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In vitro gastrointestinal digestion of pea protein isolate as a function of pH, food matrices, autoclaving, high-pressure and re-heat treatments

Laura Laguna [a, b], Pierre Picouet [c,e], M. Dolors Guàrdia [c], Catherine M.G.C. Renard [d], and Anwesha Sarkar [a]*

[a] Food Colloids and Processing Group, School of Food Science and Nutrition, University of Leeds, Leeds LS2 9JT, UK

[b] Institute of Food Science Research (CIAL), CSIC-UAM

C/ Nicolás Cabrera 9, 28049, Madrid, Spain

[c]IRTA-TA, Food Processing and Engineering, Finca Camps i Armet 17121 Monells, Girona, Spain

[d]UMR408 Sécurité et Qualité des Produits d'Origine Végétale, INRA, Avignon University, F-84000 Avignon, France

[e] USC1422 GRAPPE, Univ. Bretagne Loire, Ecole Supérieure d'Agricultures (ESA), INRA, 55 rue Rabelais, F-49007, Angers, France

*Corresponding author:

Dr. Anwesha Sarkar

Food Colloids and Processing Group,

School of Food Science and Nutrition, University of Leeds, Leeds LS2 9JT, UK.

E-mail address: A.Sarkar@leeds.ac.uk (A. Sarkar).

Abstract

This study investigated the influence of pH and processing conditions (autoclave at 93 °C/13 min or high pressure processing (HPP) at 600 MPa/5 min without/ with follow-up reheating at 80 °C/30 min) on the digestibility of pea protein isolate. Both aqueous solutions and real food matrices (apple and carrot purees) containing pea protein was examined at 37 °C. In vitro gastrointestinal digestion was followed using sodium dodecyl sulphate polyacrylamide gel electrophoresis, titrimetric techniques and theoretical calculations. Pea protein with HPP followed by re-heating showed the highest rate of proteolysis in gastric conditions. In case of sequential intestinal digestion of the gastric chyme, pea protein at pH 6.2 demonstrated higher degree and rate of digestibility as compared to that at pH 3.6, the latter being close to the isoelectric point of pea protein. However, autoclave treatments overshadowed such pH effects. Processing-induced enhancement in digestibility might be attributed to the unfolding of the globular pea protein subunits. Pea protein in the carrot puree was more digestible than in the apple puree, due to apple procyanidins binding to pea protein. These new findings might have important implications in designing the process parameters and selection of appropriate food matrices for delivering pea protein.

Key words: HPP, autoclave, digestibility, puree, pea protein

3.1 Introduction

Proteins are an essential component of the diet, however, their intake and recommendations vary with age (Chernoff, 2004). Particularly, in the elderly population, in order to improve body function, an increase in the protein intake is generally recommended (Wolfe, Miller, & Miller, 2008). Whilst for healthy adults, the recommended dietary allowance is 0.8 g/kg/d, controlled trials report protein recommendation for elderlies at 1.0-1.3 g/kg/d (Nowson & O'Connell, 2015). Despite this recommendation, protein malnutrition is a frequently encountered problem in the elderlies. This might be attributed to the lack of adequate protein intake or lower metabolism of the ingested protein type. For that, food designed for elderlies should take into account not only the nutritional composition but also the digestibility of protein.

Due to relatively low cost and reduced influence on the environment, plant proteins have captured recent research and industrial attention (Barac, et al., 2010; Sarkar & Kaul, 2014). Proteins from legumes, such pea (Pisum sativum L.) are a good source of lysine, biologically active components, such as antifungal bioactive peptides or dietary lectins with health-promoting properties (Nguyen, Gidley, & Sopade, 2015). Besides the amino acid contents, the bioavailability of the protein, which is in part governed by the digestion rate and extent, is a key determining factor of protein quality and postprandial protein gain (Dangin, et al., 2001). The digestion kinetics of a particular protein may also depend on the processing conditions, pH during such processing, interactions with other components in the food etc (Sarkar, Goh, & Singh, 2010; Sarkar, Goh, Singh, & Singh, 2009; Singh & Sarkar, 2011). Habiba (2002) studied the changes in anti-nutrients' content, protein and amino acid solubility, digestibility of vegetable pea after different cooking methods (ordinary cooking, pressure cooking and microwave). Overall, cooking improved the in vitro protein digestion rates by decreasing the levels of various anti-nutrients, such as phytic acid, trypsin inhibitor etc.

