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Assessment of the interactions between pea and salivary proteins in aqueous dispersions

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ABSTRACT

To understand and limit the unpleasant oral sensation of astringency felt during the consumption of pea-based drinks, we investigated the interaction between mixtures of salivary and pea proteins, as compared to mixtures where HEPES buffer (at pH 6.8) was used as a negative control for saliva. Since astringent compounds have the ability to bind with salivary proteins, mixes of freshly collected whole unstimulated saliva and a pea protein isolate (PPI) (a dispersion at 3.5% *w/v*) were prepared in ratios 95:5 and 1:1 saliva:PPI, to allow different stoichiometries to occur in the mouth. Samples were incubated at 37 °C during 30 min, after centrifugation at 16000*g* during 20 min to separate pellet from supernatant. Using techniques such as SEC, Native-PAGE and LC-MS, 7 pea proteins were identified as being capable of forming aggregates with at least 7 saliva proteins, some of which have been previously connected to astringency.

1. Introduction

Understanding the root-cause of astringency, also referred as "dry mouthfeel", has become of great interest for the food industry, since this rather unpleasant sensation can limit the consumer acceptance of many foods (Lawrence, Lopetcharat, & Drake, 2016; Liu, Toro-Gipson, & Drake, 2021). Nevertheless, given their complexity, the mechanisms at the origin of astringency are still to be fully elucidated (Pires, Pastrana, Fucinõs, Abreu, & Oliveira, 2020; Wu, Zhu, Wang, Grierson, & Yin, 2022). Defined as a drying-out, roughening, and puckery sensation felt in the mouth (Lawless & Corrigan, 1994), astringency is generally associated with the consumption of polyphenols, such as tannins (Bate-Smith, 1954; Joslyn & Goldstein, 1964), but also food proteins like Lysozyme and β–Lactoglobulin (Beecher, Drake, Luck, & Foegeding, 2008; Vardhanabhuti, Kelly, Luck, Drake, & Foegeding, 2010), salts of multivalent cations (i.e. aluminum salts), dehydrating agents (i.e. ethanol) (Joslyn & Goldstein, 1964), and other small molecules like flavonoids (i.e. quercetin), phenolic acids (i.e. p-coumaric acid) (Troszyńska, Amarowicz, Lamparski, Wołejszo, & Baryłko-Pikielna, 2006) and saponins (Price, Griffiths, Curl, & Fenwick, 1985; Suárez-Estrella et al., 2021). Given the large variety of molecules that can elicit astringency, it is likely that multiple mechanisms coexist and occur simultaneously, each of them accounting partially for the sensation of astringency. In the past decade, different hypotheses involving salivary protein precipitation, disruption of the salivary pellicle, interaction with the oral mucosa, decrease in oral lubrication, and mechanical perception sensed by receptors, among others, have been proposed (Canon, Neiers, & Guichard, 2018; Feron et al., 2021; Gibbins & Carpenter, 2013; Lee, Ismail, & Vickers, 2012; Lee & Vickers, 2012; Ployon et al., 2018; Schobel et al., 2014). Despite the lack of consensus among the scientific community, it is generally recognized that astringency originates from the interaction of food compounds with saliva and the structures in the oral cavity (Pires et al., 2020). Saliva is a physiological fluid that coats the oral cavity, acting as lubricant, and plays an essential role in the oral processing and the digestion of foods. It is composed of nearly 99% water and a mixture of electrolytes, fatty acids and proteins, such as proline-rich proteins (PRPs), statherins, cystatins, mucins and histatins, to cite a few (Carpenter, 2013). For many years, the most studied mechanism of astringency was focused on the interaction between astringent compounds, mainly tannins, and salivary PRPs (Bennick, 2002; Canon, 2019; Canon & Neyraud, 2017; Prinz & Lucas, 2000; S. Soares et al., 2018). Understanding the interactions of salivary proteins and astringent compounds is a key step towards the elucidation of

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List of symbols and abbreviations

Abreviation	Meaning
PRP's	Proline-rich proteins
PPI	Pea protein isolate
LC-MS	Shotgun Liquid Chromatography-Mass Spectrometry
PAGE	Polyacrylamide Gel Electrophoresis
SP	Salivary proteins
PP	Pea proteins
PF	Pea flour
SEC	Size Exclusion Chromatography
MW	Molecular weight
TCA	Trichloroacetic
NC	Negative control
Proteins	
LOX1.3	Seed linoleate 9S lipoxygenase 3
Hsp70	Heat shock protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ZG16B	Zymogen granule protein 16 homolog B
BPI-A2	BPI fold-containing family A member 2
ZA2G	Zinc-alpha-2-glycoprotein
Others	
DDM	n-dodecyl b-D-maltoside
BT	Bis-Tris

astringency mechanisms. Recently, the use of pea-based (*Pisum sativum L.)* ingredients in the formulation of plant-based foods has become very popular due to the crop's low allergenicity and high nutritional value; its protein content and quality being of particular interest. In fact, pea protein isolate (PPI) is now used as one of the main ingredients in the meat and dairy alternative industry (Middleton & Littler, 2019). However, PPI has been reported to be astringent in sensory analysis, likely due to the interaction between pea proteins and salivary proteins (Cosson, Oliveira Correia, Descamps, Saint-Eve, & Souchon, 2022; Cosson, Souchon, Richard, Descamps, & Anne, 2020; García Arteaga, Leffler, Muranyi, Eisner, & Schweiggert-Weisz, 2021). Yet, research studies aiming to characterize this interaction and the proteins therein involved are lacking. Indeed, (Brown, Mackie, He, Branch, & Sarkar, 2021) has pointed out the current literature gap regarding the study of interactions between plant and salivary proteins, which is crucial for the formulation of plant-based foods with a higher protein content without jeopardizing their sensory acceptability. In this context, the main aim of this study was to identify the proteins involved in the interaction between pea proteins and salivary proteins leading to the formation of a precipitate when PPI and saliva are mixed. The identification of the proteins involved in such interaction may give further insight about the mechanisms that underlie the perception of astringency. To the best of the authors knowledge, this is the first study to assess the interactions between pea proteins and salivary proteins using several state-of-the-art techniques, including Shotgun Liquid Chromatography-Mass Spectrometry (LC-MS), which is a method used for the separation, identification, and quantification of complex protein mixtures (Mukherjee, 2019), and Native-Polyacrylamide Gel Electrophoresis (PAGE), which allows assessing the composition and structure of native proteins, as both their conformation and biological activity remain intact during the analysis (Cresswell, 1998).

2. Material and methods

2.1. Rationale of the experiment

The general rationale behind the experiments described in the present work consisted in allowing a pea protein isolate (PPI) dispersion interact with human saliva at physiological temperature in order to form a precipitate which is then separated to identify its constitutive proteins. The saliva:PPI mixtures were prepared at different ratios (described in 2.1), and were all incubated at 37 °C during 30 min in a water bath (MB5, Julabo, Germany) in order to allow the proteins to interact and form stable complexes. The 30 min had the goal to allow enough time to reach an equilibrium after the interactions, 37 °C was used as it is the body temperature. The samples were subsequently centrifuged to separate the pellet containing the precipitated proteins from the supernatant. Except when indicated, both, pellet and supernatant were analyzed using several state-of-the-art protein identification techniques that are described in section 2.5. As a negative control, the HEPES buffer, prepared to a concentration of 0.01 M and adjusted to a pH of 6.8, was used instead of saliva. HEPES is widely used in biological research since its buffer capacity is optimal within the physiological range of 6.8 to 8.2, it cannot form complexes with metal ions and it may prevent the damage of certain proteins (Ferreira, Pinto, Soares, & Soares, 2015; Good & Izawa, 1972). HEPES has being used in the past by other authors as a control when studying the lubrication properties of pea proteins (Kew, Holmes, Stieger, & Sarkar, 2021) and to prepare solutions to an adjusted pH of 6.8 when working with whole saliva. To be able to control the PPI production process and rule out any special treatment from commercial suppliers, the PPI used in this study was prepared in the laboratory as described in 2.2. The saliva used in all experiments was collected without stimulation from healthy donors following the protocol described in 2.3.

