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ABSTRACT

In the food and feed industry, protein extraction is commonly performed under acid or basic conditions, combined with heat, in order to increase the extraction yield. Under severe processing conditions, proteins may undergo molecular modifications. Here, the effects of heating (30, 60, 90°C) at different pH values (2, 7, 9, 11, 13) were evaluated on commercial whey proteins, used as a simplified protein model. The main structure and chemical modifications concerning protein aggregation, hydrolysis, insolubilization, amino acid degradation and racemization were investigated in detail. Using *in vitro* static models, the degree of protein hydrolysis and the released peptides were determined after the digestive process. Accumulation of molecular modifications was mostly observed after basic pH and high temperatures treatments, together with a marked decrease and modification of the digestibility profile. Instead, protein digestibility increased in neutral and acidic conditions in a temperature-dependent manner, even if some modification in the structure occurs.

KEYWORDS: protein modification; protein digestion; heat treatment; basic pH; whey proteins; peptide release

1 INTRODUCTION

Proteins are one of the three main macronutrients and are essential components to be introduced into the human diet. Dietary proteins are found as animal-based (milk, meat, eggs) and plant-based (cereals, legumes). The integrity of the proteins is an important parameter closely related to the quality of their constituents, the amino acids, as well as to their biological use and their digestibility (Friedman, 1996).

The Food and Agricultural Organization of the United Nations (FAO) indicated that, with an expected population rise to 9.1 billion, it will be necessary to increase food production by 70% by 2050 (FAO, 2009). Given the demographic increase associated with socio-economic changes (e.g., recognition of the role of proteins in a healthy diet), also the demand for protein is expected to strongly increase (Henchion et al., 2017). It has been estimated that only 35% of the protein produced by the agricultural sector is used for food use, while the majority is used for feed, non-food products or wasted (Leip et al., 2014). To meet the increasing demand for proteins, food and feed industries are persistently aiming to extract additional proteins from traditional protein sources (milk protein, cereals, oil seeds) as well as new or unconventional (insects, microalgae, leaves). Additionally, many new research underscores the importance of using food waste and by-products as protein source in food and also in non-food applications (Torres-León et al., 2018). An example of the valorisation of food waste is represented by whey, the main by-product of the dairy industry, which is widely reused and appreciated for its nutritional, biological and functional properties (Mollea et al., 2013).
For this reason, many methods have been developed for the extraction and utilization of proteins from many diverse biomasses. In the food and feed industry, the most common technique used to extract proteins involves solubilisation in water under neutral, acidic, or basic conditions (Qin et al., 2018), together with heating at medium to high temperatures to increase the solubilisation, and/or possibly the use of specific enzymes. In recent studies, acid or basic conditions combined with the addition of specific enzymes (mainly proteases) are often used to hydrolyse proteins into peptides and free amino acids, and consequently increase the extraction yield (Jung, 2009; Sari et al., 2015). Alternatively, the modification of the pH around the value of the isoelectric point (e.g., 4-5 for caseins) represents an alternative technique to purify proteins after their precipitation (as in the case of the dairy industry) (Borad et al., 2017).

Alkaline treatments, on the other side, are among the most used methods for protein extraction, highly valued for their low cost and high extraction yield (Momen et al., 2021; Roberts et al., 1985). The parameters of the extraction conditions, such as type and concentration of the solvent, pH, time, and temperature, play an important role in this type of processing. Frequently, the alkaline extractions are obtained by using NaOH in water at a concentration of 0.1-1 M (Sari et al., 2015). Next to the solvent concentration, also the time-temperature combination represents a key factor (Awuah et al., 2007; van Boekel et al., 2010). Most processes use a processing temperature between 25°C and 90°C, for a time varying between 10 and 120 minutes (Sari et al., 2015). In addition, a combination with other processes/treatments is often performed. Samples are commonly pre-treated by flaking, extrusion, de-oiling, ammonia fibre expansion, lyophilization in order to eliminate the non-protein fractions (Sari et al., 2015). Severe conditions such as long processing times, heat, extreme pH increase the extraction yield and are therefore widely used in the food and feed industry (Cui et al., 2017; Zhang et al., 2014). On the other hand, they can cause adverse effects on the integrity, quality, and subsequent digestibility of proteins (Meade et al., 2005; van Lieshout et al., 2020; Wu, W.;Hettiarachchy, N.S.;Kalapathy, 1998). For this reason, more attention needs to be paid to these aspects in the field of food processing (Meade et al., 2005).

A detailed understanding of the molecular changes that occur during extraction and how different extraction methods affect the digestibility of proteins would be important to ensure the production of high nutritional value proteins. Food processing conditions, as heat treatments and pH alteration, indeed affect proteins and their constituents, leading to structural and chemical modifications. These changes generally involve all proteins, of both animal and plant origin, and alter their digestibility and absorption (Sun-Waterhouse et al., 2014). Anyway, in depth molecular studies linking specific protein modification occurring during technological processing and digestion are very limited. Thus, to gain detailed molecular insights on how different methods of processing can modify protein structure, and how this, in turn, affect protein digestibility, whey proteins are used in this work as a model protein system to check the effect of processing on protein modifications and hence digestibility. To do this, commercial whey proteins isolate are subjected to different processing conditions for 3 hours, studying the effect of pH (between 2 and 13) and heat (30°C, 60°C, 90°C), Aggregation, degradation, solubility, glycation, and racemization are determined on the processed proteins at an unprecedented level of molecular details using state-of-the art chromatographic and spectrometric

techniques. Then, on the digestate, the degree of hydrolysis of proteins and the amount and the type of the different peptides released are determined. In this way, the link between processing conditions, protein structure modification and protein digestibility is clearly outlined.

