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ORIGINAL PAPER

Glycation of pea protein isolate with different mannooligosaccharides to improve solubility

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Abstract
Due to the increasing demand for plant based proteins in food products, research is being carried out to improve the functional properties of pea protein. Here, the glycation of pea protein isolate (PPI) with mannooligosaccharides (MOS) was studied, with the aim to increase solubility. Various MOS were released by enzymatic hydrolysis of galactomannans and separated into four fractions according to their degree of polymerization (DP; DP of 2, 3, 7–9 and ≥9). MOS reacted with PPI under controlled conditions via the Maillard reaction. Different reaction times were tested for the MOS fractions to vary the extent of glycation and the progress of the Maillard reaction. Thus, the color and the decrease in free amino groups were measured, and gel electrophoresis was performed to observe the change in molecular weight of the proteins. Based on the results, MOS fractions with a DP of 7–9 and ≥9 and reactions times of 24–48 h, respectively, were classified as promising due to a moderate decrease in free amino groups and only a slight change in color. Differently, glycation with mannobiose and mannotriose resulted in advanced glycation and was considered unsuitable. The solubility of the glycated proteins was determined at a pH of 3.5 and 7.0. Under neutral conditions, solubility did not change as a result of glycation. However, under acidic conditions, glycation increased the solubility of the pea proteins from 26% to about 46%.

Keywords Plant based protein · Protein modifications · Galactomannans · Dietary fiber · Maillard reaction · Protein-oligosaccharide conjugates

Introduction

Currently, there is a heightened demand for protein enriched food products. Plant proteins are becoming increasingly a desired source of protein as their production is more sustainable and cost-effective compared to animal proteins. Therefore, there is great interest in replacing animal proteins with plant proteins in various food products [1]. After soy protein, pea (*Pisum sativum* L.) protein is the most widely used plant protein, offering high nutritional quality and health-related benefits in addition to wide availability and low-cost production. In addition, unlike soy, there are currently no concerns of pea allergenicity [2]. Pea protein consists mainly of water-soluble albumins (10-20%, 5-80 kDa) and salt-soluble globulins (70-80%), the latter being further classified into legumin (11 S storage proteins) and vicilin (7 S storage proteins) [3]. Overall, pea protein has a high content of lysine, but limited amounts of methionine and tryptophan [3]. In terms of functional properties, pea protein lags behind soy protein [1], and is therefore a plant protein with high potential for development. To be used in food products (e.g., in meat alternatives, protein drinks), proteins must generally fulfill requirements in terms of nutritional quality, flavor, and specific functionality [1]. To achieve the desired functional performance, proteins can be modified physically, biologically, or chemically. One potential chemical approach is Maillard-induced glycation [2].

The Maillard reaction is a well-known browning reaction in foods. However, the same mechanism can also be utilized for the glycation of proteins if it is stopped at an early stage. In the first step of the Maillard reaction, an amino group of the protein reacts with the carbonyl group of a reducing sugar unit in a condensation reaction. Thus, only the

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reducing unit of an oligo- or polysaccharide can react with the protein. The ε-amino group of lysine is highly reactive and represents the largest proportion of available primary amines in proteins, making it of particular interest for this reaction [4]. The N-substituted glycosylamine formed from aldoses further reacts to form the Schiff base and rearranges to form the Amadori product. In the later stages of the Maillard reaction, however, Amadori products convert to deoxyosones and are further degraded through enolization, dehydration, fragmentation, oxidation, acid hydrolysis, and/or reactions with free radicals. As a consequence, various advanced products such as furfural, hydroxymethylfurfural, pyruvaldehyde, and/or diacetyl are formed. In the last steps of the Maillard reaction, nitrogen-containing, brown-colored polymers and copolymers called melanoidins are formed [5]. Generally, products of the advanced stages of the Maillard reaction are not favored as these may be colored and/or may raise concern as potential food borne contaminants. Thus, in order to take advantage of the benefits of the Maillard reaction for the glycation of proteins, it must be carried out under controlled conditions. Relevant reaction parameters include pH, temperature, the ratio of amino to carbonyl groups, relative humidity, and the reaction time [4]. Although being a challenging task, glycation of proteins with hydrophilic poly- or oligosaccharides such as gum Arabic, pectins, and maltodextrin has been demonstrated to increase solubility, emulsifying properties, and heat stability [6, 7]. In addition, glycation may remove pea protein off-flavor [6].

Mannans are hemicelluloses of the plant cell wall, belonging to the dietary fiber complex. Mannans are based on a backbone of β-1,4 linked D-mannose units that can be substituted with side chains of α-D-galactose units attached in position *O*6 of the backbone units. In addition, D-glucose units may be incorporated into the backbone [8]. Cleavage of mannans by the enzyme *endo*-β-1,4-mannanase results in mannooligosaccharides (MOS) of different degree of polymerization (DP). MOS can also be obtained by an enzymatic treatment of mannan-rich by-products of the food industry such as coffee grounds and currant or coconut pomaces [9]. Currently used mannan-rich materials include locust bean gum (LBG), guar gum, and konjac flour. These sources are more expensive than by-products, and from a sustainability point of view, MOS generation from by-products would be preferred. However, due to their high amounts of mannans, materials such as LBG are well suited to generate high amounts of MOS for model studies. LBG originates from seeds of the carob tree and is used in food and pharmaceutical industry as thickening and gelling agent. LBG mostly consists of galactomannans, having a ratio of mannose to galactose of roughly 4:1 [10].

As the focus in past studies was mostly on the protein component of the Maillard-induced glycation [6, 7, 11–14], our study aimed to broaden the knowledge on the role of the carbohydrate used. Therefore, the focus of this study was to isolate fractions of mannooligosaccharides with defined DP and study their suitability for the glycation of pea protein isolate (PPI). From a technofunctional point of view, we aimed to increase the solubility of pea protein through glycation with mannooligosaccharides, thus enabling its use in liquid or semi-liquid food products such as protein enriched drinks.