2.1.1. Ratios of interaction

The interactions between salivary proteins (SP) and pea proteins (PP) were studied at two different ratios of saliva:PPI (1:1 and 95:5). The ratio 1:1 was chosen to represent the oral concentration at the initial consumption of a PPI based food or beverage; while the ratio 95:5 represents the hypothetic remaining concentration of PPI in the mouth after swallowing the food, therefore in this scenario the proportion of saliva is deliberately higher. Moreover, in the 95:5 (saliva:PPI) ratio the overall "true" protein ratio (SP:PP) is 2:1, which allows for a more balanced stoichiometry than in the 1:1 (saliva:PPI) ratio, where the ratio SP:PP is 1:8. The details of these calculations can be found in the Appendix.

2.2. Pea protein isolate (PPI) production

The PPI used in this study was developed starting from raw yellow pea (*Pisum sativum*, unknown variety) seeds purchased from Scharnebecker Mühle GmbH (Germany). Briefly, the protocol consisted in the following steps: milling (temperature not controlled), defatting, alkaline extraction, and freeze-drying. Pea grains (400 g) were cleaned and dry milled using a rotor mill with a 12 teeth rotor (Ultra Centrifugal Mill ZM 200, Retsch, Germany) in two steps to obtain a Pea flour (PF). First, the ring sieve of stainless steel with holes of 2 mm was used for 2 min at 8000 RPM, followed by the ring sieve of stainless steel with holes of 0.12 mm for 4 min at 8000 RPM. The PF was then defatted using a Soxhlet system with hexane as solvent for 12 h, according to the method from (Shahidi, 2005). Alkaline extraction was carried out based on the methods described by (Boye, Zare, & Pletch, 2010; Che & Lam, 2016) with some modifications. The defatted flour was solubilized 1:6 (*w*/w) in deionized water, pH was adjusted until alkaline (pH 11) with NaOH 1.0 N while being agitated at room temperature, the agitation persisted 30 min. Then, the sample was centrifuged for 10 min at 4500 x*g* at 4 °C (Centrifuge 3K30H, Sigma, Germany). Only the supernatant was

recovered. Isoelectric precipitation (pH 4.5) was caused by adding HCl 1.0 N. The sample was centrifuged for 10 min at 15000 xg at 4 °C (Centrifuge 3K30H, Sigma, Germany). Only the pellet was recovered, and it was washed by adding 30 mL of deionized water, then the centrifugation step was repeated. Pellets were collected and neutralized to pH 7.0 using NaOH 1.0 N, prior to freeze-drying. Freeze drying was done with a BenchTop Pro Manifold Lyophilizers (SP VirTis, VIRS BTP8 ZL 00×, Pennsylvania, USA) using a 12 Port Acrylic Drum Manifold; 20 cm Diameter and a bulk shelf rack with 3 shelves for 48–67 h; the actual freeze-drying time depended on sample volume. The protein content of the PF and PPI were measured in triplicates via the Dumas method (McClements, Newman, & McClements, 2019). The PF had an initial protein content of 20% ± 1.0 *w*/w (N*6.25), and the PPI was found to have a protein content of 68.5% ± 1.8 w/w (N*6.25). This yield was slightly lower than typical commercial isolates (≈80–94% *w*/w), since the protocol was not optimized for yield (Lam, Can Karaca, Tyler, & Nickerson, 2018). Likely, the remaining fraction was primarily composed of carbohydrates (starch) and dietary fiber, which are major components of the pea grain (Gueguen, 1983). The saponin and phenolics content of the isolate was quantified using spectrophotometric methods (Hiai, Oura, & Nakajima, 1976; Julkunen-Tiitto, 1985), and was found to be 9.47×10^{-2} +/− 0.0034 g of saponins/ 100 g PPI and 5.25×10^{-2} +/− 1.82×10^{-5} g of phenolics/100 g PPI. In both cases, these values are within the literature reported range for yellow pea (Heng et al., 2006; Hiai et al., 1976). The phytic acid content was quantified using an enzymatic kit (Megazyme, Ireland) which was found to be 1.054±0.008 g/100 g of PPI. Finally, the content of free calcium was determined potentiometrically and was found to be negligible.

2.3. Saliva collection

Whole human unstimulated saliva was collected from a group of healthy donors ($3 \geq n \leq 6$) in the morning of the experiment. The saliva was pooled from the same group (1 male and 5 females with ages between 23 and 32), although not every donor donated each time. All donors were asked to avoid consuming food 1 h prior to the donation. A time frame of 30 min was given to produce 5 mL of unstimulated saliva. Donors were instructed to rinse their mouth 3 times with water before collecting saliva and to allow saliva to be naturally produced in their mouths for 2–5 min without movements of tongue, after which they spitted the saliva in a plastic container until the time limit or the volume required was achieved. After each donation, the saliva collected was kept on ice and used within the next couple hours. The collected saliva was centrifuged (Centrifuge 3K30H, Sigma, Germany) to remove dead cells and food debris at 16000 x g, for 20 min at 4 °C (Condelli, Dinnella, Cerone, Monteleone, & Bertuccioli, 2006). All subjects agreed on the content of the study and signed informed consent. The protein content in saliva was estimated to be 3 mg/mL, as reported in literature (Agha--Hosseini, Mirzaii-Dizgah, Moghaddam, & Akrad, 2007; Bajec & Pickering, 2008; Nederfors, Dahlöf, & Twetman, 1994).

2.4. Pea protein isolate (PPI) dispersion preparation

The PPI dispersion used to prepare the mixtures saliva:PPI was the supernatant of a dispersion at 3.5% *w/v* (protein basis) in Milli Q water made with the PPI made in the laboratory described in 2.2. This concentration of 3.5% was used since it is similar to the concentration in milk products. The PPI was mixed with warm Milli Q (65 °C) in a volumetric flask and agitated with a magnetic stirrer for 30 min. Then the volume of the volumetric flask was completed with Milli Q water. The obtained 3.5% w/v dispersion was mixed in a Polytron (PT6000 Kinematica AG, Switzerland) at 2500 RPM for 10 min. Finally, the dispersion was homogenized with a Microfluidizer (M110EH Microfluidics, Westwood, USA) at 1500 bar (3 cycles) and at 500 bar (7 cycles). The homogenized dispersion was centrifuged (Centrifuge 3K30H, Sigma, Germany) at 16000 x g, for 20 min at 4 °C. The supernatant was recovered and divided in 1 mL aliquots. The protein concentration of the supernatant was measured in triplicates via the Dumas method (McClements, Weiss, Kinchla, Nolden, & Grossmann, 2021) and was 2.8 ± 0.2% w/v (N*6.25), which contained only soluble proteins. The aliquots were kept frozen (−20 °C) until use.

2.5. Protein identification techniques

2.5.1. Size Exclusion Chromatography (SEC)

This method was performed with a column Superdex 200 Increase 10/300 GL (Cytiva GE, New York, USA) attached to an Äkta purifier system (GE Healthcare, United Kingdom) at a flow rate of 0.5 mL/min (UV 280 nm), at room temperature. The column was equilibrated with HEPES buffer (pH 6.8 and concentration of 0.01 M), and calibrated for MW vs. elution volume (in mL). In order to estimate the MW, the obtained peaks were compared to those in a control chromatogram that is standardized with molecules of known MW in the same column (Hall, 2018). Since the column had a protein content detection range of 5–10 mg/mL, only the sample with a saliva:PPI ratio of 95:5 was investigated using this technique, since its protein content was estimated to be of 4.6 mg/mL. The calculation can be found in the Appendix. The PPI dispersion supernatant was diluted to reach a final concentration of 5 mg/mL. The protein concentration of saliva alone was estimated from literature to be between 2 and 4 mg/mL (Eva J. Helmerhorst, Sun, Salih, & Oppenheim, 2008), therefore no dilution was needed. To avoid pore blockage of the column, all samples were centrifuged (Micro Star 17R, VWR, Pennsylvania, USA) during 20 min at 16000 x g prior to being loaded to the column; only their supernatants were analyzed.

