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1 **Metal-chelating activity of soy and pea protein**
2 **hydrolysates obtained after different enzymatic treatments**
3 **from protein isolates**

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20 **Abstract**

21 Soy and pea proteins are two rich sources of essential amino acids. The hydrolysis of these
22 proteins reveals functional and bioactive properties of the produced small peptide mixtures. In
23 our study, we employed the hydrolysis of soy and pea protein isolates with the endopeptidases
24 Alcalase[®] and Protamex[®], used alone or followed by the exopeptidase Flavourzyme[®]. The
25 sequential enzyme treatments were the most efficient regarding the degree of hydrolysis. Then,
26 soy and pea protein hydrolysates (SPHs and PPHs, respectively) were ultrafiltrated in order to
27 select peptides of molecular weight \leq 1kDa. Whatever the protein source or the hydrolysis
28 treatment, the hydrolysates showed similar molecular weight distributions and amino acid
29 compositions. In addition, all the ultrafiltrated hydrolysates possess metal-chelating activities,
30 as determined by UV-spectrophotometry and Surface Plasmon Resonance (SPR). However, the
31 SPR data revealed better chelating affinities in SPHs and PPHs when produced by sequential
32 enzymatic treatment.

33

34 **Key words:** Metal-chelating peptides, enzymatic hydrolysis, soy protein, pea protein, affinity
35 constant, screening.

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39

¹*Abbreviations:* DH: Degree of hydrolysis; SPHs: Soy protein hydrolysates; PPHs: Pea protein hydrolysates; MW: Molecular weight; SPR: Surface plasmon resonance; MCPs: Metal-chelating peptides; ROS: Reactive oxygen species; SPI: Soy protein isolate; PPI: Pea protein isolate; LC-MS: Liquid chromatography-mass spectrometry; IMAC: Immobilized metal affinity chromatography.

40 **1. Introduction**

41 Transition metals, such as iron II (Fe^{2+}) and copper II (Cu^{2+}) ions, are powerful promoters for
42 the production of Reactive Oxygen Species (**ROSSs**) since they are able to donate and accept
43 electrons *via* intracellular reactions, such as the Haber-Weiss and Fenton reactions, and thus
44 induce the production of free radicals ([Hancock 1992](#)). The dietary metal-chelators brought by
45 nutrition help to reduce oxidative stress resulting from environmental toxins and modern life
46 related factors such as environmental pollution, smoking, UV exposition *etc.* The antioxidant
47 metal-chelating properties of peptides are related to their characteristic amino acid composition
48 and their proper positioning within the peptide sequence. Indeed, certain amino acids can form
49 coordination compounds with metals through their α -amino group, carboxyl group, and R side
50 chain of amino acids. The carboxylate group (COO^-) and the nitrogen atom (N) attach to metals
51 to establish metal carboxylate salt, amine complex, and 5 or 6 membered chelating rings ([El](#)
52 [Hajj, et al. 2021a](#)).

53 Over the past few decades, protein hydrolysates have been widely engaged in human nutrition
54 applications. Upon proteolysis, the obtained small-sized peptides (up to 10 amino acid residues
55 long) are considered as highly advantageous for targeting specific physiological or nutritional
56 requirements, mainly because there are less subjected to digestion in the gastrointestinal track
57 and are in the meantime faster absorbed ([Clemente, 2000](#)). Proteolysis induces structural
58 changes of proteins notably by degrading the protein primary sequence, which increases the
59 number of AA residues accessible for potential interactions. Indeed, with the loss of their native
60 structures, the produced low-molecular-weight peptides enhance their interactions with the
61 environment. Food-derived proteins have popularly undergone proteolysis to obtain a broad
62 range of bioactive and functional peptides, notably metal-chelating peptides (**MCPs**). For
63 instance, the MCPs produced from the proteolysis of milk proteins, especially casein and whey
64 proteins, have been widely reported in the literature. Calcium, iron and zinc binding motifs