Materials and methods

Materials and reagents

Yellow pea flour was kindly provided by AGT Foods and Ingredients Inc. (Regina, SK, Canada). PPI was produced by alkaline extraction at pH 7.5 and precipitation at pH 4.5 at the Joseph J. Warthesen Food Processing Center of the University of Minnesota as detailed in Hansen *et al*. [15]. Commercial pea protein isolate (cPPI, ProFam® Pea 580, 79.5% protein) was provided by Archer Daniels Midland (ADM; Decatur, IL, USA). *Endo*-β-1,4-mannanase (from *Aspergillus niger*, GH26, EC 3.2.1.78, ~ 600 U/mL) was purchased from Megazyme (Bray, Ireland). Pierce™ BCA assay kit and SnakeSkin™ Dialysis Tubing (molecular weight cut off (MWCO) of 10 kDa) were from Thermo Fisher Scientific Inc. (Waltham, MA, USA). β-Mercaptoethanol (βME), running buffer (10x Tris/glycine/SDS running buffer), Laemmli buffer, Precision Plus Protein™ molecular weight marker, and Criterion™ TGX™ Precast polyacrylamide gel (4–20%, 18-well) were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). LBG, ethanol, sodium hydroxide, sodium acetate, albumin, dextrose, L-lysine, sodium dodecyl sulfate (SDS), sodium tetraborate, o-phthaldialdehyde (OPA), ethylenediaminetetraacetic acid (EDTA), and glycine were purchased from Sigma-Aldrich (St. Louis, MO, USA or Darmstadt, Germany).

Preparation of mannooligosaccharides

LBG was dissolved in deionized water (1% (weight (w)/volume (v))) and mannanase (100 µL) was added. The suspension was incubated for 24 h at 60 °C with shaking, followed by enzyme inactivation at 95 °C for 10 min. Residual polymers were precipitated with 60% (v/v) ethanol. The suspension was centrifuged, and the supernatant containing MOS was rotary evaporated (max. temperature 50 °C). MOS were redissolved in deionized water and separated with size exclusion chromatography (SEC) using a Bio Gel

P-2 column (bed volume: 85 × 2.6 cm; BioRad Laboratories, Feldkirchen, Germany). Deionized water with a flow rate of 0.5 mL/min was used as eluent. The eluate was monitored using a Smartline RI detector 2300 (KNAUER Wissenschaftliche Geräte GmbH, Berlin, Germany) at 45 °C. The eluate was collected in 6 min fractions. Fractions were combined according to the chromatograms, resulting in four fractions (F1 - F4) (Fig. 1).

Structural characterization of mannooligosaccharides

Degree of polymerization

To get information about the purity and DP of the isolated fractions, they were measured using a system of high performance anion exchange chromatography (HPAEC) coupled with pulsed amperometric (PAD) and mass spectrometric (MS) detectors as described earlier by Steck and co-workers [16]. In brief, a CarboPac PA-200 column (6 µm particle size, 250 × 3 mm, Thermo Fisher Scientific) was used, and double deionized water (A), 0.1 M NaOH (B), and 0.1 M NaOH + 0.5 M sodium acetate (C) formed the mobile phase

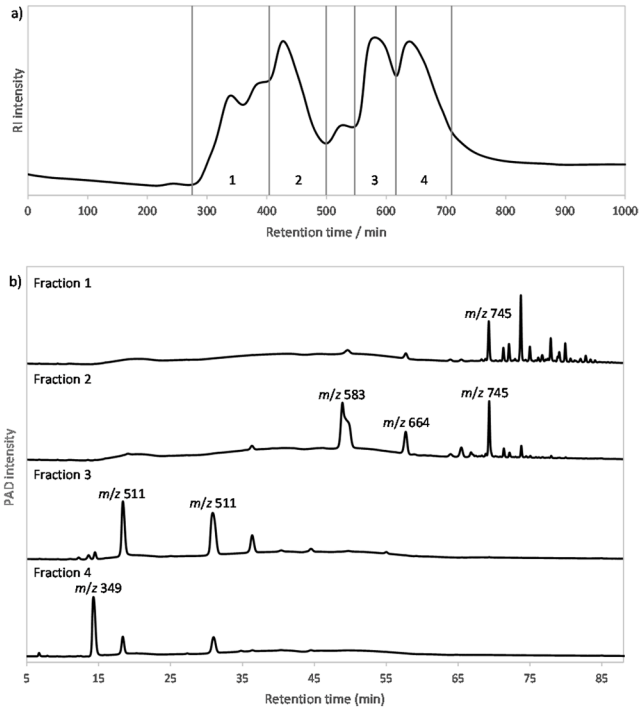

Fig. 1 **a** Refractive index (RI) chromatogram of the size exclusion chromatography (SEC) based separation of mannooligosaccharides released from locust bean gum by *endo*-β-1,4-mannanase. **b** High performance anion exchange chromatography with pulsed amperometric detection and mass spectrometry (HPAEC-PAD/MS) based chromatograms of the analysis of the SEC separated fractions of mannooligosaccharides. Mass to charge ratios (*m*/*z*) of Li adducts of selected dominant signals up to a degree of polymerization of 9 are labeled.

