three times and a blank WPI sample and blank juice sample were measured as control.

2.5.4. Quantification of hydrolysis degree

The HPSEC samples were analyzed using an Ultimate 3000 HPLC

Table 1
Number of identified proteases and protease inhibitors in each protease subclass, their cellular component and enzyme function classification, mean coverage (%), sum of unique peptides, sum of razor peptides and sum of razor intensity. n.a stands for non-applicable.

Enzyme function classification	Protein subclass	Number	Cellular component	Mean coverage %	Sum of Unique Peptides	Sum of Razor Peptides	Sum of Razor Intensity
n.a	Proteasome	17	Cytoplasm; nucleus; proteosome core complex, alpha-/beta-subunit complex	22.4	46	68	2.6E+08
Exopeptidase	(Metallo) aminopeptidase	6	Chloroplast; nucleus; cytoplasm; intracellular membrane-bounded organelle; membrane; cellular anatomical entity; unknown	16.1	14	39	1.1E+08
Exopeptidase	Serine carboxypeptidase	5	Extracellular region	7.7	15	19	6.9E+07
Endopeptidase	Aspartic peptidase	5	Unknown	11.3	18	19	8.0E+07
Endopeptidase	Clp protease	3	Chloroplast; endopeptidase Clp complex; plastid stroma	8.3	5	10	4.9E+07
Endopeptidase	Serine peptidase	1	Unknown	13.2	9	9	1.8E+07
Endopeptidase	Cysteine peptidase	1	Extracellular space; vacuole	12.4	0	3	4.2E+07
n.a	Serine endopeptidase inhibitor	2	Extracellular region; unknown	19.3	1	2	5.5E+06
	Cysteine proteinase inhibitor	2	Unknown	5.8	2	2	3.7E+06

(ThermoFisher, Waltham, USA) system. Two columns of TSKGel G3000SWXL 5 μm 300 × 7.8 mm and TSKGel G2000SWXL 5 μm 300 × 7.8 mm were used at 30 °C in combination with eluent 30 % acetonitril in MilliQ containing 0.1 % trifluoroacetic acid. A sample of 10 μL was injected each time at a flow rate of 1.5 mL/min. UV detection was at 214 nm. Data analysis was performed in Chromeleon 7.2 CDS software (ThermoFisher Scientific Inc., USA). BSA, α-lg and β-lg were identified in the commercial WPI based on retention time of individual whey protein (Supplementary material, Figure S2). To quantify the concentrations of BSA, α-lg and β-lg in the WPI control sample and juice samples, calibration curves of these individual proteins were made with concentrations of 0.4, 0.7, 1.9, 3.8, 5.5 and 7.2 mg/mL against their integrated peak area. This information was used to determine the protein composition in the WPI sample, which was 9.16 ± 0.35 % BSA, 65.67 ± 1.22 % β-lg and 23.55 ± 0.42 % α-lg. In addition, peptides that were generated from the enzymatic hydrolysis by juice proteases (Fig. 1) and from blank juice sample were measured based on the molecular weights (Möller et al., 2022; Yu et al., 2023). A calibration curve of molecular weight on a logarithmic scale against retention time was plotted for α-lg (14 kDa), aprotinin (6.51 kDa), bacitracin (1.42 kDa) and phenylalanine (165 Da) for this purpose.

The difference in each whey protein concentration between the WPI control sample and different juice samples was quantified as the hydrolysis degree of each whey protein, which was based on Akkermans et al. (2008) and Hoppenreijs, Overbeck, et al. (2023) and calculated as follows:

$$Hydrolyzation\ degree\ (\%) = 100 - \left(\frac{C_{protein,sample}}{C_{protein,WPI}} * 100\right) \quad (1)$$

Where $C_{protein,sample}$ is the concentration of each protein (i.e BSA, α-lg and β-lg) in the sample with juice incubated at different conditions and $C_{protein,WPI}$ is the concentration of each protein in the WPI control sample.

2.6. Enzymatic hydrolysis of leaf proteins

The hydrolysis of endogenous proteins by proteases in the juice was investigated by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition. The reducing sample buffer was prepared by mixing the 2x concentrated Laemmli sample buffer with 2-mercaptoethanol at ratio of 19:1 (v/v). The frozen juice was thawed at room temperature and the pH of the juice was adjusted to 7 by adding 6 M sodium hydroxide. The juice was then incubated at 20 °C and samples were taken at 0 h, 1 h and 18 h, respectively. The taken sample was immediately mixed with reducing sample buffer at 1:1 ratio (v/v), the mixtures were heated at 95 °C for 10 min and centrifuged at 10,000 g for 5 min. A 15 μL of the obtained supernatants and 10 μL of the protein marker standard were loaded in different lanes on the gel. The electrophoresis was carried out at 200 V for approximately 1 h. The gel was washed three times with MilliQ water, stained with Bio-safe Coomassie stain and detained with MilliQ water. The gel was scanned using GS-900 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, USA).

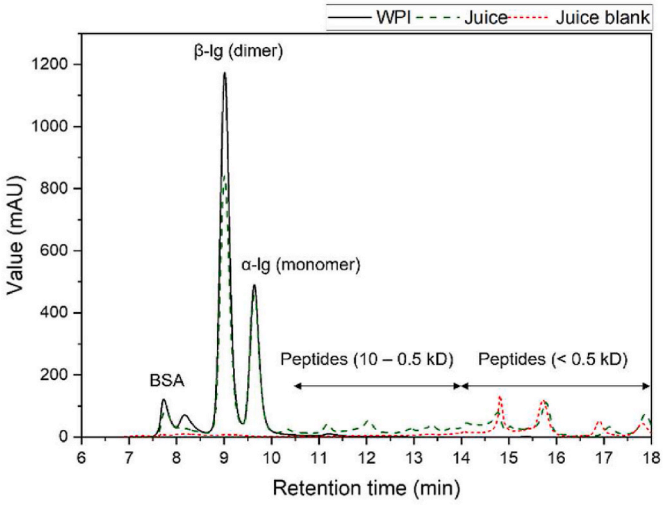


Fig. 1. Chromatograms of WPI with water (WPI) and with tomato leaf juice (Juice) and blank juice sample (Juice blank) after incubation at 20 °C for 1 h. Bovine serum albumin (BSA), beta-lactoglobulin (β-lg) and alpha-lactalbumin (α-lg) were marked, peptides with molecular weights in the range of 0.5–10 kD and <0.5 kD were highlighted with double sided arrows. Chromatograms of WPI with heated juice and with additional protease inhibitors can be found in Supplementary material, Figure S3.

2.7. Structural and thermal properties change

The (secondary) structure of WPI before and after incubation with juice was investigated with the Fourier Transform Infrared Spectroscopy (FTIR). The method was based on Hoppenreijs, Overbeck, et al. (2023). A Confocheck Tensor 2 system (Bruker Optics, Ettlingen, Germany) equipped with a thermally controlled BioATR 2 unit was used. Frozen juice was thawed and the pH was adjusted to 7. WPI was dissolved in the juice at concentration of 30 mg/mL. The mixture was incubated at 20 °C for 1 h and centrifuged at 10,000 g for 5 min. A blank WPI sample dissolved in MilliQ water and a blank juice sample (without WPI) were incubated and centrifuged in the same way. A sample of 20 μL of the supernatants was transferred onto the crystal and analyzed using 64 scans with a resolution of 4 cm^{-1}. MilliQ water was used for the background signal calibration. Each sample was analyzed in both independent and dependent duplicates. Data analysis was performed using the OPUS software (Bruker Optics, Ettlingen, Germany). The spectrum of each sample was vector normalized (1700–1600 cm^{-1}), cut (2200–1000 cm^{-1}) and baseline corrected (concave rubberband correction with 10 interactions and 64 baseline points, excluding CO_2). The normalized spectra of blank juice sample (Supplementary material Figure S4) was subtracted from the juice sample containing WPI.