2.5.2. Native PAGE (Polyacrylamide Gel Electrophoresis)

This method was performed according to the NativePAGE™ Bis-Tris Gels Manual from Novex® by Life Technologies™ (https://assets.thermoffsher.com/TFS-Assets/LSG/manuals/nativepage˙man.pdf). All materials were purchased from Thermo Fisher Scientific. Pre-cast gels with 4 to 16% gradient polyacrylamide Bis-Tris gels specific for Native PAGE were used (NativePAGE™ 4–16% BT). All buffers were kept at 4 °C prior to the sample preparation. The samples were prepared according to the NativePAGE™ Bis-Tris Gels Protocol (https://assets.thermoffsGer.com/TFS-Assets/LSG/manuals/MAN0007893˙NativePAGE˙BisTris˙Gels.pdf), except each sample was incubated on ice for 15 min after adding detergent n-dodecyl b-D-maltoside (DDM). All gels were loaded in the electrophoretic cell (XCell Sure Lock Mini-Cell). The protein concentration of each sample was measured using the Bradford assay (Bradford, 1976) and was normalized to 0.5 mg/mL. All samples (the supernatants and pellets of the 95:5 and 1:1 saliva:PPI mixtures) and negative controls (the supernatants and pellets of the 95:5 and 1:1 HEPES:PPI mixtures) were centrifuged at 13500 xg for 5 min (Micro Star 12, VWR, Pennsylvania, USA) before loading, and only their supernatant was loaded. 15 μL of sample were loaded in a separate well. 10 μL of unstained Protein Standard (NativeMark™) was loaded in a separate well. Two gels were run simultaneously during 2 h at 150 V at room temperature. The gels were later stained with Instant Blue (Expedeon, United Kingdom). A selection of bands containing proteins of interest (i.e. that were not present in the saliva or PPI alone but only in the mixtures) was excised from the gel to be digested and analyzed via Liquid Chromatography-Mass Spectrometry (LC-MS), see 2.5.3.2.

2.5.3. Shotgun Liquid Chromatography-Mass Spectrometry (LC-MS)

This experiment was carried out in collaboration with the Functional Genomics Center Zurich (FGCZ) using the technique Shotgun Liquid Chromatography-Mass Spectrometry (LC-MS). Shotgun proteomics combines Liquid Chromatography (LC) with coupled tandem Mass Spectrometry (MS), this is particularly suitable to analyze complex protein mixtures (van Vliet, 2014). The software Scaffold (versions 4 and 5, Portland, USA) was used to handle the large-scale data generated from the quantitative experiments of the MS/MS analytic. The results

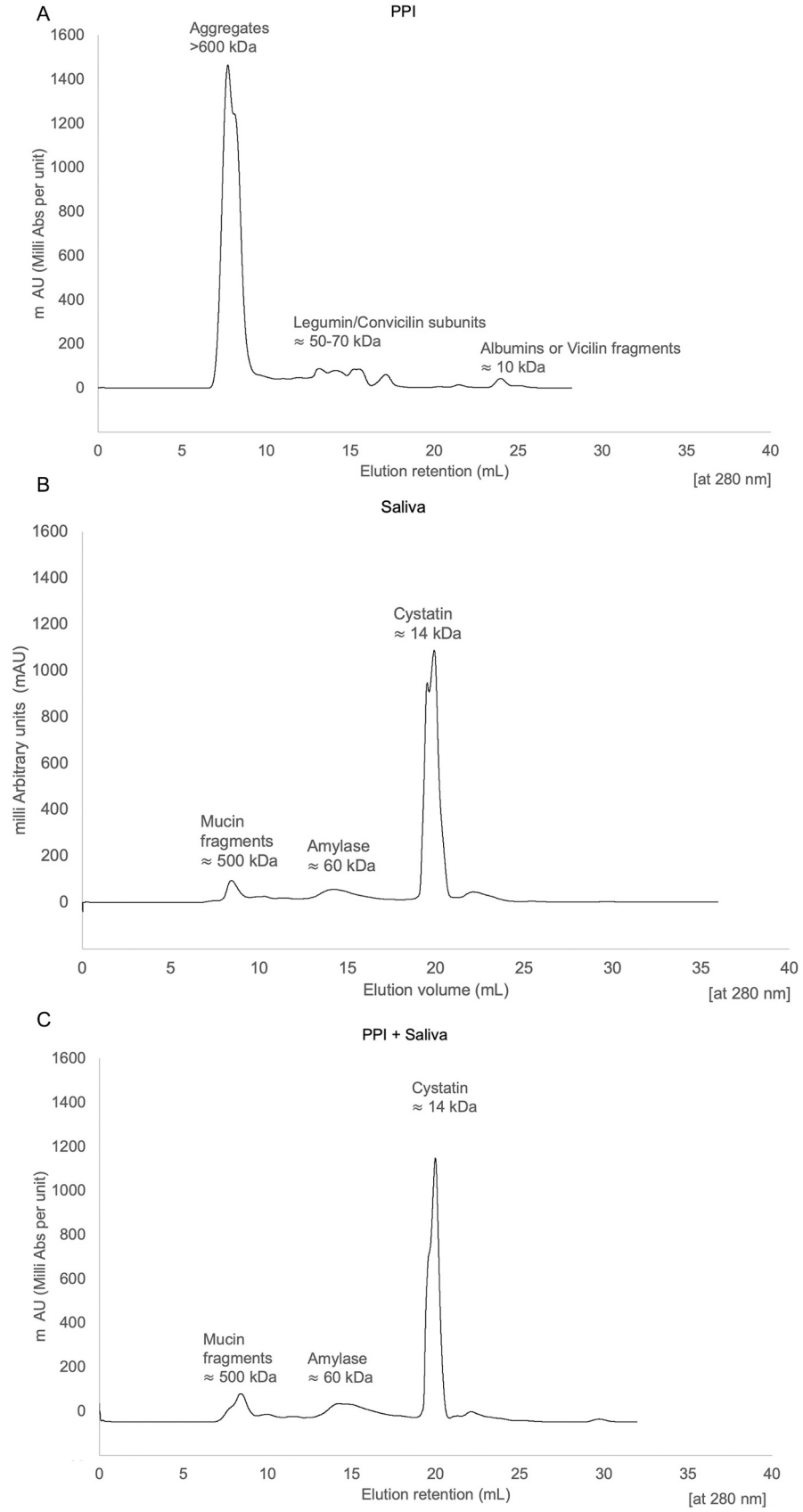

Fig. 1. Chromatograms from A) pea protein isolate (PPI), B) saliva and C) PPI-saliva mixture separated by the SEC Superdex 200 column. Conditions: flow rate of 0.5 mL/min (at UV 280 nm), run at Room Temperature, Protein content was 5–10 mg/mL. The column was equilibrated with HEPES buffer (pH 6.8 and concentration of 0.01 M).