However, traditional cooking was also postulated to result in lesser extent of digestibility. For example, high temperatures or prolonged exposure to heat has been reported to result in losses in the essential amino acids due to Maillard reactions (Satterlee & Chang, 1982), and thus might reduce the overall digestibility of the proteins.

To overcome some of these issues with conventional heat treatments, alternative processing, such as high hydrostatic pressure processing (HPP) have been proposed, which reduce microbial counts to a similar level as compared to that of the conventional pasteurization treatments (Hurtado, et al., 2017; Picouet, Sárraga, Cofán, Belletti, & Guàrdia, 2015). In meat and milk proteins, HPP promoted structural changes by protein unfolding and re-binding to form aggregates (Considine, Patel, Anema, Singh, & Creamer, 2007). Besides industrial processing, food products are often re-heated at homes in ovens, microwave oven etc before consumption, particularly the foods that are tailored for elderly population (Laguna, et al., 2016). However, rare attention has been paid in literature to understand whether such reheat treatment has any additional influence on the digestibility of the proteins ingested. Although the enzymatic hydrolysis of pea protein has been investigated (Barać, et al., 2011), to our knowledge, there has been no literature that studied systematically the impact of different processing conditions on digestibility of pea protein isolate.

Hence, this study aimed to investigate the digestibility of pea protein isolate, as a function of pH, food matrices, processing conditions (autoclave or HPP) with/ without reheating. We hypothesize that such severe processing will enhance the degree and rate of proteolysis of pea protein. Two pH conditions (pH 3.6 and pH 6.2) were selected to represent the two extreme pHs of food products in real life as well as to serve as controls for the food products being tested (apple and carrot puree), containing 50 g/L pea protein isolate, respectively. Apple and carrot purees were chosen because they are known to be widely

accepted by the elderly population (Mingioni, et al., 2016), and their digestibility can be hypothesized to be independent of the oral processing capability of the potential consumers.

3.2 Materials and methods

2.3.1 Materials

2.1.1 Protein source

Pea protein (NUTRALYS S85F, with a protein content of 840 g/kg), was kindly supplied by Roquette (Roquette, Lestrem, France).

2.1.2 Chemicals

Pepsin from porcine gastric mucosa (P7000, ≥250 units/mg protein), trypsin from porcine pancreas (85450C, ≥250 units/mg protein) and α-chymotrypsin from bovine pancreas (C4129, ≥40 units/mg protein) were purchased from Sigma–Aldrich Chemical Co., St. Louis, USA. Mini-PROTEAN® TGX™ precast polyacrylamide gels (8–16% gradient, 10×30 μL wells), Precision Plus Protein™ standards (10-250 kDa) and Proto-Safe Coomassie stain were purchased from Bio-Rad Laboratories Ltd., Hemel Hempstead, UK. Analytical-grade reagents were used for the preparation of all solutions. Milli-Q water (water purified by a Milli-Q apparatus, Millipore Corp., Bedford, MA, USA) was used as a solvent in all experiments.

2.3.2 Methods

2.2.1 Sample preparation

Fig. 1 shows the schematic representation of the sample preparation as a function of pH, processing conditions, food matrices. In order to understand the kinetics of protein digestion as a function of pH, two buffers were prepared, 0.2 mol/L Na-acetate (adjusted to pH 3.6 with 1 mol/L HCl, simulating the pH of apple puree, B3.6) and 0.05 mol/L Tris buffer (adjusted to pH 6.2 with 1 mol/L NaOH, simulating the pH of carrot puree, B6.2).

Pea protein was dispersed in each of these two buffers at 50 g/L (protein content) and stirred for 2 h at ambient temperature. Processing treatments were employed for each pH conditions: no heat treatment (N), heat treatment in autoclave (A), autoclave followed by re-heating (reheating at 80 °C/ 30 min in a water bath) (A-RH), HPP (HPP) and re-heating HPP samples (HPP samples were heated again at 80 °C/ 30 min in a water bath) (HP-RH). To study the influence of the food matrices, carrot (CP) and apple puree (AP) containing 50 g/L pea protein with/ without autoclave/ high pressure processing conditions (described in Fig. 1) in presence or absence of re-heat treatment were obtained from the pilot plant of IRTA (Girona, Spain).