(flow rate: 0.4 mL/min). For separating MOS, the column was rinsed with 100% C for 10 min and with 95% A and 5% B for 20 min prior to injection. Following injection, the eluent was kept constant for 10 min at 95% A and 5% B, followed by a linear gradient from 10 to 15 min to 90% A and 10% B, kept constant for 10 min, followed by a linear gradient from 25 to 35 min to 80% A and 20% B, from 35 to 45 min to 45% A and 55% B, and from 45 to 75 min to 40% A, 55% B, and 5% C. Finally, the linear gradient was changed to 100% C from 75 to 85 min to rinse the column. For the simultaneous detection by PAD and MS, the eluate was split and desalted by an electrolytically regenerated suppressor (Dionex AERS 500 + AXP pump, Thermo Fisher Scientific) for electrospray ionization (ESI) coupled with MS detection. To facilitate the formation of stable adducts, lithium chloride solution was added to the eluate before entering the MS detector. The Li adducts of the analytes were detected as $[M + Li]^+$ or $[M + 2*Li]^{2+}$, enabling the calculation of the DP.

Monosaccharide composition

The monosaccharide compositions of F1 - F4 were analyzed following trifluoroacetic acid (TFA) hydrolysis. MOS containing fractions were treated with 2 M TFA for 1 h at 121 °C [17], evaporated, and the samples were washed twice with ethanol and evaporated to remove TFA residues. The residues were dissolved in double deionized water and 2-deoxy-D-glucose was added as internal standard (final concentration: 25 µM). Monosaccharide analysis was performed by HPAEC-PAD using a CarboPac PA-20 column (6 µm particle size, 150 × 3 mm, Thermo Fisher Scientific) and a mobile phase consisting of double deionized water (A), 0.1 M NaOH (B), and 0.1 M NaOH + 0.5 M sodium acetate (C) (flow rate: 0.4 mL/min). Prior to injection, the column was rinsed with 100% B for 10 min and with 90% A and 10% B for 10 min. The eluent composition was linearly changed from 0 to 1.5 min to 97% A and 3% B, followed by an isocratic elution from 1.5 to 22 min and a linear change from 22 to 27 min to 100% B. The last 10 min of elution were performed isocratically with 60% B and 40% C. For calibration, galactose and mannose standards were used in concentrations of 1–125 µM. The monosaccharide composition of the fractions was measured in duplicate.

Glycation

PPI and oligosaccharide fractions F1 - F4 (1:4, w/w) were solubilized in a potassium phosphate buffer (0.01 M, pH 7.0, 1:4 w/v). After lyophilization, the mixture was ground in a mortar and distributed evenly in petri dishes (0.4 g in 23 mm diameter dishes or 2.472 g in 87 mm diameter dishes).

Samples were incubated at 60 °C and 49% relative humidity [7] for different periods of time (6–48 h). Each experiment was done in triplicate, and the samples were combined after measuring the color. This combined sample was used for further analysis.

Characterization of the glycated protein

Color

To monitor unwanted browning, the color of the samples was measured before and after glycation using the colorimeter Chroma Meter CR-221 (Minolta Camera Co., Osaka, Japan). L^* (lightness), a^* (redness), and b^* (yellowness) values of the CIE (commission internationale de l'éclairage) system were measured in triplicate [11].

Loss of free amino groups

To determine the loss of free amino groups, the OPA method was performed as described by Goodno and co-workers [18] with modification of preparing the sample solutions in 3% SDS as described by Schneider and co-workers [7]. A standard curve was generated using L-lysine. Protein contents were measured using Pierce™ BCA assay kit following the manufacturer's instructions. Finally, the loss of free amino groups was determined by comparing free amino groups per protein before and after glycation. All samples were measured in triplicate.

SDS-poly acrylamide gel electrophoresis (PAGE)

To monitor changes in the protein profile, SDS-PAGE was done using Criterion™ Cell and PowerPac™ Basic Power (BioRad Laboratories, Hercules, CA, USA), Criterion TGX Precast polyacrylamide gels (4–20%, 18-Well), and a Bio-Rad running buffer (10x Tris/glycine/SDS running buffer) at 200 V [13]. Samples (to final concentration of 12.5 µg in 5 µL) were prepared under non-reducing (100 µL sample + 100 µL Laemmli buffer) and reducing conditions (100 µL sample + 95 µL Laemmli buffer + 5 µL βME) and boiled for 5 min. A molecular weight marker was used for comparison. After completion of the electrophoresis run, the gel was stained using an imperial protein stain solution for 45 min, while shaking. After staining, the gel was washed with water. Molecular Imager Gel XR system (BioRad Laboratories, Hercules, CA, USA) was used for imaging the gels.

Protein content

An automated Leco® FP828 nitrogen analyzer (LECO, St. Joseph, MI, USA) was used to measure protein contents in duplicate, following the Dumas principle (AOAC 990.03), with a standard nitrogen-protein conversion factor of 6.25. Calibration was performed using EDTA for solid samples and glycine for liquid samples.

Purification of glycated proteins by dialysis

The mixture of MOS and PPI after glycation was solubilized in double deionized water (8% (w/v) solution) and placed in a dialysis tube with a MWCO of 10 kDa. Dialysis was performed at 4 °C with ten water changes at 3–4 h intervals, followed by lyophilizing of the retained glycated proteins.

Solubility

The protein solubility was determined as described by Wang and Ismail [19]. Suspensions of the purified glycated PPI (5% (w/v)) were prepared by stirring for 2 h at room temperature. The pH was adjusted to 7.0 or 3.4 (Eutech™ pH 700 m, Thermo Fisher Scientific Inc., Waltham, MA, USA) by using NaOH (0.1 N) and HCl (0.1 N) after stirring and was checked after 1 h of further stirring, respectively. The suspension was divided into three parts. One part was kept to represent the initial sample. Another part was heated for 30 min at 80 °C (heated sample) and the third part was left at room temperature (non-heated sample). The heated and non-heated samples were centrifuged (Microcentrifuge 5418, Eppendorf, Hamburg, Germany). The protein contents of the initial sample and the supernatants of the heated and non-heated sample were measured via the Dumas method (AOAC 990.03). The solubility was calculated as the ratio of the protein contents of the supernatants of the heated or non-heated sample after centrifugation to the protein content of the initial sample without heat treatment and centrifugation. Samples were prepared in duplicate, and the protein content of each duplicate was measured twice. For statistical analysis the mean of protein solubility for each sample was used.