65 were discovered in these proteins after submitting them to enzymatic hydrolysis (Chen et al.
66 2014; Kibangou et al. 2005; Vegarud, Langsrud, and Svenning 2000). Calcium-binding
67 peptides were derived from the hydrolysis of tilapia proteins and shrimp processing by-products
68 as well (Chen et al. 2014; Charoenphun et al. 2013; Cheung et al. 2012). Other protein
69 hydrolysates produced for instance from chickpea, rapeseed, sunflower, and bean protein
70 contain also bioactive MCPs (Xie et al., 2015; Carrasco-Castilla et al., 2012; Torres-Fuentes et
71 al., 2011; Megías et al., 2007) . As for the choice of digestive enzymes, many various
72 proteinases and their combinations – including pure (*e.g.*, trypsin, pepsin, thermolysin) and
73 crude (Alcalase[®], Protamex[®], Flavourzyme[®], *etc.*) enzymes – were listed in literature with their
74 hydrolysing optimum conditions (Korhonen and Pihlanto 2006). We hypothesized here that
75 from a same protein resource, different hydrolysis conditions can lead to the production of
76 hydrolysates with variations among their physio-chemical characteristics and bioactivities, such
77 as the metal-chelating activity.

78 Soy and peas are the two most produced legumes in France. Soy proteins, generally cheaper
79 than other proteins, are one of the vegetable protein sources that contain all the essential amino
80 acids, and are considered as relevant substitutes of animal proteins, especially for vegetarians
81 and vegans. They are linked to multiple health benefits for pregnancy, cardiovascular and
82 gastrointestinal systems, cancer prevention, and lactose intolerance condition (Barrett 2006;
83 Montgomery 2003). Similarly, pea proteins are also a low-cost source of proteins and can be
84 used as substitute for animal proteins, mainly in smoothies and shakes to increase protein
85 content. As main advantage, pea proteins aids in kidneys and heart health, weight loss, and
86 muscle growth and repair (Babault et al., 2015; Tömösközi et al., 2001). Certain studies have
87 identified MCPs with antioxidant activities in soy and pea protein hydrolysates produced by
88 different enzymatic treatments. For example, Lv et al. (2009) used Immobilized-Metal Affinity
89 Chromatography (IMAC) to identify iron-chelating peptides from soy proteins hydrolysates

90 produced by protease M. Also, [Bao et al. \(2008\)](#) used Fourier transform infrared spectrometry
91 (**FTIR**) to study complexes formed between peptides of soy hydrolysates produced after
92 pepsin, flavourzyme[®] and protease M treatments. Iron-chelating activity was determined as
93 well using UV-spectrophotometry in pea protein hydrolysates produced by thermolysin
94 ([Pownall, Udenigwe, and Aluko 2010](#)).

95 Hence, the aim of this research is to compare the effect of different enzymatic hydrolysis
96 conditions on two types of interesting protein sources (soy and pea protein isolate) on the
97 production of metal-chelating peptides.

98 **2. Material and Methods**

99 **2.1. Production of protein hydrolysates**

100 The raw materials, soy protein isolates (**SPI**) and pea protein isolates (**PPI**), were kindly
101 provided from SAS IMPROVE (Dury, France). The two protein isolates (2%, w/v) were
102 dispersed in 50 mM ammonium bicarbonate buffer and preheated at 90°C for 5 minutes. Their
103 enzymatic hydrolysis was performed in thermostatically controlled reaction vessels at 55°C
104 with various proteases. The enzymes Alcalase[®] (Protease from *Bacillus licheniformis*, ≥ 2.4
105 U/g, Sigma-Aldrich; **Alc**) and Protamex[®] (Protease from *Bacillus* sp., ≥ 1.5 U/g, Sigma-
106 Aldrich; **Prt**) were added individually to the SPI solution for a complete 1-hour and 3-hours
107 hydrolysis, carried out at pH = 8 for the Alcalase[®] treatment and pH = 7 for the Protamex[®]
108 treatment. Two sequential hydrolysis were also performed on SPI and PPI solutions using in
109 each case an additional enzymatic treatment with Flavourzyme[®] (Protease from *Aspergillus*
110 *oryzae*, ≥ 500 U/g, Sigma-Aldrich; **Flv**) to compare two sources of proteins in the most
111 interesting hydrolysis conditions. For these sequential treatments, Alcalase[®] was applied for 1
112 hour (pH = 8) followed by Flavourzyme[®] treatment for 2 hours (pH = 7; **Alc+Flv**). Similarly,