The denaturation profile change of WPI due to hydrolysis was investigated with differential scanning calorimetry (DSC). The method was based on Hoppenreijs, Overbeck, et al. (2023). Here WPI solution with a concentration of 100 mg/mL was used instead of 30 mg/mL to increase sensitivity during the measurement. The samples (WPI with water, WPI with juice and blank juice) were prepared as above-mentioned. A high volume pan was filled with approximately 50 mg of sample (exact amount was noted for enthalpy calculation). The pans were sealed and analyzed with an empty pan used as reference. The pans were equilibrated at 20 °C and heated to 140 °C at a rate of 3.5 °C/min. After cooling down to 20 °C at 10 °C/min, the pans were heated the second time to check for complete denaturation, at a rate of 3.5 °C/min (Supplementary material Figure S5). Finally, the pans were cooled to 20 °C at 30 °C/min. Data analysis was performed using the TRIOS software (TA Instruments), the denaturation enthalpy was defined as the area under the endothermic peak and was corrected for the WPI content in the sample. The onset and peak temperature for denaturation were derived from the endothermic peak.

2.8. Least gelling concentration

The least gelling concentration measurement was adapted from Jia et al. (2022) and Schlangen et al. (2022). Frozen juice was thawed at room conditions and the pH of the juice was adjusted to 7 with 6 M sodium hydroxide.

Afterwards, two stock solutions were made by dissolving WPI into MilliQ water and juice at concentration of 15 % (w/v), respectively. The stock solutions were diluted with only MilliQ water to 3, 6, 9 and 12 % (w/v), the ratio of the juice to WPI in solutions containing juice were

therefore kept constant at 100: 15 (mL: g). All solutions were incubated at 20 °C for 1 h with 40 rpm and subsequently heated at 95 °C in a water bath for 30 min. After cooling down to room temperature, all samples were inverted. The least gelling concentration was determined as the concentration where the sample did not slip or fall after inversion. Pictures of the samples were made with an iPhone 11 smartphone at room (light) conditions in a photobooth against white background. All samples were made and evaluated twice. A control sample of blank juice (pH 7, non-diluted) was also incubated and heated under the same conditions, here the sample did not form a gel (Supplementary material, Figure S6).

2.9. Rheological properties measurement

The rheological properties of samples were analyzed by small angle oscillatory shear (SAOS) and large amplitude oscillatory shear (LAOS) measurements, based on Nieuwland et al. (2021) and Schlangen et al. (2022).

2.9.1. SAOS measurement (temperature sweep)

Two 15 % (w/v) WPI stock solutions with water and with juice were made and incubated as described in section 2.7. After incubation, the rheological properties of each sample was measured with a MCR301 rheometer (Anton Paar, Graz, Austria) combined with a CC-10 concentric cylinder geometry. A 2 mL of sample was loaded, the exposed edges of the geometry was covered with a thin layer of paraffin oil to prevent water evaporation. A temperature sweep was conducted by increasing the temperature from 20 to 95 °C at a rate of 2 °C/min. The temperature was kept at 95 °C for 30 min, subsequently reduced to 20 °C at a rate of 2 °C/min and kept at 20 °C for 10 min. During the temperature sweep, the frequency and shear strain were kept constant at 1 Hz and 0.1 %, respectively. The storage (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 as follows:

$$tan\ \delta = \frac{G''}{G'} \quad (2)$$

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

2.9.2. Laos measurement (strain sweep)

Right after the temperature sweep, a (shear) strain sweep was conducted from 0.1 to 1000 % at frequency of 1 Hz and at 20 °C. The storage (G′) and loss modulus (G″) were recorded during the measurement. The end of linear viscoelastic regime was defined as the strain where the first modulus data points 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 point where G′ and G'' overlap. All samples were measured three times.

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 were analyzed with one-way ANOVA using a multivariate general linear model and with the Tukey test. Differences between the control sample and other samples were considered significant when $P \leq 0.05$, and significant difference was marked with an upper *.

3. Results and discussion

3.1. Protease composition

From a total number of 1121 different proteins (Supplementary material proteomics file), 38 proteases and 4 protease inhibitors were identified in the leaf juice. The composition and content of these proteases and protease inhibitors are presented in their subclasses, enzyme function classification and cellular component (Table 1). The majority of the identified proteases were proteasome, which is multi-subunit enzyme complex present in cells that is responsible for degradation and recycling of proteins (Adams, 2003). The rest of the identified proteases included a mixture of exo- and endopeptidases, specifically (metallo)aminopeptidases and serine carboxypeptidases and aspartic peptidase, Clp proteases, serine and cysteine peptidase, respectively. Such diverse combination of proteases provides a robust protein hydrolysis system, which is essential for leaves because proteases are involved in many important functions, such as plant defense mechanism, stress response (Pinedo et al., 2000), plant development (Messdaghi & Dietz, 2000), protein turnover and more. These protease activities are well regulated in leaves, for example by endogenous proteases inhibitors, of which 4 were identified in this study namely 2 serine endopeptidase inhibitors and 2 cysteine proteinase inhibitors. As indicated by their names, these inhibitors will have specific inhibition on proteases such as serine endopeptidases and cysteine proteases, of which two were also detected in the leaf juice (Table 1). It is important to note that in the (lysed) leaf juice, proteases are separated from their (sub) cellular environment, and therefore will exert different activity compared to its normally regulated conditions. For example, as indicated in Table 1, some proteases originate from different cellular components within the leaves and therefore might not come into contact with each other. However, when leaf tissue is lysed, these proteases are released from their cellular components and can interact, possibly resulting in self-cleavage or cleavage of other proteases (Gill & Parks, 2008).

We further investigated the characteristics of these proteases by mixing them with WPI. The enzymatic effects were analyzed using HPSEC method, of which the results are presented in Fig. 1. Three peaks were detected in the control WPI sample, which were β-lg, α-lg and BSA (comprising 65.67 ± 1.22 %, 23.55 ± 0.42 % and 9.16 ± 0.35 % of total WPI, section 2.5). In the blank juice sample, only peptides that were smaller than 0.5 kD were detected. In other words, no other components with larger molecular weight were detected. RuBisCo (ribulose bisphosphate carboxylase oxygenase), being the major leaf protein (Fiorentini & Galoppini, 1983), with molecular weight of approx. 550 kD (Santamaría-Fernández & Lübeck, 2020), was likely to be present in the juice due to its solubility. The absence of RuBisCo on the HPSEC chromatogram could be contributed to the low dry matter content in the juice (5.52 ± 0.4 %, section 2.3), therefore the RuBisCo content was below the detection limit. In addition, part of RuBisCo could be degraded by the endogenous proteases during the processing, resulting in low concentration in the juice. Interestingly, when the juice was incubated with WPI, the three whey protein peaks showed a significant reduction, while accumulation of peptides with various molecular weights (0.5–10 kD and <0.5 kD) were observed (Fig. 1). These peptides were likely to be the products of enzymatic hydrolysis of whey proteins by the proteases in the juice. It could be interesting for future research to identify these peptides, especially the ones that were smaller than 0.5 kD, since part of these peptides were overlapping with the peptides that were already present in the blank juice sample. In addition, it is clear that the hydrolysis of WPI was predominantly carried out by endopeptidases, since endopeptidases tend to cleave within one molecule (Rawlings & Barrett, 1994), rather than at the end of one molecule (exopeptidases). This action leads to a reduction of the substrate concentration and accumulation of the generated products, as shown in Fig. 1. Table 1 outlines that a mixture of endopeptidases were identified in the leaf juice. We therefore concluded that (some of) these endopeptidases exhibited activity on WPI and hydrolyzed WPI in a predominant endopeptidase manner.