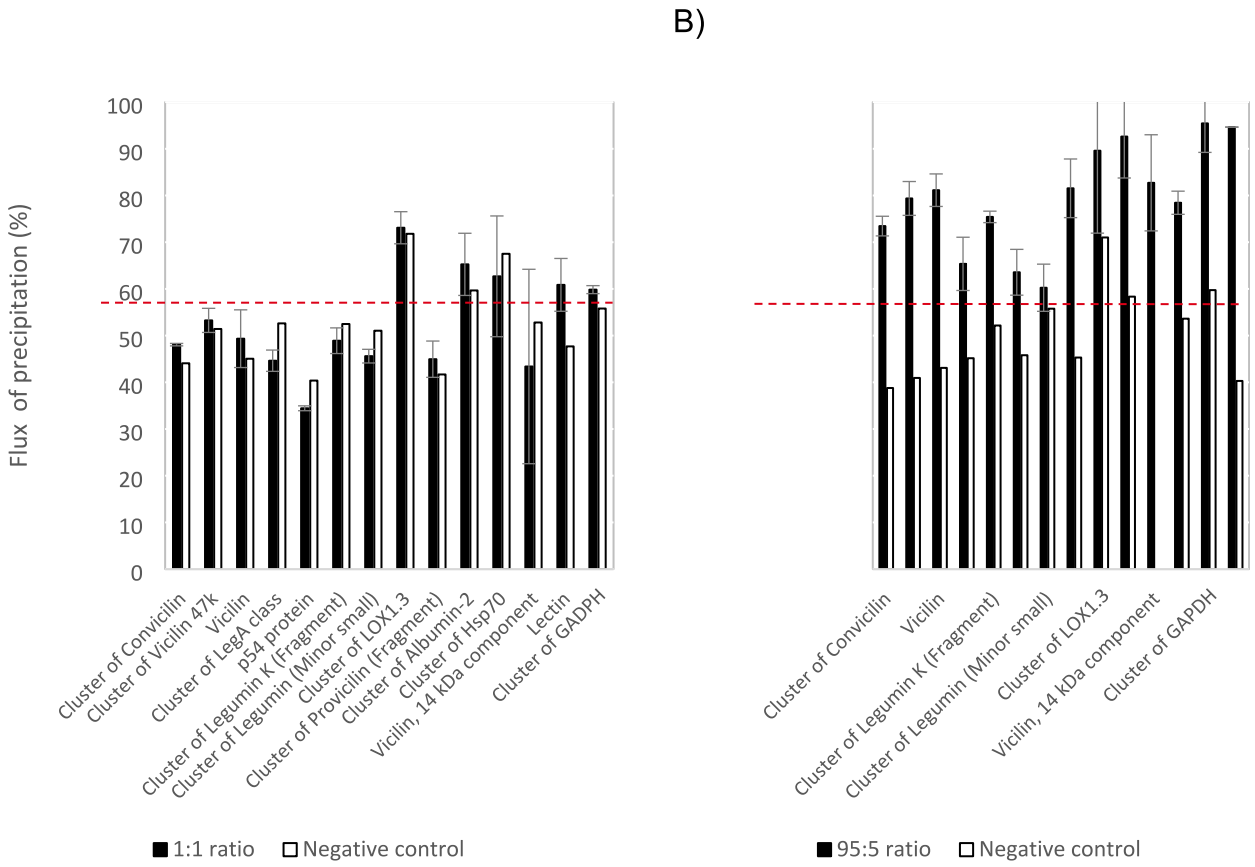

Fig. 2. Flux of precipitation of the 14 most abundant pea proteins of the saliva:PPI mixtures for the A) 1:1, and B) 95:5 ratios.

were expressed as the normalized quantitative value of the total spectral count, which is the sum of all peptide fragment spectra leading to the identification of a protein. A changing spectral count is correlated with the relative change of protein abundance between different samples (Liu, Sadygov, & Yates, 2004; Wienkoop, 2013). The normalization scheme in Scaffold adjusts the sum of the selected quantitative value for all proteins within each MS sample to a common value: the average of the sums of all MS samples present in the experiment. This is achieved by applying a scaling factor to the number of spectra for each protein in a sample, adjusting in this way the selected value to a normalized "Quantitative Value" (Scaffold User's Manual, Version 5.0). In the present study, for the sake of simplicity, clusters, which represent groups of proteins that are similar in structure (Inzitari et al., 2006), were treated as a single protein. In Scaffold a cluster is a set of proteins with overlapping peptide evidence and may be treated as a proxy for a single identification (Scaffold User's Manual, Version 5.0). This was done to facilitate data analysis and to avoid redundancy in the results, since protein clusters are represented by the protein that shows the highest associated probability. All samples (the supernatants and pellets of the 95:5 and 1:1 saliva:PPI mixtures) were prepared and run in duplicates, excepting the negative controls (the supernatants and pellets of the 95:5 and 1:1 HEPES:PPI mixtures), for which no duplicate was needed as its interpretation was based on the presence/absence of a protein criterion. The preparation of both the samples and the negative controls prior to the loading in the LC-MS is described in detail in 2.5.3.1.

2.5.3.1. Supernatants and pellets from 95:5 and ratio 1:1.

- TCA precipitation (supernatant samples)

Trichloroacetic (TCA) precipitation was performed on each sample by adding 1000 µL of sample + 53 µL of H2O + 100 µL of 100% TCA (5% TCA end concentration). Protein pellets were washed 3× with cold acetone, dried and dissolved as follows: + 50 µL of 10 mM Tris/2 mM $CaCl_2$, pH 8.2 buffer Only 20 µg per sample were taken (the whole 50 µL for the negative controls) filled up to 45 µL with 10 mM Tris/2 mM $CaCl_2$, pH 8.2 buffer +5 µL trypsin (100 ng/µL in 10 mM HCl).

- Protein digestion (pellet samples)

Samples were dissolved in 50 µL 10 mM Tris/2 mM CaCl2, pH 8.2 buffer, followed by freeze-thaw cycles 3 × 1 min using liquid nitrogen / 10 min sonication. Only 20 µg per sample were taken (the whole 50 µL for the pellet of the negative controls filled up to 45 µL with 10 mM Tris/2 mM $CaCl_2$, pH 8.2 buffer +5 µL trypsin (100 ng/µL in 10 mM HCl).

- Protein digestion

Both pellet and supernatant samples had a Microwave assisted digestion (60 °C, 30 min). The digested samples were dried and dissolved in 20 µL ddH2O + 0.1% formic acid; transferred to the autosampler vials for Liquid Chromatography-Mass Spectrometry analysis (LC-MS); 1 µL were injected on a nanoAcquity UPLC coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientifc, Waltham, USA).

2.5.3.2. Native PAGE excised bands. In-gel protein gel bands stained with Coomasie blue were cut in small pieces and washed with 100 µL of 100 mM NH_4HCO_3/50% acetonitrile (2×); + 50 µL acetonitrile (1×). All three supernatants were discarded. The protein digestion was performed adding 20–30 µL of trypsin (5 ng/µL in 10 mM Tris/2 mM CaCl2, pH 8.2); + 30/40 µL of digestion buffer (10 mM Tris/2 mM $CaCl_2$, pH 8.2); Microwave assisted digestion (60 °C, 30 min). Supernatants were collected and the peptides were extracted from the gel pieces using 150 µL 0.1% TFA/50% acetonitrile. The supernatants were combined and