Statistical analysis

Analysis of variance (ANOVA) and t-tests were performed OriginPro 2023 for Windows. A paired t-test was used to determine significant differences ($p \leq 0.05$) between the means of two paired samples (color changes). Tukey test ($p \leq 0.05$) was used to determine significant differences between means of different samples (loss of free amino groups, solubility).

Table 1 Degree of polymerization (DP) and monosaccharide composition (mol% ± range/2, $n=2$) of four fractions of mannooligosaccharides isolated by size exclusion chromatography

	DP	Monosaccharide composition	
		Mannose / mol%	Galactose / mol%
Fraction 1	≥9	65.3±0.2	34.7±0.2
Fraction 2	7–9	66.7±0.1	33.3±0.1
Fraction 3	3	79.4±0.1	20.6±0.1
Fraction 4	2	94.1±0.0	5.9±0.0

Results and discussion

Production and structural characterization of mannooligosaccharides

LBG was enzymatically hydrolyzed using an *endo*-β-1,4-mannanase. As previous studies showed that the DP of the oligosaccharides affects protein glycation under controlled conditions [7, 12, 19], we were aiming to compare MOS of different DP. Thus, the hydrolysis conditions were optimized in order to obtain MOS fractions of different DP instead of using an end point incubation resulting in a very limited diversity of the liberated MOS. Separation of liberated MOS from residual polysaccharides was achieved by precipitation of the polysaccharides using 60% ethanol. Usually 80% ethanol is used to separate oligosaccharides from soluble polysaccharides [20]. However, as some oligosaccharides with a higher DP precipitate at this high percentage of ethanol (data not shown), 60% ethanol was chosen instead to increase both yield and diversity of MOS.

The liberated MOS were fractionated using SEC resulting in the isolation of four fractions containing MOS of different DP (Fig. 1a). Before being used for glycation experiments, fractions F1 - F4 were chemically characterized. The two peaks in F1 were combined as one fraction because both peaks showed similar HPAEC-PAD/MS data. Since LBG are galactomannans, MOS substituted with galactose are also released by treatment with mannanase in addition to unsubstituted MOS. According to HPAEC-PAD/MS data (Fig. 1b) and analysis of the monosaccharide composition (Table 1), the oligosaccharides in F1 were characterized by a DP of ≥ 9. F2 contained oligosaccharides with a DP of 7-9. The ratio of mannose to galactose in both fractions was about 2. Thus, the major difference of F1 and F2 oligosaccharides was based on their DP and not on their degree of substitution with galactose. By comparison with standard compounds of commercial MOS and isolated galactomannooligosaccharides [9], F2 contained predominantly a disubstituted mannopentaose and a disubstituted mannohexaose (Fig. 2b and c). Application of previously mentioned analyses to F3 demonstrated a mannose to galactose ratio of about 5, and comparison with standard compounds revealed that F3 mainly consists of mannotriose and a monosubstituted mannobiose (Fig. 2a). F4 mainly contained mannobiose, consistent with a low galactose content of 6%. Thus, differently from previous studies, in which oligosaccharides with DP 2–5 were observed after incubating LBG with mannanase [21, 22], the mannanase used here released also MOS with a higher DP under the selected hydrolysis conditions.

Glycation using different mannooligosaccharide fractions

Color

Initially, PPI was incubated with the four MOS fractions for 24 h. Following glycation with F3 and F4, the samples showed a strong decrease in the lightness (L^*) and red (a^*)

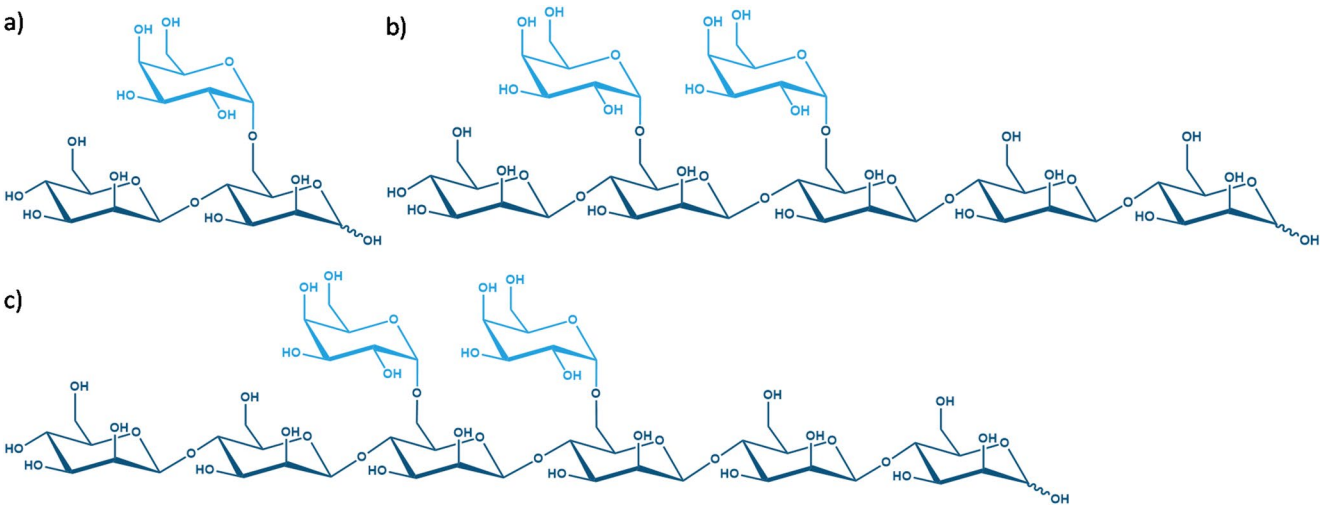

Fig. 2 Structures of some major galactomannooligosaccharides that were identified in the size exclusion chromatography fractions: **a** monosubstituted mannobiose, **b** disubstituted mannopentaose, **c** disubstituted mannohexaose, dark blue: D-mannopyranose, light blue: D-galactopyranose