3.2. Effect of incubation time

Given the fact that part of the juice proteases were active (Fig. 1), a next step is to investigate the effect of incubation time. Here we investigated the hydrolysis of both WPI and endogenous leaf proteins in the juice by the juice proteases. The results are presented in Fig. 2. The hydrolysis of all three whey proteins and endogenous leaf proteins was similar during the studied incubation time. In the case of WPI, the hydrolysis degree of these proteins increased with incubation time, peaking at around 60 min and remained relatively stable afterwards (Fig. 2a). Similar observations were made for leaf proteins, especially the large and small subunits of RuBisCo (approx. 50 and 15 kD (Santamaría-Fernández & Lübeck, 2020),), since hydrolysis of these subunits were evident at 1 h incubation time but did not significantly increase with time (Fig. 2b). Additionally, we also observed reduction of intensities for bands at 37 kD and 20 kD, respectively. These bands could be associated to thylakoids membrane (Kiskini et al., 2016) and their intensities continuously decreased as a function of incubation time (until 18 h, Fig. 2a). However, same as the observation for RuBisCo subunits, these bands did not disappear completely after long time incubation, suggesting a limited hydrolysis by the juice proteases. One of the possible reasons was that part of the protease activity was limited by the endogenous protease inhibitors present in the juice (Table 1). In addition, proteases that do not encounter with each other in leaves may potentially degrade each other, especially at longer incubation time (Gill & Parks, 2008). Both effects results in reduced enzyme activities, which could explain the incomplete (limited) hydrolysis of proteins.

When diving into the hydrolysis of WPI, BSA had the highest hydrolysis degree on an overall level, followed by β-lg and α-lg (Fig. 2a). For instance, after 5 min, the degree of hydrolysis for BSA was already 27.74 ± 0.4 %, while that for β-lg and α-lg were 9.49 ± 0.4 and 4.86 ± 0.1 % respectively. The hydrolysis degree of BSA gradually increased to 39.29 ± 0.2 % after 60 min incubation, while the hydrolysis degree of β-lg sharply increased to 32.95 ± 1.0 % after the same incubation time. This sharp increase indicated higher initial hydrolysis rate for β-lg as compared to BSA. α-lg was hydrolyzed in a similar manner as BSA with increase of incubation time to 60 min (10 ± 0.6 %) and remained as the least hydrolyzed proteins among the three whey proteins. Despite of the fact that the concentration of BSA was lower than that of β-lg and α-lg in the WPI (section 2.3), BSA was still the most hydrolyzed protein by the juice proteases (Fig. 2a). The high hydrolysis degree of BSA was possibly due to the presence of a higher quantity of proteases in the juice that specifically cleaved BSA. A next step could be to identify which enzyme in the juice cleaves specific protein in WPI. To reflect on the downstream protein extraction, it becomes clear that fast processing is required to minimize the hydrolysis of proteins. Nevertheless, completely avoiding the hydrolysis remains challenging, since all three whey proteins were already hydrolyzed within 5 min, especially BSA (Fig. 2a).

3.3. Heat deactivation

We investigated the deactivation of juice proteases by heating and the results are presented in Fig. 3. Heating effectively reduced the hydrolysis degrees of all three whey proteins. To be more specific, heating had the most profound effect on reducing the hydrolysis degree of β-lg. For instance, the hydrolysis degree for β-lg reduced from 32.95 ± 1.0 % (without heating, shown in Fig. 2) to 2.42 ± 1.4 % after heating the juice for 10 min, at 60 min incubation time (shown in Fig. 3). In the meanwhile, heating (for 10 min) reduced the hydrolysis degree of α-lg and BSA from 10 ± 0.5 % to 2.5 ± 1.4 % and from 39.29 ± 0.2 % to 27.65 ± 0.9 % at 60 min incubation time, respectively. The reduction of the hydrolysis degree suggested that heating at 98 °C efficiently reduced the protease activities in the juice, especially for the ones that hydrolyzed β-lg. In addition, the heating time did not affect the protease activities in the juice, since the hydrolysis degrees of BSA, β-lg and α-lg remained roughly constant (in average 27 %, 1 % and 1 %, respectively) with the increase of heating time from 10 min to 60 min (Fig. 3). However, it is crucial to point out the difficulty to fully eliminate the protease activities

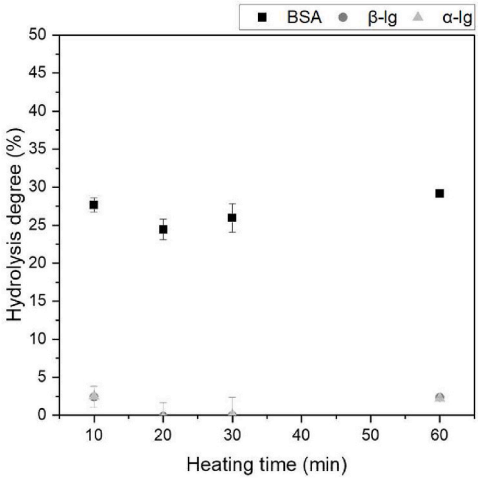


Fig. 3. Hydrolysis degree of BSA, β-lg and α-lg incubated at 20 °C for 1 h with heated juice (98 °C) for different heating time.

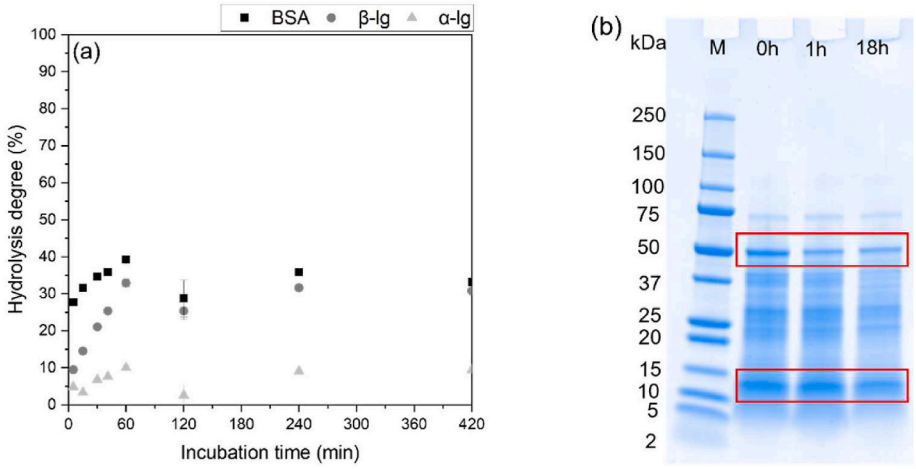


Fig. 2. Hydrolysis degree of BSA, β-lg and α-lg incubated with juice (a) and hydrolysis of endogenous proteins in the juice (b) at 20 °C at different incubation time. M represents the protein standard marker. The red line frames indicated the large and small subunits of RuBisCo.

through heating. Even after 60 min of heating at 98 °C, part of proteases in the juice remained active and therefore could still hydrolyze the whey proteins. This suggests that those proteases are thermostable, in particular the ones that were involved in the hydrolysis of BSA (Fig. 3). To conclude, heating at 98 °C for 10 min was sufficient to limit protease activities in the juice on β-lg and α-lg. However, proteases that hydrolyze BSA were thermostable, and therefore their activities could not be sufficiently limited by heating at high temperature.