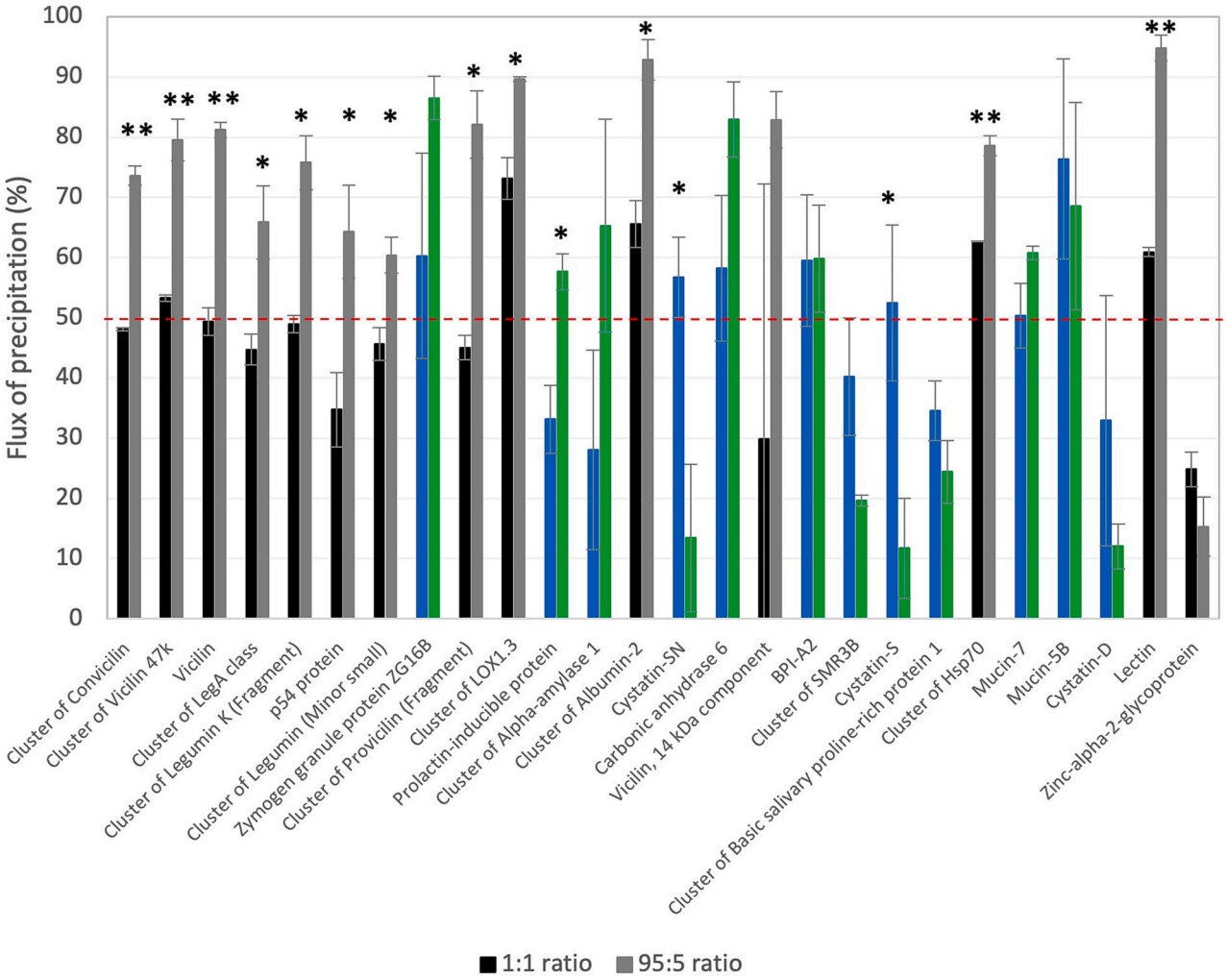

Fig. 3. Flux of precipitation of the 26 most abundant pea (black and gray) and salivary proteins (blue and green) of the saliva:PPI mixtures for the 95:5 and 1:1 ratios. The impact of ratio on the flux of precipitation was tested via the Student's *t*-test. Significance is indicated by asterisk with levels of <0.05 (*), <0.01 (**), and < 0.001 (***). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

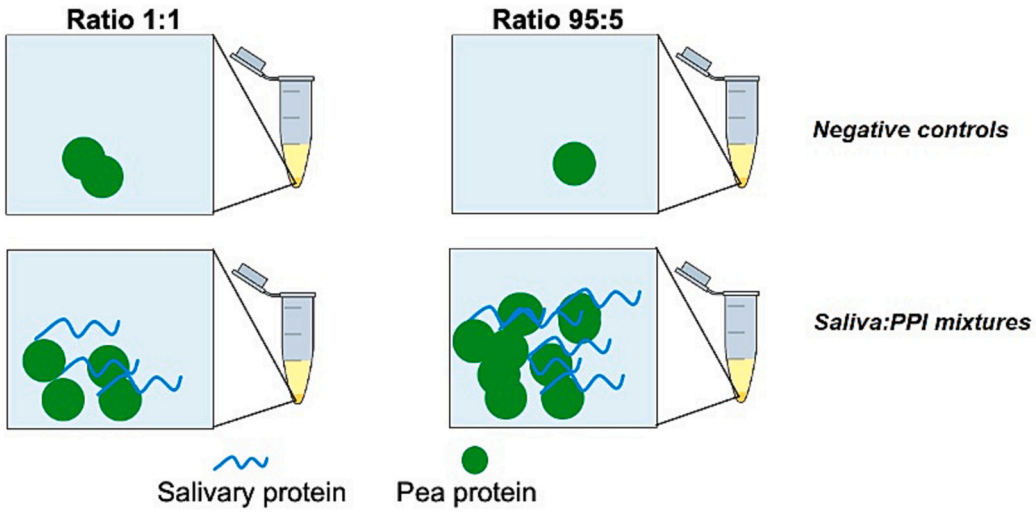

Fig. 4. Hypothesized behavior of the interaction between pea proteins and salivary proteins. Salivary proteins are able to interact and precipitate the majority of the pea proteins at the 95:5 ratio, while at the 1:1 ratio precipitation is mainly driven by self-aggregation of pea proteins (95:5 saliva:pea protein, and 1:1).

Table 1
Salivary proteins abundant in both ratios. Where G16B: Zymogen granule protein 16 homolog B, SGAR-3B: Submaxillary gland androgen-regulated protein 3B, ZBPI-A2: BPI fold-containing family A member 2, ZA2G: Zinc-alpha-2-glycoprotein.

Salivary Proteins*	Ranking ratio 1:1	Ranking ratio 95	Uniprot accession number	MW (kDa)	Function**
Cystatin-SN	1	4	P01037	16	Cysteine proteinase inhibitors (enzymes that aid the breakdown of proteins) that are immunologically related to cystatin S. Binds to Tannins (EGCG) and $Fe_2(SO_4)_3$) (Delius et al., 2017)
Alpha-amylase 1	3	3	Q5T085	58	The single most abundant protein in saliva. Generally thought to be involved in the initial digestion of starch-containing foods (Carpenter, 2013). Binds to Polyphenol, correlated in sensory (Gambuti et al., 2006)
ZG16B	6	1	Q96DA0	23	Related to carbohydrate binding, Expressed in minor salivary gland.
Prolactin-inducible protein	7	2	P12273	17	Actin-binding protein secreted in saliva
Cystatin-S	2	8	P01036	16	See Cystatin SN
SGAR-3B	4	7	P02814	8	Secreted into saliva, binds to and stops, prevents or reduces the activity of an endopeptidase
BPI-A2	8	6	Q96DR5	27	Antibacterial activity, secreted into saliva
Carbonic anhydrase 6	9	5	P23280	35	Reported to be involved in bitter taste perception, but no via precipitation (Patrikainen, Pan, Kulesskaya, Voikar, & Parkkila, 2014)
bPRP1	11	11	P04280	39	Secreted into saliva, function not clear (Canon et al., 2018, 2013)
bPRP2	10	12	P02812	41	Secreted into saliva, function not clear (Canon et al., 2018, 2013)
Mucin-5B	12	14	Q9HC84	596	Thought to contribute to the lubricating and viscoelastic properties of whole saliva. Can bind to lysozyme (Silletti et al., 2010).
Cystatin-D	16	17	P28325	16	See Cystatin SN
Mucin-7	20	13	Q8TAX7	39	May function in a protective capacity by promoting the clearance of bacteria in the oral cavity and aiding in mastication, speech, and swallowing. Capable of binding to β -Lactoglobulin, an astringent whey protein component (Silletti et al., 2010).
ZA2G	18	21	P25311	34	Stimulates lipid degradation in adipocytes, present in saliva, sweat, epithelial cells of various human glands. Capable of binding to β -Lactoglobulin, an astringent whey protein component (Silletti et al., 2010).

* Proteins in **bold** have been previously connected to astringency as they bind to astringents and precipitate.
** According to UniProt or source indicated.

dried. The digested samples were dried and dissolved in 20 μL ddH2O + 0.1% formic acid; transferred to the autosampler vials for Liquid Chromatography-Mass Spectrometry analysis (LC-MS); 7 μL were injected on a nanoAcquity UPLC (Waters Corporation, Milford, USA) coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, USA).

2.6. Statistical analysis

Student's *t*-test with a significance level of $\alpha = 0.05$ was carried out to compare the precipitation of certain proteins of interest in the saliva: PPI mixtures between the 95:5 and 1:1 ratios. The analysis was carried out with the R software (Version 1.3.1093).