2 MATERIALS AND METHODS

2.1 Reagents and solvents

Commercial whey protein, α-lactalbumin and β-lactoglobulin from Aptonia (UK) were used in the present study. 20× XT MES running buffer, 20× XT reducing agent, 4× XT sample buffer, Coomassie Brilliant Blu protein stain powder R-250, Criterion Bis-Tris Precast Gels (12% Bis-Tris, 13.3 × 8.7 × 0.1 cm), and Precision Plus Protein Standards were from Bio-Rad (Hercules, CA, USA). Quant-iT™ Protein Assay Kit was purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). Defoamer for Kjeldahl analysis was purchased from Merck (Darmstadt, Germany). Hydrogen peroxide, Kjeldahl catalyst tablets (3.5 g/tablet), and sulfuric acid (96%), were purchased from Merck Millipore (Darmstadt, Germany). Ultrapure water was obtained with Milli-Q® system (Merck Millipore, Darmstadt, Germany). The standards of β-lactoglobulin (98% purity) and α-lactalbumin (92% purity), ammonium bicarbonate (NH_4HCO_3), ammonium carbonate ($(NH_4)_2CO_3$), aspartic acid, boric acid, bovine bile, cysteic acid, DL-dithiothreitol (DTT), formic acid (>95%), hydrochloric acid (37%, HCl), L-isoleucine, methionine sulphone, NAC (N-acetyl-cysteine), DL-norleucine, OPA (o-phthaldialdehyde), pancreatin powder from porcine pancreas (8×USP), pepsin from porcine gastric mucosa (757 U/mg), SDS (sodium dodecyl sulphate), sodium chloride (NaCl), disodium phosphate (Na_2HPO_4), monobasic sodium phosphate (NaH_2PO_4), and tryptophan were purchased from Sigma Aldrich (St. Louis, MO, USA). Amino Acid Standard Mixture (2.5 mM) was purchased from Thermo Scientific (Waltham, MA, USA). Kj-tabs VS antifoam was purchased from VELP Scientifica (Usmate Velate, MB, Italy). Acetonitrile (CH_3CN) and copper (II) oxide were purchased from VWR Chemicals (Radnor, PA, USA). The AccQ•Fluor Reagent Kit for Amino Acid Analysis was purchased from Waters (Milford, MA, USA). Calcium chloride ($CaCl_2$), magnesium chloride hexahydrate ($MgCl_2(H_2O)_6$), monobasic potassium phosphate (KH_2PO_4), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), and sodium hydroxide (NaOH) were purchased from Carlo Erba (Milan, Italy).

105 **2.2 Induction of thermal and pH stress on whey protein samples**

106 Whey proteins (10 g) were solubilised in 100 mL of demineralized water and the pH was then adjusted to the
107 desired value (pH 2, 7, 9, 11, 13) using 0.1 M HCl or 0.5-5 M NaOH. The samples were then placed into oil
108 bath under stirring and heated for 3 hours to three different controlled temperatures, (30°C, 60°C and 90°C)
109 for each different pH value.
110 The processed whey samples were then lyophilized (freeze drier Lio 5P, 5 Pa, Milan, Italy) and stored at -
111 20°C until analysis.

112 **2.3 Characterization of the protein fraction**

113 **2.3.1 Characterization of intact proteins**

114 The processed whey proteins were characterized by electrophoresis (SDS-PAGE) under reducing conditions
115 as previously described by Gasparini et al., 2020 except for preliminary sample preparation and the amount of
116 protein loaded. The processed whey proteins, in lyophilized form, were solubilized in DTT/Urea/AmBic buffer
117 (5 mM, 4 M, 100 mM) under stirring for 30 minutes. The solution was then centrifuged at 4°C, 3220*g* for 10
118 minutes (5810R Eppendorf, Hamburg, Germany) and the supernatant was used for the analysis. The amount
119 of protein loaded into each well was 10 µg (quantified by QuBit fluorometer, Invitrogen, Thermo Fisher
120 Scientific, Waltham, MA, USA). 20× reducing agent and 4× sample buffer (suitably diluted) were added to
121 each sample. The sample was then incubated at 95°C for 5 minutes, and then rapidly refrigerated for 5 minutes.
122 The samples were loaded into precast 10% gel (Criterion XT Bis-Tris, Bio-Rad, Hercules, CA, USA).
123 Electrophoretic run (SDS-PAGE) was performed at a constant voltage of 150 V using a Criterion
124 electrophoretic chamber (Bio-Rad, Hercules, CA, USA). Protein bands were visualized on the gel by
125 Coomassie Blue staining. Molecular marker standards (Precision Plus Protein unstained standard, Bio-Rad,
126 Hercules, CA, USA) were used to determine the molecular weight of the proteins in the sample.

127 The whey proteins were also characterized by using UPLC-MS (Acquity Waters with a single quadrupole mass
128 spectrometer) according to a previously published procedure (Gasparini et al., 2020). Briefly, the processed
129 whey proteins, in lyophilized form, were dissolved in water (100 mg/mL), mixed and centrifuged at 4°C, 3220*g*
130 for 10 minutes. Subsequently, the supernatant was filtered through a 0.45 µm filter membrane and injected
131 into UPLC-MS system using a RP column (ACQUITY UPLC BEH 300 C4 1.7 µm 2.1×150 mm, Waters,

Milford, MA, USA) and a gradient elution. Eluent A was H_2O with 0.1% formic acid, eluent B was acetonitrile with 0.1% formic acid; gradient: 0–7 min 69% A, 7–20 min from 69% A to 64.5% A; flow: 0.2 mL/min; column temperature: 35°C; sample temperature: 18°C; injection volume: 4 µL. The samples were analysed in the Full Scan mode; ionization type: positive ions; scan range: 100–2000 m/z; capillary voltage: 3.2 kV; cone voltage: 30 V; source temperature: 150 °C; desolvation temperature: 300 °C; cone gas flow: 100 L/h; desolvation gas flow: 650 L/h. The identification was performed using external standards of pure bovine α-lactalbumin and β-lactoglobulin. The characteristic ions of α-lactalbumin and β-lactoglobulin were extracted and the chromatographic peaks corresponding to the native (unglycated) forms of whey proteins (α-lactalbumin and β-lactoglobulin isoform A and B) and their glycated forms, i.e., mono and dilactosylated, were integrated (when detected) in order to determine the relative percentage of protein glycation.