3.4. Effect of protease inhibitors and temperature

To further explore the options to limit protease activities in juice, the effect of protease inhibitors and temperature were investigated (Fig. 4). Regardless of the incubation temperature, the addition of protease inhibitors had the largest effect on reducing the hydrolysis degree of β-lg (Fig. 4b). For instance, the addition of pepstatin or higher dosage of cOmplete™ protease inhibitors at 4 °C significantly reduced the hydrolysis degree of β-lg from 29.68 ± 2.1 % to 13.1 ± 0.6 % (+EDTA + pep), 16.58 ± 1.2 % (-EDTA + pep), 9.65 ± 0.6 % (2x + EDTA) and 13.34 ± 0.2 % (2x-EDTA), respectively. Since pepstatin and cOmplete™ protease inhibitors specially inhibit aspartic proteases and serine and cysteine proteases (section 2.5), the reduction of hydrolysis degree upon addition of these inhibitors suggested the involvement of serine, cysteine and aspartic proteases in the hydrolysis of β-lg. A similar reduction of hydrolysis degree upon addition of these inhibitors were also observed for BSA, but to somewhat less extent (Fig. 4a). This suggested the involvement of serine, cysteine and aspartic proteases in the hydrolysis of BSA as well. In the meanwhile, the presence or absence of EDTA did not affect the hydrolysis of β-lg and BSA (Fig. 4a and b). Given the fact that EDTA specially inhibits the activities of metalloproteases, the absence of an effect upon addition of EDTA indicated that metalloproteases were not involved in the hydrolysis of β-lg and BSA. Our findings were in line with literature where many natural proteases were responsible for the hydrolysis of caseins, such as cysteine proteases from *Calotropis procera* (Kumar Dubey & Jagannadham, 2003), ginger (Gagaoua et al., 2015) and green fig fruits (Afsharnezhad et al., 2019), and serine proteases from red seaweed (Arbita et al., 2020), non-edible parts of fennel (Bey et al., 2018) and lettuce leaves (Lo Piero et al., 2002). The addition of protease inhibitors had limited effect on reducing the hydrolysis degree of α-lg (Fig. 4c). Under most of the conditions, the addition of inhibitors did not affect the hydrolysis degree of α-lg. Only exceptions were found to be with addition of -EDTA at 40 °C, 2x + EDTA at 4 and 20 °C, 2x-EDTA at 40 °C and +EDTA + pep at 20 °C. However, the reduction of hydrolysis degree remained limited under these conditions, since the largest reduction was from 9.35 ± 1 % to 5.4 ± 0.5 % (4 °C, with addition of 2x + EDTA). Finally, it is important to point that achieving complete inhibition of proteases using inhibitors remained challenging (Fig. 4) although the addition of some protease inhibitors led to significant decrease in hydrolysis degree of whey proteins (especially β-lg). The observations aligned with literature (Afsharnezhad et al., 2019; Arbita et al., 2020; Kumar Dubey & Jagannadham, 2003).

The hydrolysis of three whey proteins by proteases in juice was also strongly influenced by temperature (Fig. 4). It was consistently observed that higher incubation temperature resulted in higher hydrolysis degrees for all three whey proteins. In particular, incubation at 60 °C resulted in almost 100 % (98.18 ± 3.1 %) hydrolysis of α-lg, followed by β-lg (92.89 ± 2.3 %) and BSA (74.97 ± 5.3 %), respectively. These high hydrolysis degrees suggested high protease activities at 60 °C, which was also reported to be the optimal temperature for natural proteases from green fig fruits (Afsharnezhad et al., 2019), red seaweed (Arbita et al., 2020), *Calotropis procera* (Kumar Dubey & Jagannadham, 2003), ginger (Gagaoua et al., 2015) and herbaceous plant *Cynanchum otophyllum*. (Luo et al., 2018). It is however important to point out that sample coagulation was observed at 60 °C after incubation with juice (data not shown). Such coagulation might lead to loss of insoluble material after centrifuge and therefore overestimation of hydrolysis degrees

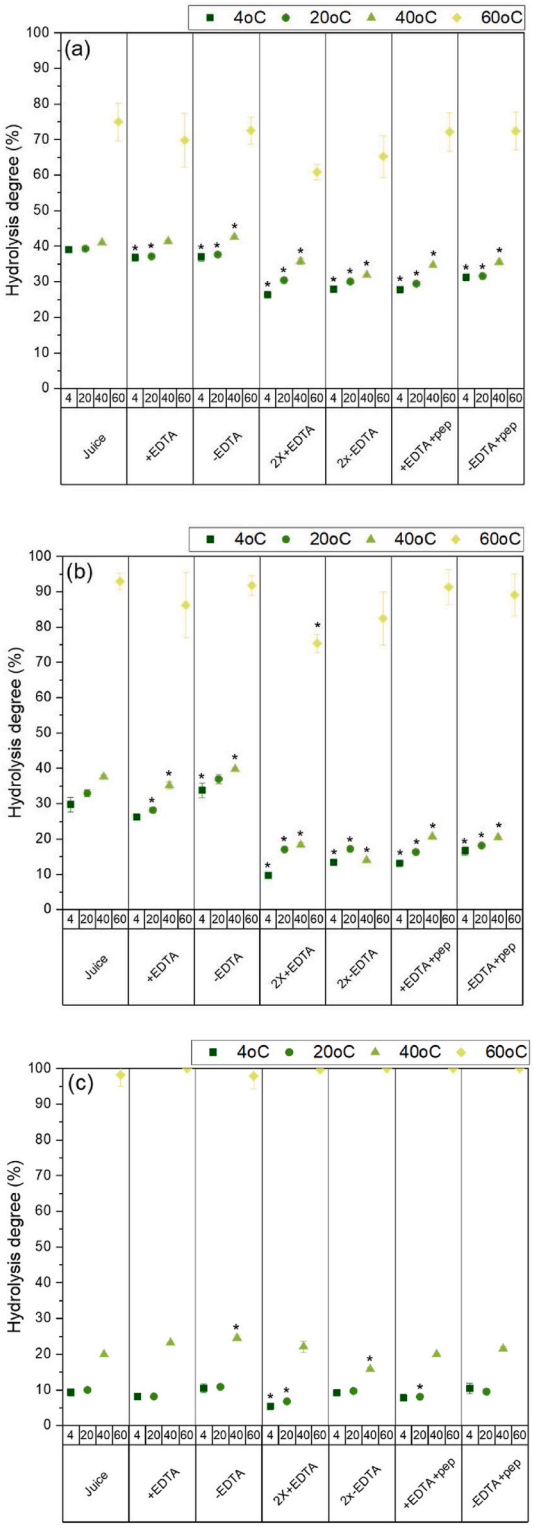


Fig. 4. Hydrolysis degree of BSA (a), β-lg (b) and α-lg (c) incubated with juice with different additional protease inhibitors at 4, 20, 40 and 60 °C for 1 h. Juice stands for blank juice, +EDTA stands for one time dosage of protease inhibitor with EDTA as suggested by the manufacturer, -EDTA stands for one time dosage of protease inhibitor without EDTA as suggested by the manufacturer, 2x stands for two times dosage as suggested by the manufacturer, +pep stands for pepstatin. * represents significant difference as compared to the Juice control at each temperature at $P \leq 0.05$.

for proteins since only soluble fractions were analyzed by the HPSEC method (section 2.5).

In summary, it is clear that full elimination of protease activity is very challenging if at all possible (Figs. 3 and 4) in downstream processing of tomato leaves. Therefore one must realize this challenge when considering tomato leaves as protein source for extraction.

3.5. Structural and thermal properties change

To further investigate the hydrolysis of WPI by juice proteases, the structural and thermal properties of WPI before and after incubations with tomato juice were measured and the results are presented in Fig. 5. In the case of secondary structure, it is often reported that peaks in the amide I zone (1600–1700 cm^{-1}) correspond to proteins with specific secondary structure in the following range: α-helix (1648–1658 cm^{-1}), intramolecular and intermolecular β-sheets (1620–1640 and 1610-1620 cm^{-1}, respectively) (Brishti et al., 2020; Kavanagh et al., 2000). A consistent reduction in the absorbance of α-helix, intramolecular and intermolecular β-sheets structure was observed for WPI after incubation with juice (Fig. 5a). Such reduction in secondary structure integrity was probably a result from the hydrolysis by juice proteases (Fig. 2a).