2.2.2 Processing conditions

Pea protein solutions or purees enriched with proteins were autoclaved in an ILPRA-Plus autoclave (Ilpra Systems, Mataro, Spain) with an initial ramp of 7 min to reach 93 °C, followed by a holding period of 13 min at 93 °C and a cooling period of 10 min to achieve 40 °C. For HPP, an industrial scale HPP equipment Wave 6500/120 of 120 L (Hyperbaric, Burgos, Spain) was used. The pressure ramp was 215 MPa/min, holding time at 600 MPa was 5 min and the total processing time was 8.05 min. Pressure measurements were made with IS-20H pressure transducers (WIKA Instrument, Lawrenceville, GA, USA), which was able to measure pressure from 0-689.5 MPa. For HPP treatment, the initial water temperature was 9-10°C and was measured by a temperature sensor (Pt100 temperature sensor, IFM Electronic, El Prat de Llobregat, Spain). Following empirical equation (Patazca, Koutchma, & Balasubramaniam, 2007), the quasi-adiabatic temperature increase (ΔT) could be estimated to be 15-18 °C in these processing conditions (600 MPa) and the maximum temperature achieved will be 25-28°C adding the initial temperature of 10 °C.

142 **2.2.3 In vitro gastrointestinal digestion**

143 Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared following the
144 harmonized protocol (Minekus, et al., 2014). Before adding the enzymes, SGF was adjusted to
145 pH 2 using 0.1 mol/L HCl and SIF was adjusted to pH 6.8 using 0.1 mol/L NaOH. Once the
146 samples were added to the SGF solution in 1:1 mL:mL, pH was readjusted to pH 2 and 320
147 mg/100 mL of pepsin was added. The simulated gastric digestion was followed for 2.5 h in a
148 shaking incubator at 37 °C. For the intestinal phase, the gastric chyme (i.e. sample:SGF
149 mixture) was mixed with SIF in 1:1 mL:mL and then neutralized at pH 6.8. Chymotrypsin and
150 trypsin were added to the SIF in the proportion of 160 mg and 310 mg, respectively per 100
151 mL of SIF. The simulated intestinal digestion was followed for 3 h in a shaking incubator at
152 37 °C.

153

154 **2.2.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) of gastric**
155 **digesta**

156 The gastric digestion of the samples was examined using reduced SDS-PAGE technique. Pea
157 protein-SGF mixtures (50 µL) were periodically sampled (0-150 min) and 50 µL of Laemmli
158 buffer (62.5 mmol/L Tris-HCl, 20 g/L SDS, 250 ml/L glycerol, 0.1 g/L bromophenol blue, 50
159 g/L β-mercaptoethanol) was added and the mixture was heated at 95° C for 5 min. After
160 cooling, 10 µL was loaded onto the SDS gels previously prepared on a Mini-PROTEAN II
161 system (Bio-Rad Laboratories). Gels were run at 100 mV/ 10 min and 200 mV/ 30 min, stained
162 with Coomassie Blue R-250 [0.5 g/L in 250 mL/L isopropanol, 100 mL/L acetic acid] for 4 h
163 and then de-stained with distilled water for 1 h. Gels were scanned using a flat-bed scanner
164 (Bio-Rad Molecular Imager, Chemi-Dco XRST) and protein band intensities were quantified
165 using Image LabTM software version 5.1 Beta.