Table 2 Color (L^* a^* b^*) of the mixtures of pea protein isolate (PPI) and oligosaccharide fractions 1 - 4 (F1 - F4) before (0 h) and after (24 h) incubation

	L^*		a^*		b^*		Visual observation	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
PPI + F1	85.81[a]	85.87[a]	0.01[a]	-0.10[a]	13.01[a]	13.53[a]		
PPI + F2	87.82[a]	84.86[b]	-0.35[a]	-0.64[b]	11.21[a]	13.26[b]		
PPI + F3	86.58[a]	53.75[b]	-0.35[a]	-1.38[b]	11.84[a]	17.38[b]		
PPI + F4	85.04[a]	45.29[b]	-0.26[a]	-0.50[a]	12.56[a]	20.15[b]		

Lowercase letters indicate significant differences between means ($n=3$) of a sample before and after incubation according to a paired t-test ($p \leq 0.05$)

color values and an increase in the yellow value (b^*). Differently, there were no or only slight color changes if F1 or F2 were used for glycation (Table 2). Decreasing L^* values indicate later stages of the Maillard reaction, in which browning occurs, whereas controlled Maillard reaction, in which progression beyond the initial/intermediate stage is largely avoided, does not result in any visible color changes [23]. Therefore, fractions F3 and F4 were deemed less suitable for the glycation of PPI than F1 and F2. Slightly greater color changes than for PPI incubated with F1 or F2 were observed by Schneider and co-workers [7] when PPI was incubated with maltodextrin with an average DP of 8.3. However, since the maltooligosaccharides were not characterized by a narrow DP range, but represented a mixture of predominantly oligosaccharides with DP from 2 to 20, the color changes probably originated from the smaller oligosaccharides as observed here when F3 and F4 were used for glycation.

Loss of free amino groups

Color changes due to Maillard reaction are usually also reflected in a more pronounced decrease in free amino groups as oligosaccharides are initially covalently bound to free ε-amino groups of lysine [6]. Also, limited glycation of the essential amino acid lysine is important to secure the biological value of PPI. Therefore, a low to moderate decrease in the loss of free amino groups between roughly 15% and 28% was targeted. The loss of free amino groups was 10.1%

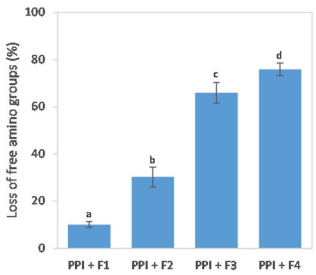

Fig. 3 Loss of free amino groups of pea protein isolate (PPI) after glycation with oligosaccharide fractions 1 - 4 (F1–4) for 24 h (% ± SD), lowercase letters indicate significant differences between means ($n=3$) of different samples according to the Tukey test ($p \leq 0.05$)

and 30.3% for PPI glycated with F1 and F2, respectively (Fig. 3). Unacceptably high losses in amino groups, up to 75.9%, were observed when F3 or F4 were used as source of carbohydrates. It was demonstrated previously that glycation is faster when monosaccharides are used compared to di- or oligosaccharides [12]. The assumption that glycation also occurs faster with di- and small oligosaccharides as compared to larger oligosaccharides can be confirmed for MOS. For the glycation experiments, the MOS to protein ratios were always calculated on weight basis. Therefore, fractions containing larger amounts of oligosaccharides with a smaller DP have more reducing ends being available for the reaction with proteins. Based on the high loss of free amino groups in combination with advanced browning, F4 was assessed as unsuitable for the glycation of PPI and was not studied further. Fraction F3 showed less (as compared

to F4) but still substantial changes in color and loss of free amino groups in the glycation experiments for 24 h; however, further incubation times were tested to see if F3 could perform better under different conditions.

Using the same incubation conditions (60 °C, 49% relative humidity, 24 h), a loss of free amino groups of 29.6% was observed for PPI glycated with maltodextrin [7]. A comparable loss of free amino groups of 25.9% was described when pea protein concentrate was glycated with gum Arabic for three days at 79% relative humidity and 60 °C [6]. However, the authors reported already a decrease in free amino groups of 11.4% when pea protein concentrate was incubated without gum Arabic. Differently, no changes were observed in our study when PPI was incubated without the addition of MOS (data not shown). These differences between the two studies might be due to different amounts of reducing carbohydrates in the starting protein samples, depending on the protein isolation process performed.