Fig. 5b showed the thermograms of WPI before and after hydrolysis by juice proteases. In both cases, a major peak was observed with peak temperature at around 76 °C, which was attributed to the denaturation of β-lg, as suggested by Boye and Alli (2000). In addition, there was a small peak on both thermograms in between 60 and 65 °C, which was possibly due to the denaturation of α-lg. This peak was however overshadowed by the large peak from β-lg, due to the fact that β-lg was the most predominant protein within WPI (section 2.5.4). There was no peak observed for the blank juice sample, which was due to the low protein content in the sample (section 2.3). The denaturation enthalpy was therefore only calculated for the major peaks at 76 °C. After the hydrolysis by juice proteases, the denaturation enthalpy of WPI slightly decreased as compared with the intact WPI (7.55 and 7.75 J/g WPI, respectively). In addition, the onset denaturation temperature also decreased after hydrolysis (64 °C–57 °C), suggesting that part of the protein structure was destabilized. Small decrease in denaturation enthalpy (5.91–4.46 J/g) was also reported for pea protein after hydrolysis at pH 7 by Alcalase (Chen & Campanella, 2022). It is possible that aggregates formed by the hydrolysis (section 3.6) restricted further conformational change of WPI (Chen & Campanella, 2022; Day et al., 2002), hence led to less significant change in denaturation enthalpy. Furthermore, it is important to note that higher WPI concentration (10 %, w/v) was used for the DSC measurement since sample with lower concentration (3 %, w/v, used for HPSEC and FTIR measurements) did not show denaturation peaks (data not shown). In this case, a higher amount of WPI dissolved in juice led to a higher substrate-to-enzyme ratio, which could lead to a lower degree of hydrolysis and therefore less significant change in the amount of energy used to denature the proteins.

3.6. Gelation behavior and rheological properties

The gelation behavior of WPI incubated with and without juice was characterized in terms of least gelling concentration and rheological properties. The least gelling concentration result is presented in Fig. 6. At neutral pH (pH 7, section 2.5), a high concentration of WPI (15 % (w/v)) was required to form a gel (Fig. 6), which was in line with literature (Ju et al., 1995; Otte et al., 1999; Otte, Ju, Færgemand, et al., 1996). Interestingly, much less material (3 % (w/v)) was required to form a gel when the WPI was incubated with juice, while a blank juice sample did not form a gel under the same conditions (Supplementary material, Figure S6). The enhanced gelation behavior was likely to be caused by limited hydrolysis (Figs. 2 and 4) induced by the proteases in juice. Limited hydrolysis of proteins led to exposure of hydrophobic groups (Creusot & Gruppen, 2007), which induced addition points of interaction between hydrolyzed products (peptides) and intact proteins, which resulted in enhanced aggregation upon heating (Chen & Campanella, 2022; Creusot & Gruppen, 2008; Ju et al., 1995). Ju et al. (1995) demonstrated that limited hydrolysis by proteases from *Bacillus lichenifonnis* led to formation of WPI gel at 3 % (w/w) at neutral pH. This phenomenon was also demonstrated on plant proteins such as pea protein, which could form gel at 5 % (w/w) and with less heat requirement upon limited hydrolysis by Alcalase (Chen & Campanella, 2022).

The rheological properties of WPI gel and WPI juice gel are presented in Fig. 7 and Table 2. A sudden increase of G′ and G'' was observed for WPI gel at 95 °C and for WPI juice gel at around 75 °C (Fig. 7a). The increase of G′ and G'' was likely caused by protein denaturation and aggregation. The lower onset gelation temperature of WPI juice gel was possibly attributed to the limited hydrolysis induced by the juice proteases. Similar results were reported, where limited hydrolysis of WPI by 1 % *Bacillus lichenifonnis* proteases resulted gelation at 50 °C at pH 7 (Ju

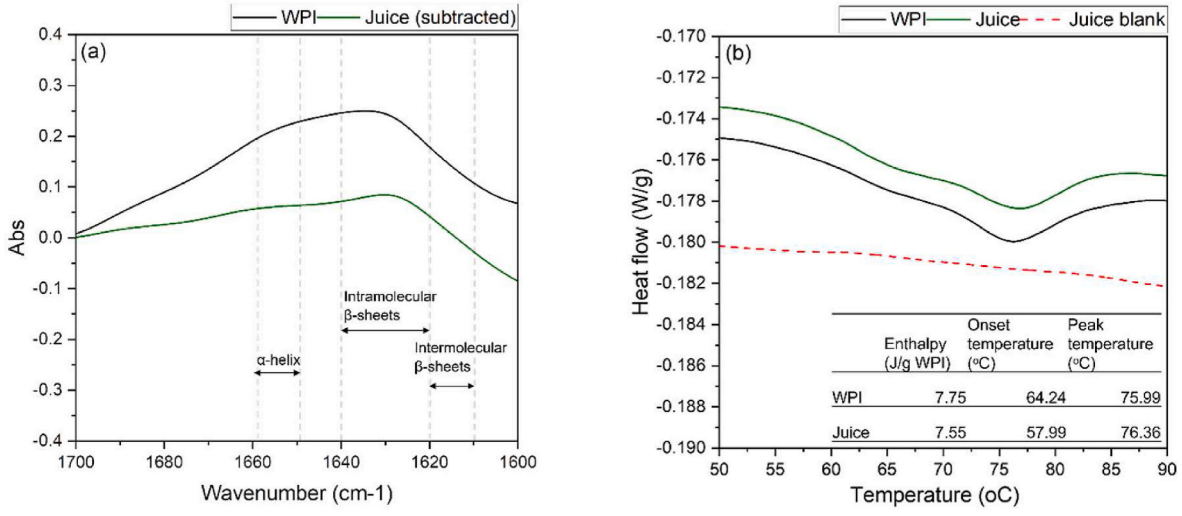


Fig. 5. Normalized FTIR spectrum (a) and DSC thermograms during the first temperature ramp (b) of WPI with water (WPI) and juice (Juice) incubated at 20 °C for 1 h. Juice blank stands for blank juice sample, of which the normalized FTIR spectra (Supplementary material Figure S4) was subtracted from the Juice sample. Different ranges for α-helix, intramolecular and intermolecular ß-sheets were highlighted with dotted grey line and double headed arrows. Error bars were expressed as light color shadow for each line.

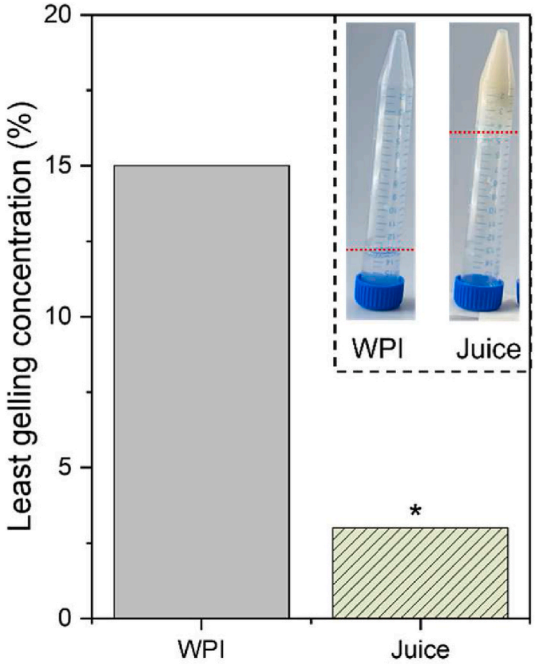


Fig. 6. Least gelling concentration of WPI with water (WPI) and with juice (Juice) incubated at 20 °C for 1 h and subsequently heated at 95 °C for 30 min. The pictures in the black dashed line area show WPI sample as a transparent liquid and Juice sample as an opaque gel after incubation and heating with a concentration of 3 % (w/w). The red round dotted lines are guidance for eyes. * represents significant difference as compared to the WPI control at $P \leq 0.05$.

et al., 1995). In addition, limited hydrolyzed pea proteins formed self-supported gel at 85 °C while intact pea proteins remained liquid under the same conditions (Chen & Campanella, 2022).