166

2.2.5 Theoretical intestinal digestibility

In vitro intestinal digestibility (without prior gastric digestion) of the pea protein isolate was assayed using the single pH-drop procedure. The theoretical digestibility assay is based on regression analyses, where tested food samples have shown strong relationship (correlation coefficient ~0.90) between in vitro digestibility (pH drop at 10 min) and in vivo apparent digestibility (Hsu, Vavak, Satterlee, & Miller, 1977). The drop in pH corresponds to the release of amino acids and peptides as digestion progresses. In this study, 10 mL of the protein (50 g/L) dispersed in the two different buffers (pH 3.6 and 6.2) were mixed with 10 mL of SIF without added enzymes. For puree samples, 10 g of purees were mixed with 10 mL of SIF without added enzymes. The pH of the sample-SIF mixture was adjusted to pH 8.0, followed by immediate addition of trypsin (3.1 mg/mL) and chymotrypsin (1.6 mg/mL). Then, the change in pH at 10 min (ΔpH_{10min}) was used to calculate the percentage in vitro protein digestibility (IVPD) using Equation (1) (Tinus, Damour, Riel, & Sopade, 2012):

$$IVPD = 65.66 + 18.10\Delta pH_{10\,min} \quad (1)$$

2.2.6 Kinetics of sequential intestinal digestion

For sequential intestinal digestion, SIF was added to the gastric chyme (i.e. samples already digested by of SGF (Section 2.2.3)), and titration measurements were performed at 37 °C with an automated pH-stat device (TitraLab, Radiometer Analytical, Copenhagen, Denmark). Titration of the amino acids was carried out using freshly prepared 0.05 mol/L NaOH solution using endpoint of pH 6.8. Three measurements were carried out and results were represented as titratable acidity (mol%), using equation (2):

$$Titratable\ acidity\ (mol\%) = \frac{mL\ of\ NaOH\ used x 0.05 \frac{mol}{L} x\ NaoH}{g\ sample} \times 100 \quad (2)$$

From the titratable acidity curve, three parameters were obtained:

- Rate of digestion (mol%/ min). Calculated from the slope of the curve, in other words, it implies the kinetics of digestion.
- Maximum extent of digestion (mol%). This factor implies the final value of of titratable acidity reached.
- Time to reach maximum extent of digestion (min). This factor represents the total time required to arrive at the maximum extent of titratable acidity.

2.2.7 Data analysis

One-way ANOVA was used to understand the difference in the IVDP between different samples. In order to know which factor (pH or processing) had more influence, two-way ANOVA with the percentage of digestibility as dependent value and pH and processing as the independent values was calculated. The least significant differences were calculated by Tukey's test ($P<0.05$). To understand the influence of processing conditions, re-heating and pH on digestibility, a multivariate analysis of variance (MANOVA) was performed using the data from the pH-stat titration. In order to study the effect of the re-heat treatment and the effect of the food matrix (non-continuous variables), a generalized linear model (GLMZ) was applied using the re-heat treatment as a factor and processing conditions, pH as covariates. Wald Chi-square test was used to study the significance of the difference. These tests were done with IBM SPSS Statistics for Windows, Version 22.0. (Armonk, NY: IBM Corp).

2. Results and discussion

3.1. SDS-PAGE of pea protein solutions during simulated gastric digestion

During simulated gastric digestion at acidic conditions, pea protein solutions at pH 3.6 and 6.2 were readjusted to pH 2 for 2 h using SGF before adding pepsin. Hence, the influence of initial pH was not considered in the SDS-PAGE experiments. Quantitative changes in protein

composition without processing (B3.6-N) or with autoclave treatment (B3.6-A) or HPP (B3.6-HP) or with/without follow-up re-heating (B3.6-A-RH, B3.6-HP-RH) during digestion were monitored (Figs. 2 and 3).

Pea protein consists of legumin (11S), vicillin (7S) and albumins (2S), with the most abundant globulins being 11S and 7S (O'Kane, Vereijken, Gruppen, & Van Boekel, 2005). Pea protein without any processing (B3.6-N) showed three sets of protein subunits i.e. convicillin (72.4-77.9 kDa), vicillin (28.7-47.3 kDa) and legumin (22.3-23.1) subunits (Fig. 2A), which is in line with the previous report (Adal, et al., 2017). When no processing was applied, most of the pea protein bands disappeared on digestion by pepsin within the first 30 min (Fig. 3A). However, 20% of convicillin (75 kDa) remained even after 150 min of digestion. A similar trend was observed for vicillin (35 kDa), which also remained after 150 min. Interestingly, the convicillin band was digested on autoclaving within the first 30 min (Fig. 2B and 3B).