2.3.2 Determination of soluble and insoluble nitrogen fraction

The soluble nitrogen content was determined using the official Kjeldahl method according to EC 152/2009 (The Commission of The European Communities, 2009). The processed whey protein was first resuspended in water (10 mg/mL), mixed with a shaker for 30 minutes, and then centrifuged at 4 °C, 3220*g* for 30 minutes. Supernatant was analysed by Kjeldahl and the content of soluble proteins was determined by applying the conversion factor for milk and dairy, 6.38 (Food and Agriculture Organisation (FAO) and World Health Organization (WHO), 1973). The insoluble fraction was then estimated from difference of the soluble protein fraction.

2.3.3 Molecular size distribution of the soluble nitrogen fraction

The molecular size distribution of the soluble protein fraction was determined by size exclusion chromatography (SEC), using an Agilent 1260 Infinity II HPLC system equipped with a Refractive Index Detector (Agilent Technologies, Santa Clara, CA, USA). For the analysis, 100 mg of processed whey protein was solubilized in 10 mL of demineralized water. Then, the samples were mixed with an overhead shaker for 30 min at room temperature and centrifuged for 30 min at 3220*g* and 4°C. The supernatant was separated from the pellet and subsequently filtered through a 0.45 µm nylon filter membrane into HPLC vials. The column used for the analysis was a PL aquagel-OH mixed-M (7.5 × 300 mm, 8 µm particle size, Agilent Technologies, Santa Clara, CA, USA) with a molecular mass range between 1 kDa and 500 kDa. The separation was

159 performed under isocratic conditions at a flow rate of 1 mL/min with milli-Q water as the mobile phase,
160 keeping the column temperature set at 30°C. Some samples, in which proper protein identification was
161 dubious, were also spiked with a known amount of β-lactoglobulin in order to confirm the retention time of
162 the native whey proteins, avoiding matrix effects leading to misidentification of the peaks. Peaks having
163 molecular mass higher than whey proteins were considered aggregated proteins. Peaks having molecular mass
164 lower than whey proteins were considered degraded proteins. For every sample, the relative percentage of the
165 different forms (aggregated, native, degraded) was obtained starting from the integrated areas of the
166 corresponding peaks compared to the total area of all detected peaks.

167

168 **2.4 Amino acid profile**

169 **2.4.1 Determination of total amino acids**

170 The total amino acids content was analysed using a previously published procedure (Leni et al., 2019) with
171 some modifications. 100 mg of treated sample were subjected to acidic hydrolysis with 6 M HCl for 23 hours
172 at 110°C. Once the acid hydrolysis was completed, the samples were brought back to room temperature and
173 7.5 mL of 5 mM water-solubilized norleucine was added. Subsequently, the samples were filtered and made
174 up to volume in 250 mL volumetric flasks.

175 A calibration curve for amino acid quantification was prepared by mixing 2.5 mM amino acid standard H
176 (Thermo Scientific, Rockford, IL, USA) and 2.5 mM of norleucine, cysteic acid, methionine-sulphone in 0.1
177 M HCl solution. Dilutions were performed to obtain the following concentrations: 0.625 mM, 0.3125 mM,
178 0.156 mM, 0.078 mM. Then, the standards, as well as the samples, were derivatized for their composition
179 using the AccQ-Tag Ultra Derivatization Kit (Waters, Milford, MA, USA) according to the manufacturer's
180 instructions.

181 Finally, total amino acids were detected and quantified by UPLC-MS (UPLC Acquity with a single quadrupole
182 detector; Waters, Milford, MA, USA) as previously reported by Buhler et al. (2019). Chromatographic
183 separation was performed using a C18 RP column (ACQUITY UPLC Peptide BEH C18 Column, 300 Å, 1.7
184 µm, 2.1 mm × 150 mm, Waters, Milford, MA, USA). H_2O+0.1% formic acid was used as eluent A, while

acetonitrile with 0.1% formic acid was used as eluent B. The flow rate was 0.2 mL/min. Gradient: 0–7 min 100% A, 7–30 min from 100% A to 73.3% A; flow: 0.2 mL/min; column temperature: 35°C; sample temperature: 18°C; injection volume: 10 µL. The samples were analysed in the SIR Scan mode; ionization type: positive ions; capillary voltage: 3.2 kV; cone voltage: 30 V; source temperature: 150 °C; desolvation temperature: 300 °C; cone gas flow: 100 L/h; desolvation gas flow: 650 L/h.

2.4.2 Determination of free amino acids

For the determination of free amino acids, 50 mg of lyophilized sample was added with 10 mL of 0.1 M HCl and 100 µL of 5 mM norleucine, mixed and centrifuged at 3220g for 10 min at room temperature. The free amino acids (10 µL) were derivatized and quantified by UPLC-MS system as reported in the previous paragraph.

2.4.3 Determination of the enantiomeric purity of amino acids

The enantiomeric purity of the amino acids was determined following the previously described procedure (Prandi et al., 2019). 40 mL of acid hydrolysed samples (as described above in the determination of total amino acids) were dried and derivatized before injection into an Agilent Technologies 7820° gas-chromatograph coupled to an Agilent Technologies 5977B mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), equipped with a chiral capillary column (CHIR-L-VAL, 25 m x 0.25 mm I.D., film thickness: 0.12 µm Varian,Agilent Technologies, Santa Clara, CA, USA) for separation of amino acid enantiomers. The percentage of D-amino acid formed after different processing conditions was calculated as D/D+L % for Ala, Asp, Phe, Glu, and Lys.