3. Results and discussion

3.1. Size Exclusion Chromatography (SEC)

This method served to separate the proteins and protein complexes formed in the different saliva:PPI mixtures in native conditions. The obtained chromatograms are shown in Fig. 1. The first peak in a SEC chromatogram represents the void volume, which are molecules that are too large to enter the column (Eriksson, Persson, Zhang, & Wieslander, 2009; Hall, 2018). For PPI alone, (Fig. 1A) the first peak is also the largest, meaning it contains most of the proteins in the sample. Pea proteins are mainly composed of globulins (≈70%), i.e. Legumin, Vicilin and Convicilin; their MW ranges between 150 and 410 kDa (Boye et al., 2010; Gueguen, 1983; Tzitzikas, Vincken, de Groot, Gruppen, & Visser, 2006). Therefore, it is likely that this peak reflects the presence of protein complexes or protein aggregates containing pea globulins. Furthermore, it has been shown that these proteins tend to form high MW aggregates (>700 kDa) when heated above 90 °C (Mession, Sok, Assifaoui, & Saurel, 2013), which is a temperature that could be encountered during the PPI extraction process, i.e. during milling. Moreover, other processing steps, such as alkaline extraction and freeze-drying can also lead to the formation of aggregates (Gao et al., 2020; Mession et al., 2013; Vanbillemont, Carpenter, Probst, & De Beer, 2020). A few other peaks are visible, although much smaller, which likely contain the subunits of Legumin (≈60 kDa) or Convicilin (≈70 kDa), pea albumins and/or Vicilin fragments (≈10 kDa) (Tzitzikas et al., 2006). For saliva alone (Fig. 1B), the largest peak appeared at a much larger elution volume (≈20 mL), which represents a MW of approximately 14 kDa. Accordingly, this peak could correspond to Cystatins (Carpenter, 2013; E. J. Helmerhorst & Oppenheim, 2007). Two other smaller peaks that could correspond to Mucin fragments (≈500 kDa) and Amylases (≈60 kDa) are visible (E. J. Helmerhorst & Oppenheim, 2007). Interestingly, the largest peak did not correspond to any of the latter two proteins, even though they are two of the most abundant found in saliva (Carpenter, 2013). One possible explanation is part of them could have been lost due to the centrifugation step that was carried out before the injection of the sample to prevent clogging of the column. It has been shown that centrifugation can precipitate high MW (<200 kDa) proteins (Zhang, Zheng, Zheng, & Zhou, 2016). Moreover, several proteins, including Mucins and Amylases, are known to be assembled in complexes known as salivary micelles (R. V. Soares et al., 2004), which reinforces the hypothesis of their precipitation during saliva centrifugation. In Fig. 1C, the chromatogram of the saliva:PPI mixture at a 95:5 ratio is presented. In comparison to the PPI sample alone (Fig. 1A), the absence of the largest peak which contained most of the proteins is remarkable, even if the PPI concentration in the mixture much lower. This suggests that these proteins or protein aggregates were likely involved in an interaction between pea proteins and salivary proteins, precipitated and were found in the pellet of the sample. Since saliva was much more present in the mixture than in PPI, it is not surprising the chromatogram is closer to that of saliva alone (Fig. 1B). In both cases, the largest peak was located at the same elution volume; however, only a single well-resolved peak can be observed in the chromatogram of mixture, instead of two overlapped peaks. The absence

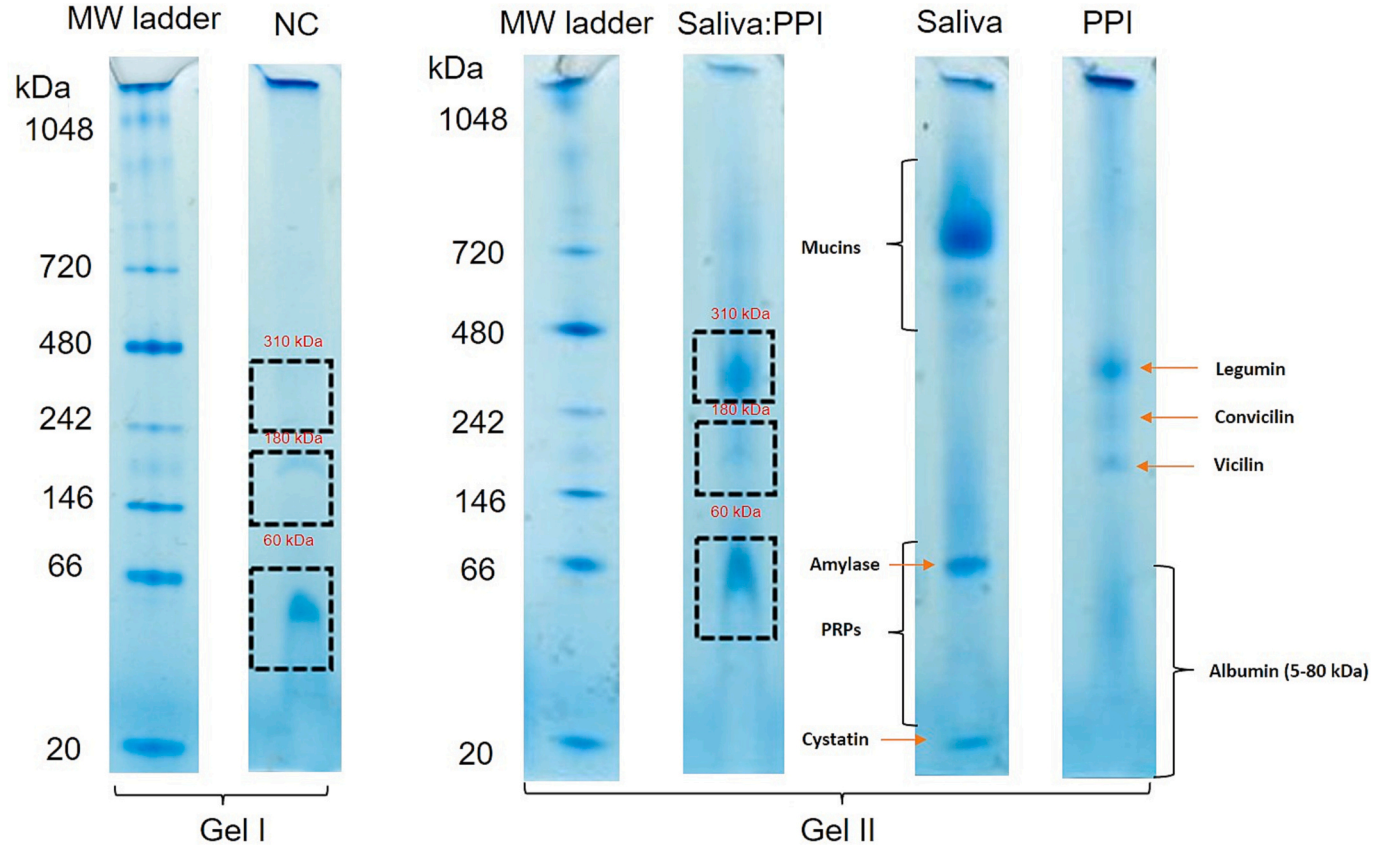

Fig. 5. Native polyacrylamide gel electrophoresis gels of Saliva alone, Pea protein isolate (PPI) and pellets of the saliva:PPI mixture at 1:1 ratio and negative control (NC). In red are the estimated molecular weights (MW) in kDa for each band detected. The pointed black squares mark the bands that were excised and analyzed via LC-MS, with estimated MW of 310, 180 and 60 kDa. Samples were centrifuged at 13500 xg for 5 min (Micro Star 12, VWR, Pennsylvania, USA) before loading, and only their supernatant was loaded. 15 μL of each sample was loaded in a separate well. Two gels were run simultaneously for 2 h at 150 V at room temperature. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of one of these peaks could be explained once again by an interaction occurring between pea proteins and salivary proteins with a MW close to 14 kDa, i.e. Cystatins. However, since the pellet of the saliva:PPI mixture could not be analyzed via SEC due to its insolubility, the confirmation of the proteins found in both pellet and supernatant could be only achieved via LC-MS (Liquid Chromatography-Mass Spectrometry). The results are presented in 3.2.

3.2. *Proteins found in pellet and supernatant via LC-MS*

Fig. 2A shows the flux of precipitation of the 14 most abundant pea proteins that precipitated in the saliva:PPI mixture at 1:1 ratio compared to the negative control. These proteins cover approximately 70% of all the identified proteins at this ratio. The full list of all the identified proteins ($n = 517$) is available in the Appendix. The flux of precipitation represents the percentage of a protein that is found in the pellet in comparison to the whole sample (pellet + supernatant). Therefore, the flux of precipitation of a protein is significant only when this value is higher than 50%. Thus, in the 1:1 saliva:PPI ratio, the flux of precipitation did not seem to be significant for most proteins. Only five proteins showed a flux of precipitation of 50 or higher: Seed linoleate 9S lipoxygenase 3 (LOX1.3), Albumin-2, Heat shock protein (Hsp70), Lectin and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). However, even if these proteins precipitated importantly in the sample, they also did in the negative control. This suggests their precipitation is not triggered by saliva exclusively and could be induced by the slight pH change that occurs when the HEPES buffer is added (pH changes from 7.2 in the PPI dispersion to 6.8 when HEPES is added).