Upon heating, both WPI and WPI juice gels exhibited solid-like properties, as evidenced by higher G′ values than G'' values (Fig. 7a). The solid-like properties were further evidenced by the loss factor (tan δ), which is the ratio between G'' and G′ at the end of temperature sweep. Schurz (1967) described that tan δ < 1 indicating a solid material (Grossmann et al., 2019; Ikeda & Nishinari, 2001). As shown in Table 2, both WPI gel and WPI juice gel had tan δ values close to 0.1 (0.12 ± 0.02 and 0.13 ± 0.00, respectively), suggesting that both gels behaved as solid-like materials. In addition, the last G' value of WPI juice gel was significantly higher (82 ± 8 kPa) than that of WPI gel (5.5 ± 1.4 kPa) (Table 2), suggesting firmer gel structure of the WPI juice gel than the WPI control gel. This finding was in line with previous studies, which described that stronger and more elastic WPI gels were obtained after incubation with *Bacillus lichenifonnis* proteases (Ju et al., 1995; Tarhan et al., 2016). As discussed above, limited hydrolysis leads to additional interactions between peptides and intact proteins and therefore aggregation (Creusot & Gruppen, 2008; Ju et al., 1995). Such aggregation resulted in a particle gel, rather than fine-stranded gel, which is commonly seen in gel formed with intact proteins (Otte, Ju, Færgemand, et al., 1996; Wang et al., 2022). Stading and Hermansson (1990) demonstrated that particle gel had much higher storage modulus than a corresponding fine-stranded gel, explaining firmer structure of gel with limited hydrolysis than gel with intact proteins (Tarhan et al., 2016). Such phenomenon however was dependent on the enzymes, since trypsin prevented WPI gelation at pH 7 while Neutrase® induced gel was weaker than intact WPI gel at neutral pH (Ju et al., 1995). Our findings in this study therefore showed the potential of tomato leaf juice as natural gelling agent for WPI demonstrated by its ability to induce stronger gelation with less material at neutral pH (Figs. 6 and 7 and Table 2).

After the temperature sweep, a strain sweep was performed, in which the rheological stability of a gel against oscillatory deformation at a constant frequency and temperature were determined (Fig. 7b). This measurement was used to determine the linear viscoelastic regime, the critical strain (γ_c) and the crossover strain ($\gamma_{G' = G''}$), which all are presented in Table 2. Within the linear viscoelastic regime, the G′ was always higher than the G'' value, indicating a solid-like behavior over the full measured strain range. The end of linear viscoelastic regime was defined as 5 % deviation from the first modulus measure point (Schlangen et al., 2022), and expressed as the γ_c. After the γ_c, the initial

Table 2
Summary of storage modulus (G'), loss factor (tan δ), critical strain (γ_c) and crossover strain ($\gamma_{G' = G''}$) of the gelled WPI with water (WPI) and with juice (Juice) incubated at 20 °C for 1 h and subsequently heated at 95 °C for 30 min * represents significant difference as compared to the WPI control at $P \leq 0.05$.

Rheological parameters	G' (Pa)	tan δ	γ_c (%)	$\gamma_{G'=G''}$ (%)
WPI	5483.60 ± 1406.91	0.12 ± 0.02	5.04 ± 2.19	343.67 ± 47.06
Juice	82363.67 ± 8212.96*	0.13 ± 0.00	1.47 ± 0.18*	87.98 ± 141.18*

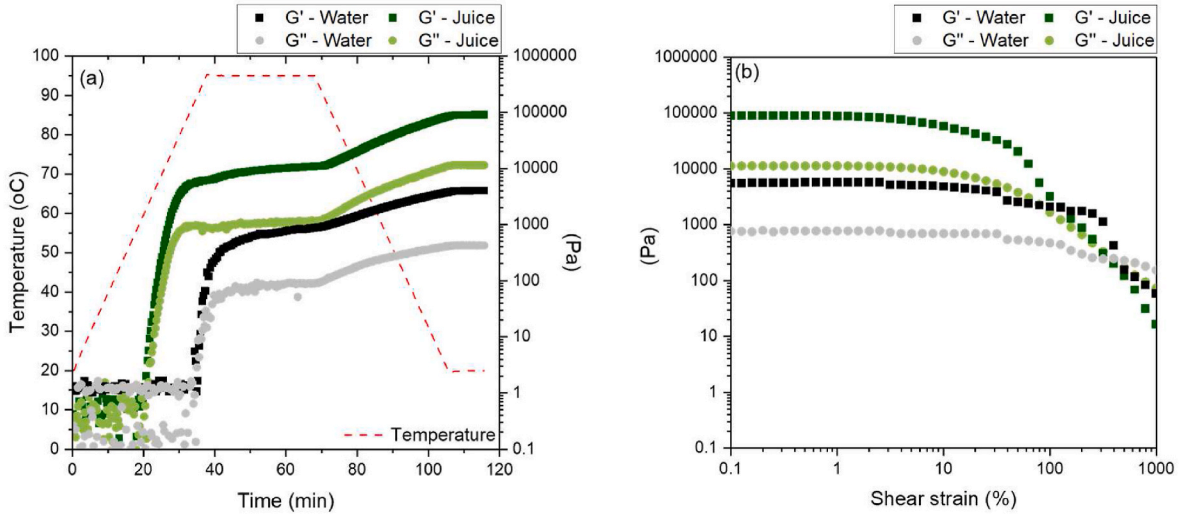


Fig. 7. Temperature sweep (20–95 °C, f = 1 Hz, •γ = 0.1 %) (a) and strain sweep (20 °C, f = 1 Hz, •γ = 0.1–1000 %) (b) of whey protein isolate with water (WPI) and with juice (Juice) incubated at 20 °C for 1 h. G′ and G'' represent storage modulus and loss modulus, respectively.

gel structure is interrupted. A longer linear viscoelastic regime, hence the higher the γ_c value, indicates that the gel can better withstand deformation. The WPI juice had significantly lower γ_c value (1.5 ± 0.2 %) than that of WPI gel (5.0 ± 2.2 %). One could interpret that the WPI juice gel was more brittle and hence could withstand less deformation than the WPI gel. Finally, both WPI gel and WPI juice gel had a crossover point where G′ and G'' overlap at certain shear strain, also expressed as $\gamma_{G' = G''}$. After the crossover strain, material becomes more liquid-like. The WPI juice gel had lower $\gamma_{G' = G''}$ value (87.98 ± 141.18 %) than the WPI gel (343.67 ± 47.06 %) (Table 2). This confirmed that the WPI juice gel was a firmer and more brittle material that yields sooner than the WPI gel.

3.7. Reflection on downstream protein extraction from tomato leaves

To reflect on the downstream protein extraction from tomato leaves, our study demonstrated the challenge due to the presence of endogenous proteases. These proteases from tomato leaves were active (on both WPI and leaf proteins) (Fig. 2) and could not be fully eliminated by means of heating and addition of inhibitors (Figs. 3 and 4). One of the challenges of leaf protein hydrolysis is reduced protein yield. It was observed in our recent study that only about 0.01 g/g leaf proteins could be extracted from old leaves at the bottom part of the tomato plant, while 0.35 g/g leaf proteins were extracted from younger leaves at the top part of the plant (Kleuter et al., submitted). Such significant reduction in protein yield was suspected to be attributed to leaf senescence (Havé et al., 2017), a process catalyzed by endogenous proteases, of which showed higher activity in older leaves (Zavaleta-Mancera et al., 1999). Notably, our study also demonstrated an alternative application for the proteases in tomato leaves. The proteolytic activity of tomato leaf juice resulted in limited hydrolysis of WPI (Figs. 2–4), which improved the gelation behavior and rheological properties of WPI (Figs. 6 and 7).