2.5 *In vitro* peptic digestion and simulated gastrointestinal digestion

2.5.1 Digestion with pepsin

250 mg of each processed whey protein sample was solubilized in 5 mL of 0.01 M HCl, and the pH value was instantly adjusted to 2. The samples were added with 12.5 mg of pepsin (727 U/mg), in an enzyme to substrate ratio 1:20 (López-Fandiño et al., 2010), and were incubated under shaking for 2 h at 37°C. Subsequently, the pH was raised to 7 and the samples were heated for 15 minutes at 90°C to completely inactivate the enzyme.

The supernatant was separated from the pellet by centrifugation at 4°C, 3220*g* for 10 min and stored at -20°C for further analysis.

2.5.2 *In vitro* simulated gastrointestinal digestion

Processed whey proteins were submitted to *in vitro* gastrointestinal digestion according to the previously published INFOGEST static model system (Brodkorb et al., 2019). Using this procedure, three digestive compartments are simulated *in vitro*: mouth, stomach and duodenum. 75 mg of processed whey protein was added to 0.5 mL milli-Q water and immediately underwent simulated digestion. The sample was mixed with simulated saliva fluid (1:1 v/v) and incubated at 37°C for 2 minutes. Then, the simulated bolus was added with simulated gastric fluid (1:1 v/v), pepsin (2000 U/mL) and mixed for 120 min at 37ºC, pH 3. Finally, the gastric chime was added with simulated intestinal fluid (1:1 v/v), pancreatin (trypsin activity of 100 U/ mL) and bile salts (10 mM) and mixed for 120 min at 37ºC, pH 7. At the end of the duodenal phase, the samples were heated at 90°C for 15 minutes to stop the enzymatic reaction, the supernatant was separated from the pellet by centrifugation at 4°C (3220g for 45 min) and stored at -20°C until analysis.

2.5.3 Determination of the degree of hydrolysis

The degree of hydrolysis (DH%) was determined in each sample after peptic digestion and gastrointestinal digestion using the previously reported OPA method (Leni et al., 2019; Spellman et al., 2003) with some modifications. The OPA/NAC mixture solution was stirred for 1 h, protected from light, by mixing 50 mM OPA solubilized in methanol, 50 mM NAC solubilized in water, 20% (w/v) SDS and 0.1 M borate buffer at pH 9.5. The OPA assay was performed by adding 3 μL of the digested (or digestion blank) sample to 17 μL of milli-Q water and 2.4 mL of OPA/NAC reagent. The absorbance was measured on the UV–Vis-spectrophotometer at 340 nm (B530 JASCO, Oklahoma City, OK, USA). Degree of hydrolysis was obtained by calculating the relative percentage between moles of free nitrogen groups determined by OPA assay, and moles of bound nitrogen groups, the latter calculated from the protein content.

2.5.4 Peptide analysis

Peptide analysis was performed on pepsin digested and gastrointestinal digested samples. For the analysis, each digested sample was filtered through a 0.45 μm filter membrane then a 1 mM solution of (L,L)-

phenylalanyl-phenylalanine (Phe-Phe) was added, used as an internal standard for semiquantification, at a final concentration of 50 μM in vial.

Samples were analysed in low resolution using the UPLC system coupled with ESI and MS (UPLC Acquity with a single quadrupole detector; Waters, Milford, MA, USA). Chromatographic separation was performed using a reversed phase column (ACQUITY UPLC Peptide BEH C18 Column, 300 Å, 1.7 μm, 2.1 mm × 150 mm, Waters, Milford, MA, USA). Eluent A was Milli-Q H_2O + 0.1% HCOOH, eluent B was CH_3CN + 0.1% HCOOH. The analysis parameters were as follows: flow rate was maintained at 0.2 mL/min; the applied gradient was: 0−7 min, 100% A; 7−50 min, from 100% A to 50% A; 50−-52.6 min, 50% A; 52.6−53 min, from 50% A to 0% A; 53−58.2 min, 0% A; 58.2−59 min, from 0% A to 100% A; 59−72 min, 100% A. The run time was 72 min; the column temperature was 35°C; the autosampler temperature was set at 18°C; the injection volume was 4 μL. The following parameters were used for the detection: type of ionization, positive ion mode; capillary voltage 3.20 kV; cone voltage 30 V; source temperature 150°C; desolvation temperature 300°C; cone gas 100 L/h; desolvation gas 650 L/h. Acquisition parameters: acquisition time, 7-58.2 min with a scan range from 100 to 2000 m/z.

Finally, the samples were also analysed by UHPLC/ESI-MS/MS using a UHPLC Dionex Ultimate 3000 (Sunnyvale, CA, USA) coupled to a TSQ Vantage triple quadrupole (Thermo Scientific, Waltham, MA, USA), using a method previously described (Prandi et al., 2017). The experimental molecular masses were obtained by low-resolution mass spectrometry and the peptide sequences were identified using online sources (Uniprot, Expasy - FindPept, and Proteomics Toolkit – Fragment Ion Calculator).