113 Protamex[®] was applied for 1 hour followed by Flavourzyme[®] treatment for 2 hours, both at pH
114 = 7 (**Prt+Flv**). The enzyme to substrate ratio (E/S) was always set at 1% (v/v). Whatever the
115 enzymatic treatment, the mixture was stirred while monitoring and maintaining the temperature
116 and pH. The first enzyme was always inactivated at 95°C for 15 min before adding the second
117 enzyme. Following the hydrolysis by the second enzyme, this latter was also inactivated at 95°C
118 for 15 min and the samples were centrifuged (10 000 g; 15min) in order to precipitate and
119 remove both large unhydrolyzed proteins and both enzymes. Finally, the six SPHs and the two
120 PPHs prepared were lyophilized to obtain powders, subsequently stored at -20°C until further
121 analysis. The hydrolysates were named by their respective protein source (**SPH** or **PPH**)
122 followed by their enzymatic treatment (**Alc**, **Prt**, **Alc+Flv**, **Prt+ Flv**) and the time defined for
123 their hydrolysis.

124 **2.2. Ultrafiltration**

125 After lyophilization, ~3 g of hydrolysates were dispersed in 50 mL MilliQ water (18 mΩ·cm⁻¹)
126 ¹) and fractionated under stirring, using consecutively 10 kDa and 1 kDa Ultracel[®] ultrafiltration
127 membranes (Millipore, Jaffry, USA) in a 100 mL-ultrafiltration cell (Millipore, Jaffery, USA).
128 Ultrafiltration through 10 kDa membrane was first required in order to facilitate then the
129 ultrafiltration of the hydrolysates through the 1 kDa membrane. The retentates were frozen at -
130 20°C and denoted as > 10 kDa fraction, while the permeates were passed through the 1 kDa
131 membrane, thus forming two fractions for each hydrolysate: a first fraction comprised within
132 1-10 kDa (frozen at -20°C) and a second one ≤ to 1 kDa (lyophilized for further assays).

133 **2.3. Efficiency of the different hydrolysis protocols**

134 *2.3.1. OPA quantification*

135 The molar concentration of each hydrolysate defined as the primary amino groups revealed by
136 hydrolysis was determined by the OPA (for *o*-phthaldialdehyde) quantification. This method
137 was adapted from [Canabady-Rochelle et al., \(2018\)](#). The OPA solution was prepared by
138 dissolving first 40 mg of OPA (ThermoFisher Scientific, Loughborough, UK) in 1 mL of pure
139 methanol and then 100 mg of *N,N*-dimethyl-2-mercaptoethyl ammonium (ThermoFisher
140 Scientific) in 20 mL of Borax buffer (sodium tetraborate 100 mM, 1% w/v sodium dodecyl
141 sulfate, pH 9.3, Sigma-Aldrich), then these two former solutions were mixed in a volumetric
142 flask of 50 mL-final volume and completed with Borax buffer to give the desired OPA solution.
143 Each solution of peptide hydrolysate (20 μ L, 1 mg/mL) prepared in Borax buffer was mixed
144 with the OPA solution (200 μ L) directly in a 96-well plate (Nunclon™ Delta Surface,
145 ThermoFischer Scientific) in 5 replicates. The absorbance was read at 340 nm (Multiskan Go
146 spectrophotometer, ThermoFischer Scientific) after 3 min of incubation under stirring at room
147 temperature. The molar concentration of each peptide hydrolysate was calculated as average
148 mean \pm standard error mean in mM eq. glycine with the help of a glycine calibration curve (1-
149 5 mM).