4. Conclusion

In this study, we investigated the abundance and composition of endogenous proteases in tomato leaf juice and hydrolysis of endogenous leaf proteins by these proteases. We further investigated the effect of incubation time, temperature, heat deactivation and protease inhibitors on the protein hydrolysis by juice proteases using WPI as substrate. In addition, we characterized the gelation behavior, the rheological properties and structural and thermal properties change of WPI incubated with and without leaf juice. Our results demonstrated that tomato leaf juice contains many different types of proteases, of which 38 were identified, together with 4 protease inhibitors. These proteases were active on both endogenous leaf proteins and WPI, and could hydrolyze the latter in a predominant endopeptidase manner. Furthermore, the protein hydrolysis was positively influenced by the incubation time and temperature. Among WPI, BSA had the highest hydrolysis degree, followed by β-lg and α-lg. The addition of protease inhibitors significantly reduced the hydrolysis degree of β-lg, but the effect was limited for BSA and especially α-lg. Heating at high temperature (98 °C) was more efficient in reducing the hydrolysis degree, but the effect was limited on thermostable enzymes (that hydrolyzed BSA). As a result, the protease activities in the juice could not be fully eliminated. The protein hydrolysis however remained limited (max. 43 %) under all conditions below 60 °C. Furthermore, the protein hydrolysis resulted in small changes in both secondary structure and thermal properties of WPI. The limited hydrolysis also resulted in WPI gelation at lower concentration (3 %, w/v) and temperature (75 °C) as compared to the control gel (gelation at 15 % (w/v) and at 95 °C). In addition, the gel with limited hydrolysis had higher storage modulus and more brittle structure than the control gel. In conclusion, the complex and active nature of endogenous proteases in tomato leaf juice poses challenges for downstream protein extraction from tomato leaves. However, limited hydrolysis induced by the leaf proteases provides ideas for new application since tomato leaf juice could be used as natural gelling agent for WPI.

CRediT authorship contribution statement

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

Declaration of competing interest

None.

Data availability

Data will be made available on request.

Acknowledgement

The authors would like to thank Marieke Bruins and Willemijn Liese for their insightful discussions and thank Maurice Strubel, Jos Sewalt, Wouter de Groot and Jarno Gieteling for their technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2024.109812.

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

Adams, J. (2003). The proteasome: Structure, function, and role in the cell. *Cancer Treatment Reviews, 29*(SUPPL. 1), 3–9. https://doi.org/10.1016/S0305-7372(03)00081-1

Afsharnezhad, M., Shahangian, S. S., & Sariri, R. (2019). A novel milk-clotting cysteine protease from Ficus johannis: Purification and characterization. *International Journal of Biological Macromolecules, 121*, 173–182. https://doi.org/10.1016/j.ijbiomac.2018.10.006

Akkermans, C., Venema, P., van der Goot, A. J., Gruppen, H., Bakx, E. J., Boom, R. M., & van der Linden, E. (2008). Peptides are building blocks of heat-induced fibrillar protein aggregates of β-lactoglobulin formed at pH 2. *Biomacromolecules, 9*(5). https://doi.org/10.1021/bm7014224

Alkoaik, F., & Ghaly, A. E. (2006). Influence of dairy manure addition on the biological and thermal kinetics of composting of greenhouse tomato plant residues. *Waste Management, 26*(8), 902–913. https://doi.org/10.1016/J.WASMAN.2005.11.023

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). https://www.researchgate.net/publication/282002103.

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*. https://doi.org/10.1016/j.foodchem.2020.127324

Bey, N., Debbebi, H., Abidi, F., Marzouki, M. N., & Ben Salah, A. (2018). The non-edible parts of fennel (Fœniculum vulgare) as a new milk-clotting protease source. *Industrial Crops and Products, 112*, 181–187. https://doi.org/10.1016/J.INDCROP.2017.10.050

Boye, J. I., & Alli, I. (2000). Thermal denaturation of mixtures of α-lactalbumin and β-lactoglobulin: A differential scanning calorimetric study. *Food Research International, 33*(8). https://doi.org/10.1016/S0963-9969(00)00112-5

Brishti, F. H., Chay, S. Y., Muhammad, K., Ismail-Fitry, M. R., Zarei, M., Karthikeyan, S., & Saari, N. (2020). Effects of drying techniques on the physicochemical, functional, thermal, structural and rheological properties of mung bean (Vigna radiata) protein isolate powder. *Food Research International, 138*. https://doi.org/10.1016/j.foodres.2020.109783

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

cOmplete Protease Inhibitor Cocktail tablets Roche. n.d. https://www.sigmaaldrich.com/NL/en/product/roche/coro?gclid=Cj0KCQjwmouZBhDSARIsALYcouqaFp9n