Adjustment of glycation time and scale-up

Additional incubation times were tested to achieve only moderate color changes and to adjust for the loss of free amino groups as a result of glycation (Table 3). Longer incubations were carried out for F1 and shorter incubations for F2 and F3, followed by analyses of color change and loss of free amino groups. In spite of a short incubation time of 6 h, an unacceptable color change and a loss of free amino groups of 40.3% were observed when PPI was incubated with F3, excluding also F3 from being a candidate to glycate PPI. Although glycation with oligosaccharides of a larger DP requires a longer incubation time, these oligosaccharides, in contrast to mono-, di-, and small oligosaccharides, offer the possibility of performing Maillard-induced glycation in a controlled manner. As mentioned before, fractions with a lower degree of polymerization, such as F3 and F4, have more reducing ends (on a weight basis) available to react with the protein. Experiments in which dextrans of different molecular sizes were used for glycation of whey protein also showed a higher degree of glycation when smaller dextrans were used (based on carbohydrate/protein weight ratios) [24]. However, Chevalier and coworkers [25] also observed small differences of the reactivity for monosaccharides with carbon skeletons of different lengths, i.e. arabinose and glucose, when these were used in the same molar ratio for the glycation of β-lactoglobulin. Monosaccharides with longer carbon chains were less reactive with amino groups. Since we used MOS fractions instead of individual MOS, it was not possible to perform the experiments on a molar basis here. However, we hypothesize that besides the increased number of reducing ends in F3 and F4, steric effects impact the glycation rate. The reaction may be limited by steric hindrance of the oligo- and polysaccharides to be attached. Thus, mono- and disaccharides potentially have more positions to which they can bind. However, if mono- and disaccharides are used for glycation the Maillard reaction usually progresses beyond the initial stage and may even result in insoluble aggregates [26]. Furthermore, highly branched polysaccharides such as dextrans are suggested to be less reactive than less branched polysaccharides such as galactomannans [27]. However, it is difficult to compare data from experiments with different proteins and polysaccharides under different glycation conditions and draw unambiguous conclusions.

Different from F3 and F4, the fractions F1 and F2 were deemed suitable for glycation of PPI without restrictions, given that the extent of loss of amino groups was below 10% when the incubation was performed with F1 for 18 h only. Based on these data, incubation times of 48 h for F1 and 24 h for F2 were chosen for scale-up experiments in order to obtain sufficient sample amounts for the determination of solubility. As desired, only minor changes in color occurred also after scaling up the glycation process (Table 4) with a slight decrease in L^* and a slight increase in b^*; a^* showed no relevant change. The loss of free amino groups after glycation with either F1 or F2 was around 25% and

Table 3 Color (L^* a^* b^*) of the mixture of pea protein isolate (PPI) and oligosaccharide fractions 1 - 3 (F1 - F3) before and after different incubation times and loss of free amino groups (% ± SD, $n=3$) of the mixture after incubation

	Incubation time (h)	L^*		a^*		b^*		Loss of free amino groups (%)
		before	after	before	after	before	after	
PPI+F1	18	85.64^{a}	85.82^{a}	-0.04^{a}	0.05^{a}	13.19^{a}	13.59^{a}	4.9 ± 0.1^{A}
	24	85.81^{a}	85.87^{a}	0.01^{a}	-0.10^{a}	13.01^{a}	13.53^{a}	10.1 ± 1.2^{B}
	36	85.64^{a}	85.74^{a}	0.09^{a}	0.09^{a}	13.12^{a}	14.00^{b}	13.2 ± 0.6^{C}
	48	85.54^{a}	85.62^{a}	0.00^{a}	-0.02^{a}	13.11^{a}	14.05^{b}	16.0 ± 0.2^{D}
PPI+F2	12	88.22^{a}	86.28^{b}	-0.27^{a}	-0.36^{a}	11.73^{a}	13.51^{b}	15.0 ± 3.1^{A}
	24	87.82^{a}	84.86^{b}	-0.35^{a}	-0.64^{b}	11.21^{a}	13.26^{b}	30.3 ± 4.2^{B}
PPI+F3	6	86.93^{a}	57.99^{b}	-0.26^{a}	-1.01^{b}	11.92^{a}	13.75^{b}	40.3 ± 1.7^{A}
	24	86.58^{a}	53.75^{b}	-0.35^{a}	-1.38^{b}	11.84^{a}	17.38^{b}	65.9 ± 4.3^{B}

Lowercase letters indicate significant differences between means ($n=3$) of a sample before and after incubation according to a, paired t-test ($p\leq0.05$)Uppercase letters numbers indicate significant differences between means (n=3) of different samples according to the Tukey test ($p\leq0.05$)

Table 4 Color (*L* a* b**) of the mixtures of pea protein isolate (PPI) and oligosaccharide fraction 1 (F1) and fraction 2 (F2) after large-scale incubation and loss of free amino groups (% ± SD, $n=3$) of the mixtures after large-scale incubation

	Incubation time (h)	L*		a*		b*		Loss of free amino groups (%)
		before	after	before	after	before	after	
PPI+F1	48	84.61[a]	84.53[a]	0.36[a]	0.34[a]	10.73[a]	12.39[a]	23.5±1.0
PPI+F2	24	86.04[a]	81.93[b]	0.06[a]	-0.47[b]	10.01[a]	11.68[b]	26.3±0.4

Lowercase letters indicate significant differences between means ($n=3$) of a sample before and after incubation according to a paired t-test ($p \leq 0.05$)

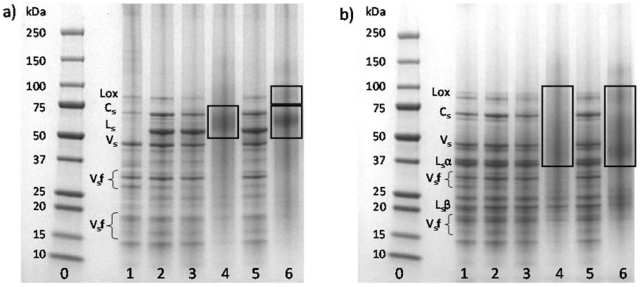

Fig. 4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of different glycated and non-glycated pea protein isolates (PPI) as performed under non reducing (**a**) and reducing conditions (**b**), lane 0: molecular weight marker, lane 1: commercial PPI, lane 2: PPI, lane 3: PPI+fraction 1 before glycation, lane 4: PPI+fraction 1 after glycation (48 h), lane 5: PPI+fraction 2 before glycation, lane 6: PPI+fraction 2 after glycation (24 h), Lox: Lipoxygenase, C_s: Subunits of convicilin, L_s: Subunits of legumin, V_s: Subunits of vicilin, V_sf: fractions of vicilin subunits result from posttranslational cleavages, $L_s\alpha$: acidic peptides cleaved from legumin subunits, $L_s\beta$: basic peptides cleaved of legumin subunits. The unedited gels are shown in the supplementary data (Figure S1).