The samples were also analysed with high-resolution mass spectrometry. Detection was obtained by VIon IMS QTof Mass Spectrometer (Waters, Milford, MA, USA), following the chromatographic parameters described above (except the injection volume, which was 1 μL and the autosampler temperature that was set at 10°C) and with the following acquisition parameters: Experiment type, peptide map (IMS); MSe; source type, ESI; polarity, positive; analyser mode, sensitivity; mode, standard transmission; capillary, 3.00 kV; sample cone voltage, 40 V; source offset voltage, 80 V; source temperature, 120°C; desolvation temperature, 450°C; cone gas, 50 L/h; desolvation gas, 800 L/h. MSe mode, high definition MSe; acquisition time, 7-58.2 min; scan range, 100-2000 m/z; scan time, 0.4 s; low collision energy, 6V; high collision energy ramp, 20 to 45 V;

automatic lock correction (leucine enkephalin). Data processing was performed using UNIFI software (Waters, Milford, MA, USA). The list of expected components includes the following protein accession numbers (Uniprot): P00711, P07254, P02662, P02663, P02666, P02668, P24627. The permitted variable amino acid modifications are deamidation (N, Q), oxidation (M), pyroglutamic acid N-term (E, Q), phosphorylation (S, T, Y). Digestion reagent: nonspecific; minimum sequence length: 3 amino acids. To obtain the final list of peptides, data filtering was performed using the following criteria: i) peptides with more than 10% matched ions; ii) at least 2 first generation fragment ions; iii) elimination from the list of peptides which were visually identified as in source fragments and have been instead erroneously identified by the software as independent peptides.

2.6 Statistical analysis

Data were analysed using SPSS version 26.0 software (SPSS Inc., Chicago, IL., USA). The data were subjected to one-way analysis of variance (ANOVA), Tukey's b test and LSD test (when variance was homogeneous) to determine significant differences between samples ($p < 0.05$). When the sample variance was not homogeneous, a non-parametric test, the Kruskal-Wallis test and median test, were used. Spearman's test was used to assess the correlation between samples and the effect of temperature and pH treatment ($p < 0.05$; $p < 0.01$). All experiments were performed in duplicate.

3 RESULTS AND DISCUSSION

3.1 Heat treatment and pH stress on whey proteins

The whey proteins in solution (10 g/100 mL) were subjected to different pH and thermal stresses, with temperature ranging from low (30°C), to medium (60°C) to high (90°C), at different pHs (2, 7, 9, 11, and 13). More pH conditions were evaluated in the basic range than in the acidic range, given the greater effects induced on protein structures by the basic environment (van Lieshout et al., 2020). The longest time (3h) and the highest temperature (90°C) were chosen considering the longest time and the highest temperature generally used for protein extractions. The possibility that major structural modifications were already present in the commercial protein could be ruled out by the absence of such modifications in the proteins treated at neutral pH and room

289 temperature (conditions that do not induce any major modifications). As an exception, as will be demonstrated
290 below, lactosylation appeared to emerge as the only already present modification. Thus, all the subsequent
291 observed modifications can be considered exclusively the consequence of the applied treatments.

292

293 **3.2 Chemical changes induced by heat/pH**

294 **3.2.1 Protein profile**

295 The SDS-PAGE of processed whey proteins, treated at three different temperatures (30°C, 60°C and 90°C)
296 and five different pH values (2, 7, 9, 11 and 13) is shown in Figure 1.
297 Neutral samples, samples treated in acidic conditions and samples treated in basic conditions at pH 9 and pH
298 11, at all tested temperatures, showed two well-defined bands at 14 and 18 kDa (Agyare & Damodaran, 2010),
299 corresponding to α-lactalbumin (α-LA) and β-lactoglobulin (β-LG), which therefore appeared to be stable
300 under these conditions. Instead, the intensities of the bands at pH 2/90°C, pH 11/60°C and pH 11/90°C were
301 lower, especially for α-LA, suggesting protein degradation and/or aggregation induced by the combination of
302 high temperature and extreme pH. Consistently, samples treated under basic conditions at pH 13, at all
303 temperatures, did not show defined bands suggesting a strong degradation/aggregation effect induced by the
304 basic pH.
305 Overall, as expected, these data indicate a higher propensity of whey proteins for molecular modifications at
306 basic pH, and a lower one at acid pH. Furthermore, according to the data reported by Galani and Apenten,
307 (1999) this propensity to molecular modifications increased by high temperatures.
308 << Insert Figure 1>>
309 The same samples were analysed by UPLC/ESI-MS (Figure 2). The chromatographic method applied allowed
310 to distinctly identify the corresponding peaks for α-LA and β-LG, the latter in the two respective isoforms A
311 and B (Buhler et al., 2019). In perfect agreement with the SDS-PAGE results, all these peaks were observed
312 in all the samples treated in neutral and acidic conditions, and in samples treated in basic conditions at pH 9
313 and pH 11 at temperatures of 30°C and 60°C. In samples treated in extreme basic conditions (pH 13), at all
314 temperatures, and in samples treated at pH 9 and pH 11 at 90°C, no protein peak could be identified. These

315 results confirmed the degradation and/or aggregation induced by the extreme alkaline processing conditions
316 combined with high temperature.
317 <<Insert Figure 2>>
318 From the α-LA and β-LG mass spectra (the latter in the two respective isoforms), the glycated form can also
319 be seen in the multicharged pattern. In fact, the monolactosylated (+324 Da) and dilactosylated (+648 Da)
320 forms were quite evident, as shown in Figure 2B. Quite obviously, no mass spectra corresponding to whey
321 proteins were identified in the samples not showing any protein peak in the chromatogram. From the mass
322 spectra it was possible to calculate the relative percentage of the lactosylated forms, as compared to the native
323 unglycated proteins. Overall, the relative amount of the lactosylated and native forms, when detected, did not
324 show differences between the samples processed under different pH and heat conditions, indicating that
325 glycation was already present in the starting proteins, and was basically not affected by processing (data are
326 reported in Table S1a of the supplementary material). However, when extreme pH values were reached, both
327 forms were simply not detectable anymore.
328 **3.2.2 Solubility, aggregation and degradation of the protein fraction**
329 Protein solubility is an important property for evaluating whey protein denaturation (Kilara & Vaghela, 2018).
330 The percentage of the soluble and insoluble fraction in all samples was calculated as reported in the
331 experimental section. As far as the soluble fraction was concerned, aggregation and/or degradation of whey
332 proteins were evaluated by size exclusion chromatography (SEC, Figure 3) (Fekete et al., 2014).
333 The percentage distribution of insoluble and soluble proteins, and the distribution of soluble aggregated,
334 soluble native and soluble degraded proteins is reported in Figure 3C.
335 << Insert Figure 3>>
336 According to Pelegrine and Gasparetto, (2005) a positive correlation between temperature and protein
337 insolubility was observed on whey protein. In fact, insolubility increased after high temperature processing,
338 reaching its maximum near neutral pH, probably due to the proximity to the isoelectric point of whey proteins
339 (Ding et al., 2011; Lucy et al., 2008; Pelegrine & Gasparetto, 2005). On the other hand, at acidic and basic pH,
340 the insolubilization induced by the process increased with the temperature in a much more limited way.
341 As far as the soluble fraction is concerned, SEC analysis indicated that degradation appears to be prevalent in
342 acid- and basic-treated samples. In addition, basic-treated samples also showed a consistent amount of