viqANqWBbPGOUvfTbv5oqTASCZJAvPpVBoaYSI718yNU1pQaAgdfEALw_wcB&gclsrc=aw.ds. (Accessed 20 June 2023)
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
da Veiga Leprevost, F., Haynes, S. E., Avtonomov, D. M., Chang, H. Y., Shanmugam, A. K., Mellacheruvu, D., Kong, A. T., & Nesvizhskii, A. I. (2020). Philosopher: A versatile toolkit for shotgun proteomics data analysis, 2020 17:9 *Nature Methods, 17*(9), 869–870. https://doi.org/10.1038/S41592-020-0912-Y.
Day, R., Bennion, B. J., Ham, S., & Daggett, V. (2002). Increasing temperature accelerates protein unfolding without changing the pathway of unfolding. *Journal of Molecular Biology, 322*(1). https://doi.org/10.1016/S0022-2836(02)00672-1
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/FIGURES/5
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
Gagaoua, M., Hoggas, N., & Hafid, K. (2015). Three phase partitioning of zingibain, a milk-clotting enzyme from Zingiber officinale Roscoe rhizomes. *International Journal of Biological Macromolecules, 73*(1), 245–252. https://doi.org/10.1016/J.IJBIOMAC.2014.10.069
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
Grossmann, L., Hinrichs, J., Goff, H. D., & Weiss, J. (2019). Heat-induced gel formation of a protein-rich extract from the microalga Chlorella sorokiniana. *Innovative Food Science & Emerging Technologies, 56*, Article 102176. https://doi.org/10.1016/J.IFSET.2019.06.001
Guo, X., Zhou, H., Yu, Z., & Zhang, Y. (2007). Changes in the distribution of nitrogen and plant enzymatic activity during ensilage of lucerne treated with different additives. *Grass and Forage Science, 62*(1), 35–43. https://doi.org/10.1111/J.1365-2494.2007.00559.X
Hadidi, M., Ibarz, A., Conde, J., & Pagan, J. (2019). Optimisation of steam blanching on enzymatic activity, color and protein degradation of alfalfa (Medicago sativa) to improve some quality characteristics of its edible protein. June 2018 *Food Chemistry, 276*, 591–598. https://doi.org/10.1016/j.foodchem.2018.10.049.
Havé, M., Marmagne, A., Masclaux-Daubresse, C. (2017). Nitrogen remobilization during leaf senescence: Lessons from Arabidopsis to crops. *Journal of Experimental Botany, 68*(10), 2513–2529. https://doi.org/10.1093/jxb/erw365
Hazra, S., Guha, R., Jongkey, G., Palui, H., Mishra, A., Vemuganti, G. K., Basak, S. K., Mandal, T. K., & Konar, A. (2012). *Modulation of matrix metalloproteinase activity by EDTA prevents posterior capsular opacification*. http://www.molvis.org/molvis/v18/a175.
Hoppenreijs, L. J. G., Brune, S. E., Biedendieck, R., Krull, R., Boom, R. M., & Keppler, J. K. (2023). Fibrillation of β-lactoglobulin at pH 2.0: Impact of cysteine substitution and disulfide bond reduction. *Food Hydrocolloids, 141*. https://doi.org/10.1016/j.foodhyd.2023.108727
Hoppenreijs, L. J. G., Overbeck, A., Brune, S. E., Biedendieck, R., Kwade, A., Krull, R., Boom, R. M., & Keppler, J. K. (2023). Amyloid-like aggregation of recombinant β-lactoglobulin at pH 3.5 and 7.0: Is disulfide bond removal the key to fibrillation? *International Journal of Biological Macromolecules, 242*. https://doi.org/10.1016/j.ijbiomac.2023.124855
Ikeda, S., & Nishinari, K. (2001). "Weak gel"-type rheological properties of aqueous dispersions of nonaggregated κ-carrageenan helices. *Journal of Agricultural and Food Chemistry, 49*(9), 4436–4441. https://doi.org/10.1021/JF0103065
Jia, W., Sethi, D. S., van der Goot, A. J., & Keppler, J. K. (2022). Covalent and non-covalent modification of sunflower protein with chlorogenic acid: Identifying the critical ratios that affect techno-functionality. *Food Hydrocolloids, 131*. https://doi.org/10.1016/j.foodhyd.2022.107800
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
Kavanagh, G. M., Clark, A. H., & Ross-Murphy, S. B. (2000). Heat-induced gelation of globular proteins: Part 3. Molecular studies on low pH β-lactoglobulin gels. *International Journal of Biological Macromolecules, 28*(1). https://doi.org/10.1016/S0141-8130(00)00144-6
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
Kleuter, M., Yu, Y., Pancaldi, F., Nagtzaam, M., van der Goot, A. J., & Trindade, M. L. (n. d.). Cell wall as a barrier for protein extraction from tomato leaves: A biochemical study. Submitted.
Kong, A. T., Leprevost, F. V., Avtonomov, D. M., Mellacheruvu, D., & Nesvizhskii, A. I. (2017). MSFragger: Ultrafast and comprehensive peptide identification in mass spectrometry–based proteomics, 2017 14:5 *Nature Methods, 14*(5), 513–520. https://doi.org/10.1038/NMETH.4256.
Kumar Dubey, V., & Jagannadham, M. V. (2003). Procerain, a stable cysteine protease from the latex of Calotropis procera. *Phytochemistry, 62*(7), 1057–1071. https://doi.org/10.1016/S0031-9422(02)00676-3
Lo Piero, A. R., Puglisi, I., & Petrone, G. (2002). Characterization of "lettucine": A serine-like protease from Lactuca sativa leaves, as a novel enzyme for milk clotting. *Journal of Agricultural and Food Chemistry, 50*(8), 2439–2443. https://doi.org/10.1021/JF011269K/ASSET/IMAGES/LARGE/JF011269KF00006.JPEG
Luo, J., Xiao, C., Zhang, H., Ren, F., Lei, X., Yang, Z., & Yu, Z. (2018). Characterization and milk coagulating properties of Cynanchum otophyllum Schneid. proteases. *Journal of Dairy Science, 101*(4), 2842–2850. https://doi.org/10.3168/jds.2017-13888
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
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
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
Otte, J., Ju, Z. Y., Færgemand, M., Lomholt, S. B., & Qvist, K. B. (1996). Protease-induced aggregation and gelation of whey proteins. *Journal of Food Science, 61*(5), 911–916. https://doi.org/10.1111/j.1365-2621.1996.tb10900.x
Otte, J., Ju, Z. Y., Skriver, A., & Qvist, K. B. (1996). Effects of limited proteolysis on the microstructure of heat-induced whey protein gels at varying pH. *Journal of Dairy Science, 79*(5), 782–790. https://doi.org/10.3168/jds.S0022-0302(96)76426-3
Otte, J., Schumacher, E., Ipsen, R., Ju, Z. Y., & Qvist, K. B. (1999). Protease-induced gelation of unheated and heated whey proteins: e!ects of pH, temperature, and concentrations of protein, enzyme and saltsJ. Otte (Ed.). *International Dairy Journal, 9*.
Pardossi, A., Tognoni, F., & Incrocci, L. (2004). Mediterranean greenhouse technology. *Chronica Horticulturae, 44*(2), 28–34.
Pinedo, M. L., Hernández, G. F., Conde, R. D., & Tognetti, J. A. (2000). Effect of low temperature on the protein metabolism of wheat leaves. *Biologia Plantarum, 43*(3), 363–367. https://doi.org/10.1023/A:1026765504374/METRICS
Rawlings, N. D., & Barrett, A. J. (1994). [2] Families of serine peptidases. *Methods in Enzymology, 244*(C), 19–61. https://doi.org/10.1016/0076-6879(94)44004-2
Sanchez, A. C., & Burgos, J. (1997). Factors affecting the gelation properties of hydrolyzed sunflower proteins. *Journal of Food Science, 62*(2), 284–288. https://doi.org/10.1111/J.1365-2621.1997.TB03986.X
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*(April), 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., 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*. https://doi.org/10.1016/j.ifset.2022.103018
Schurz, J. (1967). Rheological methods. *Polymer Fractionation*, 317–339. https://doi.org/10.1016/B978-1-4832-3245-4.50016-9
Stading, M., & Hermansson, A. M. (1990). Viscoelastic behaviour of β-lactoglobulin gel structures. *Food Hydrocolloids, 4*(2), 121–135. https://doi.org/10.1016/S0268-005X(09)80013-1
Tamayo Tenorio, A., Gieteling, J., De Jong, G. A. H., Boom, R. M., & Van Der Goot, A. J. (2016). Recovery of protein from green leaves: Overview of crucial steps for utilisation. *Food Chemistry, 203*, 402–408. https://doi.org/10.1016/j.foodchem.2016.02.092
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. August 2017 *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*. 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
Teo, G. C., Polasky, D. A., Yu, F., & Nesvizhskii, A. I. (2021). Fast deisotoping algorithm and its implementation in the MSFragger search engine. *Journal of Proteome Research, 20*(1), 498–505. https://doi.org/10.1021/ACS.JPROTEOME.0C00544/SUPPL_FILE/PR0C00544_SI_002.ZIP
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

Yu, F., Haynes, S. E., & Nesvizhskii, A. I. (2021). IonQuant enables accurate and sensitive label-free quantification with FDR-controlled match-between-runs. *Molecular & Cellular Proteomics: MCP, 20*. https://doi.org/10.1016/J.MCPRO.2021.100077

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*. , Article 135072. https://doi.org/10.1016/J.FOODCHEM.2022.135072

Zavaleta-Mancera, H. A., Franklin, K. A., Ougham, H. J., Thomas, H., & Scott, I. M. (1999). Regreening of senescent Nicotiana leaves. I. Reappearance of NADPH-protochlorophyllide oxidoreductase and light-harvesting chlorophyll a/b-binding protein. *Journal of Experimental Botany, 50*(340). https://doi.org/10.1093/jxb/50.340.1677

Zhang, W., Grimi, N., Jaffrin, M. Y., & Ding, L. (2015). Leaf protein concentration of alfalfa juice by membrane technology. *Journal of Membrane Science, 489*, 183–193. https://doi.org/10.1016/J.MEMSCI.2015.03.092

Zhang, C., Jiang, S., Zhang, J., & Li, S. (2021). Effect of enzymatic hydrolysis on the formation and structural properties of peanut protein gels. *International Journal of Food Engineering, 17*(3), 167–176. https://doi.org/10.1515/IJFE-2018-0356/ASSET/GRAPHIC/J_IJFE-2018-0356_FIG_005.JPG