On the other hand, there was overall more precipitation of pea proteins in the 95:5 ratio than in the 1:1 ratio, as shown in Fig. 2B, which presents the percentage of precipitation of the 14 most abundant pea proteins found in the pellet of the PPI:saliva mixture for the 95:5 ratio compared to the negative control. These proteins cover approximately 60% of all the identified proteins at this ratio, which is lower than in the 1:1 ratio, but this is expected since the 95:5 ratio contains more salivary proteins. Interestingly, the most abundant proteins that precipitated are the same between the two ratios, but more proteins had a flux of precipitation above 50, including Convicilin, several Vicilins and Legumins, which is in line with was observed in the SEC chromatogram (3.1). In the 95:5 ratio, most of the proteins that precipitated with saliva, also did in the negative control. However, even if it cannot be considered quantitatively strictly speaking, the precipitation level of the negative control was consistently lower than in the 1:1 ratio. Moreover, one protein stood out for being absent in the negative control at this ratio: Vicilin 14 kDa component. This protein can therefore be considered as a possible astringent, meaning its precipitation is exclusively mediated by saliva, at least in one of the studied ratios. Surprisingly very few proteins met this condition and most of them were not abundantly present (normalized number of spectra<10); the full list is provided in the Appendix. Moreover, like Vicilin 14 kDa, their precipitation behavior was not consistent between ratios. A more detailed analysis of the impact of the ratio on precipitation is given in the following section (3.2.2).

3.2.1. *Impact of ratio*

Fig. 3 shows the flux of precipitation of the 26 most abundant proteins that precipitated in the two studied ratios of saliva:PPI mixtures (1:1 and 95:5). Together, these proteins account for 80% of the total precipitated proteins in the 1:1 ratio, and 71% in the 95:5 ratio. Some

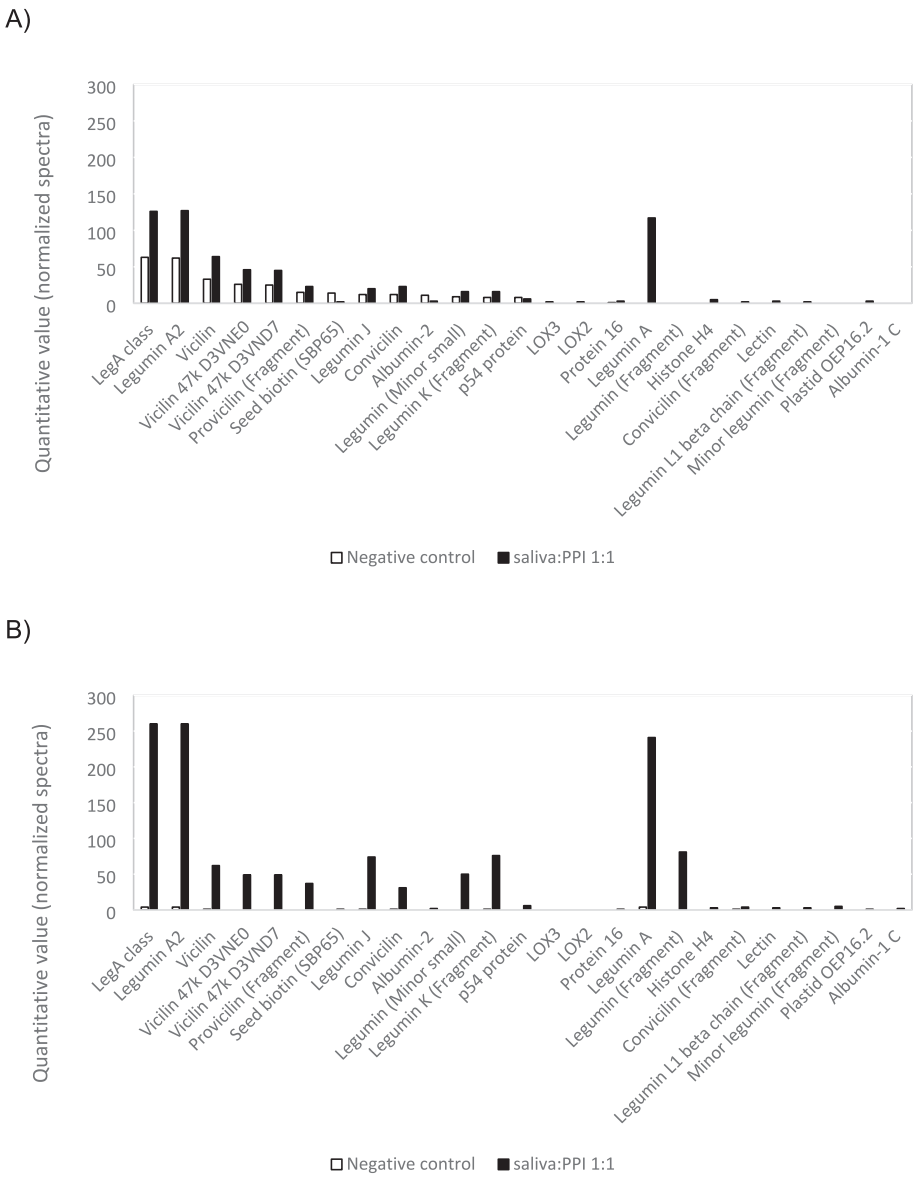

Fig. 6. Identified pea proteins present in bands of pellet sample compared to the pellet of the negative control for the A)180 and B)310 kDa excised bands from Native-PAGE gels identified via LC-MS.

proteins such as the Lipoxygenase 3 (LOX1.3), Albumin-2, Heat Shock protein 70, and Lectin stand out for their high precipitation in both ratios. However, the precipitation of these and other pea proteins was heavily affected by ratio (Fig. 3, gray and black bars). Indeed, most pea proteins precipitated significantly ($p < 0.05$) more in the 95:5 than in the 1:1 ratio. This may be due to the more balanced stoichiometry found in the 95:5 ratio compared to the 1:1 ratio. We hypothesize that the salivary proteins are able to interact and precipitate the majority of the pea proteins at the 95:5 ratio, whereas in the 1:1 ratio, a saturation might be reached, and the exceeding pea proteins cannot interact with saliva anymore. Instead, in the 1:1 ratio the precipitation may be mainly driven by self-aggregation of pea proteins (Fig. 4), which also explains the higher pea protein flux of precipitation observed in the negative controls at this ratio. Additionally, this highlights the importance of investigating more than one ratio when studying the interactions between food and salivary proteins.

Unlike pea proteins, the precipitation was not affected by ratio for many salivary proteins (Fig. 3, blue and green bars). Only three proteins were significantly impacted: Prolactin-inducible protein, Cystatin-S and Cystatin-SN. Interestingly, for the latter precipitation occurred more in the 1:1 ratio, despite its lower concentration of saliva. This is consistent with the results observed in SEC (3.1) and support the involvement of Cystatins in the salivary and pea protein interaction. Some proteins showed a flux of precipitation above 50 that was consistent in both ratios, such as Mucin-5B, Zymogen granule protein 16 homolog B (ZG16B), Carbonic anhydrase 6 and BPI fold-containing family A member 2 (BPI-A2). It was also observed that several proteins known to be involved in astringency were able to precipitate in presence of pea proteins, which are described in Table 1. These include Alpha-amylase (De Freitas & Mateus, 2001; Gambuti, Rinaldi, Pessina, & Moio, 2006) and Cystatin-SN (Ployon et al., 2018; Silletti, Vitorino, Schipper, Amado, & Vingerhoeds, 2010) which were also consistently found among the top 6 most abundant salivary proteins for both ratios. Furthermore, Mucin-5B, Mucin 7 (Biegler, Delius, Käsdorf, Hofmann, & Lieleg, 2016; Gambuti et al., 2006; Lee et al., 2012; Silletti et al., 2010) and basic Proline-rich-proteins (b-PRP-1, bPRP-2) (Canon et al., 2018, 2013; Delius, Médard, Kuster, & Hofmann, 2017) were also found among the top 20 most abundant salivary proteins in both ratios. In order to confirm the interaction between salivary proteins and pea proteins, the pellets of the saliva:PPI mixture and the negative control at 1:1 ratio were also