degraded proteins, and as a consequence a smaller amount of native protein. Despite the paucity of studies in this field, our data are in agreement with other works that report the correlation between the formation of soluble aggregates and heating (Le Bon et al., 1999; Nicolai et al., 2011; Pouzot et al., 2005).

Overall, the above data hint at very different effects on the protein structure according to pH. At neutral pH, insolubilization is the most evident consequence of processing at high temperature. At acidic pH the most important modification is degradation of the protein fraction, whereas at basic pH both degradation and the formation of soluble aggregates are observed, beside insolubilization. Thus, in agreement with the previous data, basic pH seems to have the most profound impact on the integrity of the protein fraction.

3.2.4 Total content of amino acids and free amino acids

The amount of total amino acids (Figure 4A), analysed as reported in the experimental section (detailed profile reported in Table S1b of the Supplementary material), showed a marked decrease in the samples treated in alkaline conditions compared to neutral and acidic conditions, supporting the hypothesis of protein degradation due to the alkaline treatment. In particular, the loss in the samples treated under basic conditions mostly concerned some specific amino acids (Lys, Arg, Thr, Ser), whose side chains are notoriously more reactive in warm/basic conditions (Borad et al., 2017). Basic pH is also expected to promote hydrolysis of Asn and Gln side chains to Asp and Glu respectively, but since those side chains are anyway hydrolysed during sample preparation, this possible modification cannot be detected by standard amino acid analysis.

The amount of free amino acids was very low in the proteins treated in acidic and neutral environment, never exceeding 0.33 % of the total amount of AA. This percentage increased to a still quite low 1.57 %, in a temperature dependent manner, for alkaline treated proteins. This very low amount of free amino acids observed for whey proteins, even those treated under basic conditions, indicates that simple hydrolysis of the peptide bond is not the prevalent protein degradation process.

In general, also the amino acid content mostly showed a susceptibility to protein degradation mostly occurring at basic pH.

3.2.5 Enantiomeric purity of amino acids

The percentage of the D-enantiomer of some amino acids was determined by chiral GC in order to evaluate the racemization of whey proteins due to the heat treatment combined with the pH. The percentage of the D-enantiomer for Ala, Asp, Phe, Glu and Lys, as shown in Figure 4B, clearly showed an increase because of the combined exposure of whey proteins to extreme basic pH and heat. As expected, alkaline treated samples (pH 13) at 60°C and 90°C led to a percentage of D-enantiomers ranging between 25 and 46% (therefore almost complete racemization). These results are in agreement with previous work suggesting that the racemization mechanism is influenced by heating and alkali treatment (Friedman, 2010). In addition, a positive correlation between Ala, Asp, Phe and temperature was observed by the Spearman correlation test.

Neutral samples, acid treated and basic treated samples from pH 9 to pH 11 showed the lowest percentage of D-amino acids, with a percentage of D-enantiomers ranging from 3 to 10%. The very low quantity of free amino acids in the basic samples (see previous paragraph 3.2.4) indicates that this racemization mostly happens inside the proteins, when amino acids are still bound together through peptide bonds. The increase of D-amino acids within proteins, in addition to generating amino acids that cannot be used anymore for protein synthesis (thus leading to a decrease in the nutritional value), certainly hinders the digestibility of proteins by preventing enzymatic cleavage, due to the "wrong" configuration which hampers protease recognition.

<<Insert Figure 4>>

3.3 Evaluation of the digestibility of proteins in differently treated samples

3.3.1 Simulated digestion protocols

Digestion of the processed samples was performed using two different protocols. A simplified digestion procedure was first performed in order to estimate digestibility, by solubilizing processed whey proteins at pH 2 and then adding pepsin, followed by mixing at a constant temperature of 37°C for 2 hours. In addition to that, a validated static consensus method of simulated gastrointestinal digestion *in vitro* was then secondly used in order to assess the degree of hydrolysis of the proteins and the peptides released at the end of complete digestion (end of the duodenal digestion tract) (Brodkorb et al., 2019). The latter model system is characterized by the use of salts and other components which recreate simulated salivary fluid, simulated gastric fluid and simulated intestinal fluid, specific digestive enzymes such as pepsin from porcine gastric mucosa, pancreatin from porcine pancreas and bovine bile extract, and it is performed at controlled pH.