150 *2.3.2. Determination of the degree of hydrolysis*

151 The degree of hydrolysis (**DH**) defined as the proportion of cleaved peptide bonds in a protein
152 hydrolysate was evaluated by the OPA quantification of the primary amino groups liberated
153 during hydrolysis. The value of DH (%) was calculated according to the following formula
154 conducted from [Romero-Garay et al., \(2020\)](#).

155
$$DH(\%) = \frac{(NH_{2(t)} - NH_{2(0)})}{(NH_{2(max)} - NH_{2(0)})} \times 100 \quad [1]$$

156 Where $NH_{2(t)}$ = concentration of amino groups in 1g/L hydrolysates at the end of hydrolysis,
157 $NH_{2(0)}$ = concentration of amino groups of SPI (without hydrolysis), and $NH_{2(max)}$ =
158 concentration of amino groups after a complete chemical hydrolysis of SPI, performed with
159 6N HCl at 130°C for 24 h (Romero-Garay et al., 2020).

160 Note that the DH was only determined for whole soy protein hydrolysates, and not for those
161 submitted to an ultrafiltration step. Indeed, upon ultrafiltration, some peptides are lost, which
162 do affect the DH determination.

163 **2.4. Physico-chemical characterization of the produced hydrolysates**

164 *2.4.1. Size exclusion chromatography (SEC)*

165 The molecular weight distribution of the 1 kDa-ultrafiltrated SPHs and PPHs was determined
166 by GFC (for gel filtration chromatography) using a Waters 515 HPLC pump, a degazer and a
167 RID 10-A Shimadzu detector. The mobile phase was composed of water containing sodium
168 nitrate at 0.1 M and sodium azide at 0.2% (w/v). The stationary phase was composed of a
169 Phenomenex PolySep-GFC-P 2000 (7.8 x 35 mm) guard column to help remove contaminants
170 and free amino acids, followed by a same phase column (7.8 x 300 mm) that has a separation
171 range comprised between 0.1 and 10 kDa. Each sample (200 μ L) was injected at 5 mg/mL and
172 separated at room temperature with a flow rate of 0.7 mL/min. PolyEthylenGlycol (PEG: 200,
173 600, 1000, 3000 and 8000 Da, Sigma Aldrich, USA) were used as standards for GFC
174 calibration. Data were collected and analysed with the software ASTRA (Wyatt Technology).

175 2.4.2. Identification of amino acid composition

176 Quantitative analysis of amino acids was realized on a UHPLC system (Thermo Fisher
177 Scientific) in-line with an Orbitrap ID-X Tribrid mass spectrometer (Thermo Fisher Scientific)
178 equipped with an atmospheric pressure ionization interface. Some aminoacids were not
179 considered for the aminogram due to their susceptibility to oxidation (*i.e.*, Tryptophane, Trp,
180 W; Cystein, Cys, C) under the working conditions. Besides, Methionine (Met, M) was
181 investigated but not detected, due to its low concentration in the soy and pea proteins as reported
182 in their sequences presented in UniProtkb databases.

183 Five microliters of samples (4 hydrolysates: 1kDa-Ultrafiltrated SPH and PPH, either prepared
184 by Alcalase[®] followed by Flavourzyme[®] or by Protamex[®] followed by Flavourzyme[®]) were
185 separated on C18 Alltima reverse phase column (150 x 2.1mm, 5 μ m – Grace/Alltech,
186 Darmstadt, Germany) equipped with a C18 Alltima pre-column (7.5 x2.1mm, 5 μ m) at 10°C.
187 The flow rate was set at 0.2 mL/min and the mobile phases consisted in water supplemented
188 with nonafluoropentanoic acid (20 mM) as ion-pairing reagent for A and pure acetonitrile for
189 B. Amino acids were eluted using a linear gradient from 5 % to 25 % of B for 12 min, and then
190 an isocratic step at 25 % of B for 18 min. Mass analysis was carried out in heated electrospray
191 positive ion mode (H-ESI⁺) and mass spectrometry conditions were as follows: spray voltage
192 was set at 4.0 kV; source gases were set (in arbitrary units/min) for sheath gas, auxiliary gas
193 and sweep gas at 30, 5, and 5, respectively; vaporizer temperature and ion transfer tube
194 temperature were both set at 300°C. MS scans were performed from 70 to 210 m/z at 60 K
195 resolution (full width of the peak at its half maximum, fwphm, at 200 m/z) with MS parameters
196 as follows: RF-lens, 35%; maximum injection time, 50 ms; data type, profile; AGC target:
197 custom; normalized AGC target: 25 %. The mass spectrometer calibration was performed using

198 the Pierce FlexMix calibration solution (Thermo Scientific). MS data acquisition was carried
199 out utilizing the Xcalibur v. 4.3 software (Thermo Scientific).