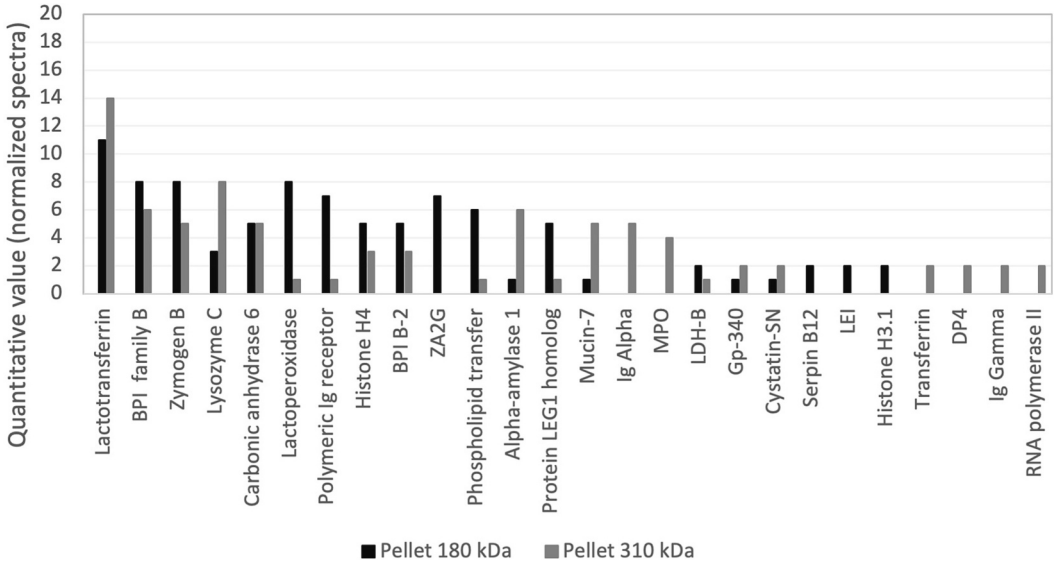

Fig. 7. Identified salivary proteins present in bands of pellet sample PS compared to the pellet of the negative control (PNC) where 180 and 310 are the estimated MW of the bands, via LC-MS.

analyzed via Native-PAGE. The results are presented in 3.3.

3.3. Native PAGE (Polyacrylamide Gel Electrophoresis) combined with LC-MS

Unlike the commonly used SDS-PAGE, which involve extensive protein denaturation, the Native-PAGE is an innovative method that allows to keep the samples in their native forms. Fig. 5 shows the resulting gel, where the main proteins in pea (Legumin, Convicilin, Vicilin) were visible in their native forms in the PPI alone sample with MW of 370, 250 and 188 kDa, respectively (Tzitzikas et al., 2006). One can also remark the upmost band that shows some proteins were not able to enter the gel, likely because of their very large size, which is in line with what was seen in SEC, which was also carried out in native conditions (see 3.1). Regarding the saliva sample alone, the three thickest bands were located at 789, 69 and 21 kDa, and could correspond to Mucins, Alpha-amylase and Cystatins, respectively (E. J. Helmerhorst & Oppenheim, 2007). In the saliva:PPI mixture, which was carried out only at 1:1 ratio, both the sample and the negative control, also three main bands were observed at 60, 180 and 310 kDa. None of these bands are visible in the PPI or the saliva samples alone, although the bands at 60 and 180 kDa are also visible in the negative control. Therefore, they are thought to be complexes formed by the interaction of pea proteins from the PPI sample with salivary proteins, especially the band at 310 kDa since it is much stronger in the sample than in the Negative control. To confirm the identity of the proteins observed in the gels of the sample and negative control, bands at estimated Molecular weight (MW) 60, 180, and 310 were excised of Native PAGE gels and analyzed via LC-MS.

Fig. 6 shows the 25 pea proteins identified in the 180 and 310 kDa bands. Since the band at 60 kDa contained a very small number of pea proteins ($n = 7$), and their presence was low (normalized number of spectra <10, data not shown), it was not further analyzed. In the 180 kDa band, the majority of the identified pea proteins (17 out of 20) had a higher presence in the sample than in the negative control. In this band, only the Legumin A was not present in the negative control. In the band at 310 kDa, all the identified pea proteins ($n = 23$) were more present in the sample than in the negative control. A few proteins were absent from the negative control, including Vicilin 47 kDa (D3VNEO, D3VND7), Provicilin (fragment), Albumin-2 and Albumin-1C. Nevertheless, the last two were not very abundant.

Regarding the salivary proteins, 26 different proteins were identified in the 180 and 310 kDa bands (Fig. 7). No salivary protein was detected in the band of 60 kDa, which further demonstrates that no aggregates involving salivary proteins were formed at this MW. Five of the most abundant salivary proteins detected have been previously connected to astringency including Lactotransferrin (Gambuti et al., 2006), Zinc-alpha-2-glycoprotein (ZA2G) (Bateman et al., 2021; Vardhanabhuti et al., 2010), Alpha-amylase (De Freitas & Mateus, 2001; Gambuti et al., 2006), Mucin 7 (Biegler et al., 2016; Gambuti et al., 2006; Lee et al., 2012; Silletti et al., 2010) and Cystatin SN (Ployon et al., 2018; Silletti et al., 2010). All of them were present in the two aggregates, except for ZA2G which interestingly was only found at 180 kDa. Lastly, two proteins that were also previously detected in the saliva:PPI mixtures via LC-MS (3.2), namely Zymogen granule protein 16 homolog B and Carbonic anhydrase 6 (CA-VI) were also detected in the Native-PAGE bands, confirming their capability to form aggregates with pea proteins; nevertheless, their role in the astringency perception is less clear and has not been documented in the literature.

4. Conclusions

The goal of the present study was to identify the pea and salivary proteins involved in the precipitate formation that results when PPI and saliva are mixed. It was seen that most of the identified pea proteins that precipitated in the presence of saliva, also did in the negative control (HEPES buffer), meaning their precipitation is not exclusively triggered by saliva and could be driven by self-aggregation. However, it can be concluded that the precipitation of these proteins is higher in the presence of saliva. Although the precipitation in the negative control was not assessed quantitatively, the analysis of the precipitation between the two studied ratios, showed that when more saliva is present, these proteins tend to precipitate more. This proves that salivary proteins play an active role in pea protein precipitation. Moreover, it was concluded for the first time that aggregates between pea and salivary proteins can be formed. These aggregates have a MW of 180 and 310 kDa. In addition, seven pea proteins were found in these aggregates which were less abundant (aggregate 180 kDa) or absent (aggregate 310 kDa) in the negative control, which are listed below:

- Legumin A2,
- Legumin A,
- Legumin K,
- Legumin J

- Vicilin 47 k D3VND7,
- Vicilin 47 k D3VNE0,
- Albumin-2

Furthermore, proteins from the Cystatin family were consistently identified in the precipitates and aggregates studied, specifically, Cystatin-SN stood out in two different techniques. Mucins (i.e. Mucin 7) also appeared to be involved in the formation of aggregates with pea proteins. Other salivary proteins that were found to interact and precipitate with pea proteins were Zymogen granule protein 16 homolog B and Carbonic anhydrase 6 (CA-VI); nevertheless, their role in the astringency perception is not recognized and remains to be investigated.

In conclusion, this study has shown for the first time that pea and salivary proteins are able to form a complex and precipitate, which might be responsible for the perception of astringency. These findings set the basis of future research that should focus on the confirmation of the role of the identified pea proteins in astringency perception by their purification and subsequent sensory analysis. Furthermore, in silico protein analysis could allow to detect common structural characteristics shared between the proteins and provide a deeper insight on the molecular origin of astringency mechanisms.

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CRediT authorship contribution statement

Assad-Bustillos, M.: Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing - Reviewing and Editing. Cázares-Godoy, A.C.: Data curation, Investigation, Writing - Original draft preparation. Devezeaux de Lavergne, M.: Conceptualization, Methodology, Supervision, Writing - Reviewing and Editing.Schmitt, C.: Supervision, Validation, Writing - Reviewing and Editing. Hartmann, C.: Supervision, Validation, Writing - Reviewing and Editing. Windhab, E.: Funding acquisition, Project administration, Supervision, Validation, Writing - Reviewing and Editing.

Declaration of Competing Interest

None.

Data availability

Table of all proteins is available

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2023.103290.

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