In case of the autoclave treatment (B3.6-A), a 15 kDa band appeared, which was rapidly digested within 30 min (Fig. 3B). Re-heating pea protein after autoclaving (B3.6-A-RH) resulted in complete digestion of this vicillin band (Fig. 2C and 3C). High-pressure treatment increased the gastric digestibility of pea protein, as reported in case of other proteins (Hoppe, Jung, Patnaik, & Zeece, 2013). With HPP treatment (B.3.6-HP), bands appeared between 100-75 kDa and between 50-25 kDa, which dissapeared within the first 30 min of digestion (Fig. 2D and 3D). About 20% of the vicillin bands at 35 kDa remained even after 150 min of pepsin digestion in the B.3.6-HPP samples (Fig. 3D). Interestingly, in the samples with HPP followed by re-heating (B3.6-HP-RH), intact protein bands disappeared almost instantaneously on addition of pepsin (Fig. 2E and 3E). The bands showed appearance of low molecular weight peptides (<10 kDa) (Fig. 2E). With HPP and further re-heating, the globular pea proteins might have been fully unfolded, allowing the otherwise buried hydrophobic groups to be exposed to

pepsin (Considine, et al., 2007). Therefore, in comparison with autoclaving, HPP followed by re-heating showed highest kinetics and extent of gastric digestion (Fig. 2E and 3E).

3.2. Theoretical digestibility (IVDP) of pea protein solutions during in vitro intestinal phase - pH and processing treatment dependence

Table 1 presents the IVDP of pea protein solutions (without prior gastric digestion). The IVDP follows a single pH-drop procedure, drop in pH corresponds to the release of amino acids due to trypsin and chymotrypsin-mediated protein digestion. The IVDP of B3.6-N was 10% higher than that of B6.2N suggesting influence of initial pH ($P<0.05$). Although this was not expected as both the samples were re-adjusted to pH 8.0 before the pH drop was assessed, this can be explained based on the stronger buffering capacity of the pea protein samples at pH 3.6, which led to the pH drop rather than the amino acids release. Such buffering capacity of protein interfering with the pH drop method has also been previously reported (O'Hare, Curry, & Allen, 1984).

At pH 6.2. there was no statistically significant difference between samples that underwent autoclave and HPP treatments (B6.2-A, B6.2-HP) ($P<0.05$), with B6.2-A-RH showing lowest IVDP (74 ± 1%). The highest IVDP (95.3±0.3 %) was shown by pea protein solution at pH 3.6 after being autoclaved and re-heated (B3.6-A-RH). Also, B3.6-HP had higher IVDP than that of samples at pH 6.2. Although pH and processing treatment were both significant ($P<0.05$), comparing F-values ($F_{pH}= 91.20$ and $F_{processing\ conditions} = 4.61$), the IVDP was more influenced by pH as compared to processing conditions, which can be attributed to the buffering effects as described before.

Linsberger-Martin, Weiglhofer, Phuong, and Berghofer (2013) studied the IVDP in dry split peas submitted to different HPP conditions (100 and 600 MPa; holding times of 30 and 60 min; at 20 and 60 °C). They found that IVDP was higher for samples that were pressurized

at 600 MPa at 60°C in comparison with traditional cooking. In the current work, industrial-scale equipment was used with holding time comparable with real-life industrial situation, while in Linsberg et al. (2013), a pilot-scale equipment was used with much longer holding times of 30-60 min and temperature of 20-60°C. Combined with difference in pea powder protein versus dry split pea, these different processing parameters might explain the difference observed in IVDP.

3.3. Sequential in vitro intestinal digestibility of pea protein gastric chyme - pH and heat treatment dependence

In the Fig. 4A and 4B, kinetics of titratable acidity of the released amino acids (mol%) for pea protein gastric chyme are shown. The proteolysis in sequential gastrointestinal digestion was highly dependent on the initial pH. The kinetics parameters of digestibility were extracted from Fig. 4 and presented in Table 2.