3.3.2 Degree of hydrolysis after digestion with pepsin and after gastrointestinal digestion simulated *in vitro*

The degree of hydrolysis represents the percentage of the total number of peptide bonds in a protein that have been cleaved during hydrolysis. The degree of hydrolysis, in percentage (DH%), was calculated with the OPA method, both after digestion with pepsin and after total gastrointestinal digestion (GD), as detailed in the experimental section. For all processed samples, as expected, DH% increased from the pepsin only digestion to the full gastrointestinal digestion. The maximum DH% was reached in the samples treated at pH 7 and 90°C, clearly indicating that the insolubilization of the proteins (see data above) did not hinder the digestibility of the proteins. In fact, a gradual increase in DH% at pH 7 was observed in a temperature-dependent manner from 30°C to 90°C (Figure 5A), thus, despite the increased insolubilization, the temperature treatment seems to favour the digestibility of the proteins. The same effect, albeit with slightly lower DH% values, was observed at acidic pH (data not shown). The increase in digestibility observed for neutral- and acidic-treated samples processed at high temperature might indicate that the loss of the tertiary structure can make the protein more accessible to digestive enzymes, provided that marked protein aggregation or amino acid degradation is not present

On the other hand, for basic-treated samples processed at high temperature we observed a marked decrease in digestibility, as reported in Figure 5B. This indicates that the changes seen above induced by basic pH (aggregation, protein degradation, amino acid racemization) strongly influence the accessibility of enzymes and the subsequent digestibility. For proteins treated at pH 13, the apparent bioaccessibility observed, measured by DH%, was less than 50% of the value observed for proteins treated at neutral or acidic pH, suggesting a dramatic decrease of the digestibility in heavily processed protein at basic pH.

<<Insert Figure 5>>

3.3.3 Peptidomic analysis after digestion with pepsin and after gastrointestinal digestion simulated *in vitro*

Peptidomic analysis was performed to investigate the molecular details of pepsin digestion and gastrointestinal digestion performed on whey proteins stressed by heat and pH treatments. Using a non-targeted approach, a peptidomic analysis was performed combining low- and high-resolution mass spectrometry. First, from the chromatographic peaks and from each associated mass spectrum, the most abundant peptides were detected,

423 estimating their amount by comparison with the internal standard Phe-Phe. Then, the molecular masses
424 associated to those peptides were determined, and the possible sequences associated with those molecular
425 masses were defined inside the target proteins (α-LA and β-LG). Through MS/MS fragmentation, the
426 theoretical fragmentation pattern of every possible sequence having compatible mass was compared with the
427 observed one, allowing to have a peptide sequence lists for each sample including all and only the most
428 abundant peptides. Finally, the same samples were analysed in high resolution by Q-Tof Mass Spectrometry
429 in order to confirm and complete the list, also including peptides nor previously identified and peptides
430 carrying modifications in the side chains. The peptide sequences identified for each sample are reported in
431 Table S2 of the Supplementary material. Some casein peptides were also identified, but they have not been
432 reported.
433 Global descriptive data for all analysed samples (number of peptides identified, percentage of peptides from
434 β-LG and α-LA, percentage of coverage of β-LG and α-LA, average length of identified peptides, hydrolysis
435 of the side chains of Asn and Gln, the cleavage site) are shown in Table 1.
436 <<Insert Table 1>>
437 The number of the identified peptides turned out to be quite variable, and with no clear relation with the
438 treatments. It is to be reminded that this number is generated by two complete opposite phenomena, since the
439 peptides are at the same time the product and the substrate of every enzymatic action. The actual sum of the
440 semiquantitative data in every sample, which gives a rough estimate of the peptide amount, indicated a
441 progressive increase associated with the temperature from 30 to 60 ° C according to degree of hydrolysis and
442 pH from 2 to 11 which is totally reversed starting from pH 11 at 90°C after pepsin digestion. The decreasing
443 trend continues also in the gastrointestinal phase, becoming drastically low at pH 13. The collapse of the sum
444 of the semiquantitative data of the identified peptides suggest a more bounded accessibility of the enzymes to
445 the cleavage sites in extremely basic condition.
446 For all the digested samples, more peptides derived from β-LG rather than α-LA were detected, which was
447 expected due to the higher abundance of β-LG in our samples (and in bovine milk; Layman et al., 2018).
448 Also expected, for all processed samples the average length of the identified peptides decreased going from
449 the pepsin digestion to the full gastrointestinal digestion, considering the more extensive enzymatic cleavage
450 taking place in the latter. Interestingly, the mean length after the full gastrointestinal digestion slightly

increased in basic digested samples (pH 9, 11, 13) if compared to the samples treated at neutral and acidic pH, indicating a more limited accessibility of the enzymes to the cleavage sites, thus a decrease in digestibility, in agreement with the data above reported on the degree of hydrolysis. Indeed, in basic treated samples, an increase prevalence of longer peptides on the same sequence of peptides present in neutral/acidic samples, were detected, indicating failed enzymatic cleavages. Moreover, the percentage of deamidation increased at basic pH, indicating a strong deamidation of Asn and Gln at basic pH as a side effect.

The preferential cleavage sites of the released peptides were also evaluated considering the recurrent amino acid at the C-terminus. Almost all samples after pepsin digestion contained mostly peptides with a Leu residue at the C-terminus (from 18 to 40% of the total peptides identified in each sample), consistently with the known pepsin specificity for lipophilic amino acids. Interestingly, and again indicating a definite change in enzyme action, Lys was the preferred C-terminal residue (26% of the total number of peptides detected) at pH 13 at 90°C. Leu and Glu were the preferred cleavage sites after gastrointestinal digestion (which includes a gastric phase with pepsin, followed by a duodenal phase with pancreatin). Again, in the sample processed at pH 13 at 90 °C, Lys was the most recurrent residue attached to the C-terminal. This analysis confirmed that the mode of action of the digestive enzymes undergo some changes in basic-treated samples, likely due to the chemical modifications induced on proteins, and also mirroring the decrease in digestibility observed above.

4 CONCLUSIONS

The reported in-depth molecular characterization of whey proteins, used as model system, stressed by heat and pH treatments similar to the ones found in literature for protein extraction, and the link between the molecular changes and protein digestibility, allow to draw some conclusions on the best methods for protein extraction, having in mind not only the protein yield (as it is often the case), but also the nutritional value of the extracted proteins.