thus in the targeted range. As mentioned before, a loss of 25% lysine does not decrease the biological value of PPI as demonstrated in a previous study, in which the protein digestibility-corrected amino acid score (PDCAAS) of glycated PPI was analyzed [7]. For commercial PPI (cPPI), PPI, and glycated PPI, the in vitro protein digestibility of all samples was around 100%. Moreover, the content of bioaccessible lysine at a 30% blockage was not reduced. The authors explained that despite the blockage, lysine (determined by amino acid analysis) was not degraded due to the Maillard reaction being limited to the initial stage [7].

Protein profiling of glycated PPI using SDS-PAGE

In order to demonstrate PPI glycation with MOS and changes of the molecular weight, protein profiling by SDS-PAGE was carried out (Fig. 4). In addition to the glycated PPI, PPI, the mixture of PPI and the MOS fraction before incubation, and a cPPI were analyzed. The samples were treated with SDS to denature and unfold the proteins as well as to mask their intrinsic charge. Under reducing conditions, βME cleaves disulfide linkages. Thus, separation is based on molecular size and less on charge and structure of the protein [28, 29]. Under reducing conditions, no visual differences can be observed between the cPPI and the used PPI. In contrast, under non-reducing conditions, PPI showed an additional band between 50 and 75 kDa, corresponding to legumin, and different intensities of other bands. Under reducing conditions, however, the acidic and basic subunits of legumin were clearly visible for cPPI. This, together with the light smearing of cPPI in the high molecular weight areas (> 250 kDa) under non-reducing conditions, indicated protein polymerization during the production and processing of cPPI, specifically legumin polymerization via disulfide linkages [15]. No differences were visible between PPI and the mixtures of PPI with the MOS fractions before incubation. Also, the bands for vicilin and convicilin were similar under non-reducing and reducing conditions because these protein components do not form disulfide linkages [15]. Under non-reducing conditions, PPI glycated with F1 showed a broad band in the range of 50 to 75 kDa, along with a reduced intensity of the bands of individual protein subunits compared to those in the PPI and the mixture of PPI and MOS. The broad band was also observed for PPI glycated with F2. In the latter case, an additional band in the range of 75 and 100 kDa was observed. The shift of the bands towards larger molecular weights suggested that subunits of the globulins legumin, convicilin, and vicilin were glycated.

Under reducing conditions, both PPI glycated with F1 and with F2 showed protein bands in the range of 37 to 100 kDa, corresponding to protein subunits that shifted to higher molecular weights compared to the PPI before glycation. This observation is attributed to the glycation of the subunits of convicilin and vicilin and the acidic subunits of legumin. The shift in molecular weight was greater than that observed of the basic subunits of legumin (at 25 kDa). This observation is supported by the fact that 7 S vicilin and 8 S convicilin have higher lysine contents than 11 S legumin [3, 12]. For the PPI glycated with F2, all the bands that shifted to a higher molecular weight range had a slightly higher intensity than those of PPI glycated with F1, indicating a higher quantity of glycated proteins. The observed broad bands of the glycated proteins indicated heterogeneity of the molecular weight distribution as a result of the attachment of different numbers of oligo-/polysaccharides [30] rather than due to different DP within the fractions. In this study, the molecular weight distribution of the MOS fractions is comparably narrow. Therefore, broad bands are

mostly attributed to the different number of oligosaccharides linked to the protein subunits within PPI. Furthermore, protein bands that correspond to very large polymers were not observed, suggesting the absence of aggregates formed through disulfide and other covalent interactions which can be formed during the advanced stage of the Maillard reaction [7]. To sum up, interpretation of protein profiling by SDS-PAGE and the determined loss of free amino groups demonstrated successful and controlled glycation of PPI with MOS fractions, resulting in PPI being modified to a likely favorable extent.

Purification of glycated pea protein isolate by dialysis

After successful glycation, the mixtures of glycated PPI and excess oligosaccharides were dialyzed to increase the protein purity to facilitate the evaluation of PPI solubility. Since SDS-PAGE observations confirmed that all relevant proteins had molecular weights larger than 10 kDa, a dialysis membrane with a MWCO of 10 kDa was selected. Although the MOS are much smaller than 10 kDa, their complete removal was not possible. In addition to size-related and steric effects potential, non-covalent interactions with the protein may contribute to incomplete removal of the MOS. Partial purification was demonstrated by an increase of the protein purity from 20.0% before dialysis to 29.8% after dialysis (PPI glycated with F1). For the PPI glycated with F2, the protein purity was further increased from 18.6% to 49.4%. This is in line with F2 being the fraction containing smaller MOS than F1 (Table 1). Another reported method for the purification of glycated proteins is hydrophobic interaction chromatography (HIC) and subsequent dialysis. Using this method, the protein purity was increased from around 18% to around 56% for PPI glycated with maltodextrin and to around 60% for glycated whey protein with dextran [7, 19]. In comparison to this more complex and cost-intensive method, dialysis using a 10 kDa MWCO membrane provides only slightly lower purification efficiency (in case of glycation with F2), thus representing a suitable alternative for the purification of glycated proteins on a large scale. It should also be mentioned that MOS are categorized as dietary fiber. Therefore, depending on the application of the glycated proteins, excess MOS contents can also be used to enrich food products with dietary fiber. In these cases, (excessive) purification of glycated protein samples might not be necessary. In case there is a need for a higher protein purity, membrane filtration could be coupled with conditions that might disrupt non-covalent interactions between the carbohydrates and the proteins, such as using salt.