3.3.1 Rate of digestion.

For autoclaved protein (B3.6-A, B6.2-A) and re-heated samples at low pH (B3.6-A-RH), rate of digestion was approximately 1% mol/min higher than the rest of the samples. Processing condition*pH had a significant effect on the rate of digestion ($P<0.05$). Samples with no processing had a higher digestion rate at high pH ($B6.2\text{-}N_{slope} > B3.6\text{-}N_{slope}$), whilst samples with reheating had lower rate of digestion at close to neutral pH ($B6.2\text{-}A\text{-}RH_{slope} < B.3.6\text{-}A\text{-}RH_{slope}$). The pH effects on digestibility can be related to the preferential solubility of pea protein at pH 6.2, thus providing better accessibility to the proteases. In contrast, the sample at pH 3.6 was less soluble as it was close to the isoelectric point (pI) of pea protein (pH 4.0) explaining the lower digestibility (Adal, et al., 2017).

3.3.2 Time to reach maximum extent of digestion.

The processing condition*pH were the key factors influencing the time to reach maximum extent of digestion. The shortest time was needed for B3.6-A and B6.2-A-RH.

3.3.3 Maximum extent of digestion.

There was no significant difference in the maximum extent of digestion ($P<0.05$) (Table 2), except the initial pH. Absence of overall significant changes might be because samples were already digested in the gastric phase (pH 2) by pepsin. Hence, by the time the samples arrived at the intestinal phase, protein hydrolysis was nearly complete. The maximum rate of digestion occurred in the intestinal regime for the pH 6.2 samples. This can be partly attributed to B6.2N chyme in intestinal regime, which might have arrived with less degree of proteolysis from the gastric regime. Such low degree of gastric proteolysis in B6.2N may be due to its buffering capacity that restricted reaching the optimal pH for pepsin activity. Furthermore, the higher protein solubility at pH 6.2 (as discussed before) allowed maximum extent of digestion in the intestinal regime for B6.2N. It is worth noting that such in vitro gastrointestinal digestion behaviour of pea protein might not represent the actual extent of bioavailable protein in human physiology, the later requires validation of in vitro results with in vivo data which was not within the scope of this study.

3.4. Influence of food matrices on IVDP

Table 3 presents the IVDP (without prior gastric digestion) of the different food matrices (carrot and apple puree) containing pea protein under different processing conditions. Overall, significant differences were found among the different purees with and without processing ($P=0.01$). Contrasting to IVDP results in buffered systems (Table 1), apple puree (pH 3.6) appeared to be less digestible than carrot puree (pH 6.2) (IVDP~68%, ~98% respectively), when no processing was applied. This might be attributed to comparatively more affinity of

316 apple polyphenols to bind to pea protein, making it less accessible to the proteolytic enzymes.
317 It is well recognized that most polyphenols can bind to proteins, but with variables affinities.
318 Tannins have the highest affinities and capacity to precipitate proteins. Apples and apple puree
319 are rich in condensed tannins, specifically procyanidins (> 0.5 g/kg FW) which are well known
320 for their high degree of affinity to bind to other plant macromolecules (Le Bourvellec, et al.,
321 2011; Le Bourvellec & Renard, 2012). In contrast, in carrot, the polyphenols are mostly
322 phenolic acids and some anthocyanins, the later being present only in black carrots (Kamiloglu,
323 et al., 2017), which have comparatively less affinity for proteins. However, once processing
324 was applied, there was no significant difference in digestibility of these two food matrices
325 (P=0.791). This further validates the hypothesis that processing played a significant role in
326 increasing digestibility of pea protein which overshadowed matrix effects.
327
328 **3.5. Conclusions**
329 In vitro pea protein digestibility was highly influenced by processing and pH. It was clearly
330 demonstrated that HPP treatment enhanced the degree and rate of proteolysis as compared to
331 autoclave, this effect was further enhanced with a follow up re-heating. The initial pH showed
332 a strong effect on extent and degree of digestibility particularly in the sequential gastrointestinal
333 digestion where pea protein at pH 6.2 was significantly more digestible owing to higher
334 solubility of pea protein at that pH. In case of the product application, protein digestibility was
335 lower in apple puree than carrot puree due to the potential binding of the pea protein to apple
336 procyanidins, reducing its accessibility for the proteolytic enzymes. However, such matrix
337 effects were not observed when processing conditions were applied. These new findings might
338 have important implications in designing the process parameters and selection of food matrices
339 for delivering pea protein in optimized food for elderlies.
340

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