A first conclusion is that the set of molecular modifications induced by the processing clearly changes according to pH and temperature. In this sense, pH seems to trigger different set of modifications, whereas temperature has the role of “enhancing” the molecular modifications typical for acid, neutral or basic conditions.

Neutral pH conditions are clearly the least affecting, only inducing precipitation of the proteins in a temperature dependent manner, without major protein and amino acid degradation or racemization, or peptide bond hydrolysis, thus preserving the nutritional value of the proteins quite intact. Quite interestingly, the digestibility of the proteins is not at all impaired by their precipitation, which appears then to be perfectly reversible, and susceptibility to digestive enzymes is even improved in samples treated at high temperature.

At acidic pH also no extensive amino acid degradation or racemization was observed, thus again nutritional quality was preserved, even if proteins showed a tendency to generate compounds having lower molecular weight, also in a temperature dependent manner. The fact that the total amino acid amount was unchanged, but also that free amino acids were not extensively present, can lead us to conclude that only a limited peptide bond hydrolysis takes place in these conditions, partially generating large polypeptides. Accordingly, and quite interestingly, digestibility seemed to be even better in these samples than in neutral-treated proteins.

At basic pH, with an increasing tendency according to the pH value, a full array of chemical modifications appears in the protein structure. Formation of soluble aggregates, protein degradation, and racemization (which reaches massive values at very basic pH and high temperatures) are mostly present. The total amino acid amount drops down to a value corresponding to about 20-30% less than the original value, indicating that not only peptide hydrolysis is responsible for protein degradation (the amount of free amino acids remains quite limited), but also other forms of amino acid degradation are present, mostly affecting the most susceptible side chains. These findings alone would immediately indicate a loss of the nutritional value, but linked to those, and clearly caused by those, a significant drop in digestibility was also observed, with a strong reduction in the degree of hydrolysis after simulated digestion (with a drop exceeding 50% in the worst case), and a change in the composition, modifications, and length of the released peptides, suggesting a difficult accessibility/recognition of the digestive enzymes to the modified proteins and a difficult cleavage of the peptide bonds.

Taken all together, the above data indicate that neutral and acid treatments, even at very low pH and even at very high temperature, can be safely applied for protein extraction without any major damage to the nutritional value of the protein biomass to be extracted, and in case of the acidic extraction might even lead to an increased digestibility. On the other side, basic extraction conditions should be applied with extreme caution, when the prese3rvation of the nutritional values is a target, and if needed by certainly avoiding extreme pH, high

506 temperatures, and long extraction times, given the quick propensity of protein at basic pH to undergo
507 modifications which impair the amino acid content, the amino acid integrity, and the processability by digestive
508 enzymes, ultimately resulting, in the most extreme cases, in a biomass having a highly degraded nutritional
509 value. Our data also indicate that the measurement of amino acid racemization can be used as fast molecular
510 marker of the damages induced to proteins, which can be easily linked to their integrity and digestibility.
511 As per the general validity of these results, some considerations can be made. It is certainly true that plant-
512 derived proteins are found to be subjected to extreme extraction conditions much more often than animal-based
513 proteins, and is certainly true that in some cases the effect might be expected to be different. Anyway, amino
514 acids in a soluble protein (belonging to the albumin or globulin class), being all proteins made of the same 20
515 amino acids, are expected to react to the external environmental stressors in a very similar way, irrespective of
516 the protein origin. The structural modifications here reported (aggregation, degradation, racemization, amino
517 acid destruction) are intrinsically linked to the amino acid chemistry.
518 On this ground, we believe that our results have a general validity that goes beyond the origin of the proteins
519 used, and that can be useful for every person working on proteins, also plant-based proteins. Anyway, they
520 will have certainly to be further confirmed on a larger range of proteins, also including plant-based proteins.
521 These future experiments will shed more lights on the general validity of the results here presented.
522

SUPPLEMENTARY MATERIAL

The supplementary material as reported in the text presents in detail:

Table S1a: Percentage of native and lactosylated proteins in the differently treated samples

Table S1b: Total amino acid content in the differently treated samples

Table S2: The peptide sequences identified after peptic (a, c) and gastrointestinal (b, d) digestion for alpha-lactalbumin (a, b) and beta-lactoglobulin (c, d).

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Figure captions:

Figure 1 Electrophoretic pattern of whey proteins after being treated at different pH/temperatures for three hours.

Figure 2 A) LC-MS chromatograms of protein samples after treatment at 30°C and pH 2 (top left), 7 (middle left) and 13 (bottom left); B) MS spectra of α-lactalbumin, β-lactoglobulin A and β- lactoglobulin B taken from the sample treated at pH 2, 30°C. The asterisks indicate the glycated forms of the proteins.

Figure 3 SEC chromatogram of the samples treated at pH 2 at 30°C (A) and pH 11 at 60°C (B). The putative distribution of aggregated, native and degraded protein is showed in figure. C) Putative distribution of the insoluble and soluble fraction (native, aggregated and degraded protein).

Figure 4 A) Total amino acid content in samples treated at different pH and temperature. Different letters mean statistically different samples ($p < 0.05$) (one-way ANOVA, LSD test). B) Formation of D-amino acids in samples treated at different pH and temperatures. The amino acid Ala, Asp, Phe, Glu and Lys were evaluated for their significant differences in each sample, different letters mean statistically differences between samples ($p < 0.05$) (one-way ANOVA, Tukey's b test).

Figure 5 A) Effect of different temperature conditions at pH 7 on the DH% of samples subjected to peptic and gastrointestinal digestion. B) Effect of the different pH on the DH% of samples subjected to peptic and gastrointestinal digestion.