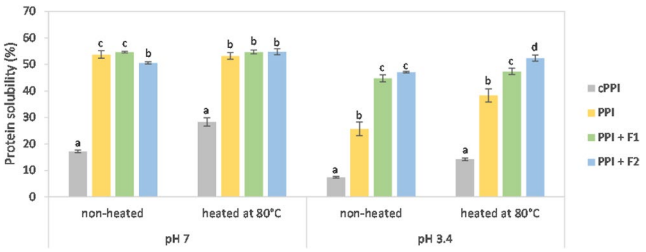

Fig. 5 Solubility of pea protein isolate (PPI) glycated with fraction 1 (F1) and fraction 2 (F2) in comparison to commercial PPI (cPPI) and non-glycated PPI in 5% protein solution (% ± range/2, $n=2$), lowercase letters indicate significant differences between means ($n=2$) of different samples according to the Tukey test ($p \leq 0.05$)

Protein solubility

Despite residual MOS contents in the samples after dialysis, protein solubility of the partially purified, glycated proteins was analyzed in comparison to the non-glycated PPI. Suspensions containing 5% protein were investigated as a model for high-protein beverages, which usually contain more than 4.2% protein [7]. When protein solubility of cPPI and PPI were compared, PPI showed a significantly higher solubility under all four conditions studied, but the solubility was generally low (< 55%) (Fig. 5), demonstrating the need for improvement. Industrial production of cPPI involves harsh extraction and processing conditions resulting in poor solubility caused by protein denaturation and polymerization [15]. High surface hydrophobicity, low surface charge, and aggregation were described as a consequence of protein denaturation [7]. At pH of 3.4, the solubility was lower as compared to that at pH of 7.0, because the acidic pH was near the isoelectric point (4.5) of PPI [15]. Hydrophobic and hydrophilic groups on the protein surface influence the protein solubility [31]. Therefore, protein glycation, which increases the net negative charge, thus hydrophilicity on the surface, can result in enhanced solubility. However, PPI and PPI glycated with F1 or F2 also exhibited a comparable solubility of around 50% at a pH value of 7.0, independent of the temperature used. Thus, solubility at pH 7.0 was not increased by glycation with MOS. In the past, an increase in solubility from 25.7% to 37.8% was shown by glycation of PPI with maltodextrin, possibly due to the initially lower solubility of the non-glycated PPI [12]. However, MOS and maltodextrins clearly show structural differences, which might have contributed to differences in the observed solubility.

At pH 3.4, however, the solubility of PPI increased from 25.7% to 44.8% when glycated with F1 and to 47.1% when glycated with F2 (non-heated conditions). Under heated conditions, there was also a substantial increase in solubility, from 38.3% to 47.4% and to 52.4% when glycated with F1 or F2, respectively. There was also an increase in

the solubility of PPI at pH 3.4 upon heating. The main proteins in pea, vicilin and legumin denature at ~ 82 °C and 90 °C, respectively. This observation was similar to that of Scheider and co-workers [7] and Bu and co-workers [11]. Heating just under the temperature of denaturation could have caused partial unfolding, likely contributing to enhanced interactions between water and some of the exposed functional groups.

Results, therefore, indicated that the solubility at pH 3.4 can almost be doubled under non-heated conditions and be increased by a factor of about 1.5 under heated conditions. Under both conditions, PPI glycated with F2 (MOS with a DP 7–9) was slightly more soluble than PPI glycated with F1 (MOS with a DP ≥ 9). Comparable increases were achieved by glycating PPI with maltodextrins [7, 14]. Based on these results, glycation with MOS that had a DP of 7–9 contributed a slightly better solubility of PPI than the glycation with MOS with a DP ≥ 9. Based on the findings, it was not clear whether the enhanced solubility resulted from the glycation conditions, the extent of loss of free amino groups, or the slightly smaller DP of the MOS. It has been suggested that an increase in protein solubility is a result of synergetic effects of the molecular mass and the amount of conjugated oligosaccharides [12]. Glycated proteins are more hydrophilic and attached oligosaccharides represent a steric hindrance that could limit the interaction among glycated proteins [6]. In addition, it must be noted that the unreacted MOS may have an impact of the measured solubility. Generally, low molecular weight carbohydrates could compete for water, and thus decrease protein solubility [32, 33]. Accordingly, the effect of protein glycation on the solubility might have been underestimated due to the incomplete removal of free, unreacted MOS. Although we see comparable effects when MOS and maltodextrins are used for glycation, these results should not be transferred to all oligosaccharides. Depending on the structure (linear vs. branched) and the monomer composition (configuration of the monomers, neutral vs. acidic monomers) of the oligosaccharides, the effects on both solubility and steric hindrance may differ, potentially resulting in different solubility of the glycated proteins.

To sum up, it was demonstrated that glycation with MOS of a DP≥7 contributed to an increase in the solubility of pea protein at pH 3.4. This outcome makes glycated pea protein more suitable to be used in acidic, high-protein beverages than non-glycated PPI. Differently, small oligosaccharides (MOS with a DP of 2-3) were less suitable for protein glycation, thus the hydrolysis of galactomannans must be well controlled. An advantage of MOS glycated PPI is that MOS with a DP≥3 are considered dietary fiber compounds. Thus, both protein bound MOS and excess MOS in the partially purified samples also enrich food products with dietary fiber. In addition, since MOS can be produced from by-products of the agri-food industry, their use for protein glycation contributes to a sustainable food production.

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Data availability Data are made available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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