200 **2.5. Determination of the metal-chelation properties**

201 *2.5.1. UV-spectrophotometry*

202 The Cu²⁺ chelation properties of the 1 kDa-ultrafiltrated SPHs and PPHs were determined as
203 an indirect antioxidant capacity and measured by spectrophotometry using murexide as colour
204 indicator, similarly as in [Canabady-Rochelle et al., 2018](#). Hydrolysates were prepared in
205 hexamine buffer between 0.42 and 40 g/L, and then, their respective concentration was
206 expressed in mM eq. NH₂. Besides, as good complexing agents, EDTA and carnosine were
207 both considered as positive controls and prepared in a range of 0.42–40 mM. EDTA, carnosine
208 and the 1 kDa-ultrafiltrated hydrolysate solutions were directly diluted in a microplate with
209 hexamine buffer for a total volume of 143 μL. Then, 143 μL of a 3 mM CuSO₄ solution in
210 hexamine buffer and 14 μL of a 1 mM murexide solution were added in 5 replicates in each
211 well (total volume: 300 μL). The 96-well plate was incubated for 3 min at room temperature
212 and the absorbance was measured at two wavelengths, *i.e.*, 485 nm and 520 nm, for the copper-
213 murexide complex and the murexide alone, respectively. The ratio of absorbance (A₄₈₅/A₅₂₀)
214 was considered proportional to the free copper ion (Cu²⁺) concentration.

$$215 \quad \text{Cu}^{2+} \text{ complexation (\%)} = \frac{[(A_{485}/A_{520})_0 - (A_{485}/A_{520})_s]}{(A_{485}/A_{520})_0} \times 100 \quad [2]$$

216 With (A₄₈₅/A₅₂₀)₀ = ratio of absorbances measured in the absence of sample, and (A₄₈₅/A₅₂₀)_s =
217 ratio of absorbances measured in the presence of sample (EDTA, carnosine or hydrolysate).
218 Results were presented as average mean ± standard error mean for 5 replicates.

219 2.5.2. Surface Plasmon Resonance

220 The surface plasmon resonance (SPR) experiments were carried out similarly to [Canabady-](#)
221 [Rochelle et al., \(2018\)](#). The binding affinity of protein hydrolysates for Ni²⁺ was analysed by
222 SPR at 25°C using a Biacore X100 instrument (GE Healthcare, Uppsala, Sweden) equipped
223 with Ni²⁺-NTA sensor chips. All binding experiments were performed at a flow rate of 20
224 μL.min⁻¹.

225 First, Ni²⁺ was injected for 1 min onto the NTA chip using a NiCl₂ solution (500 mM, Biacore
226 kit) and followed by a 1-min stabilization. The NTA flow channel uncharged in Ni²⁺ was used
227 as a reference channel in order to determine the importance of aspecific interactions. Then, each
228 peptide sample was injected on both channels for 270 s followed by 270 s undisturbed
229 dissociation time. The chip was regenerated between each studied concentration with a two
230 steps protocol involving successively imidazole (500 mM) at a flow rate of 20 μL.min⁻¹ for 1
231 min and SDS (0.5 % v/v) at a flow rate of 40 μL.min⁻¹ for 1 min. Buffer blanks and
232 concentration duplicates were used in each set of samples for dual reference during data
233 processing.

234 The sensorgrams obtained from the SPR experiments were processed with BIAevaluate
235 software. The isotherms obtained were expressed in Resonance Unit (RU, corrected by the
236 offset value) as a function of the concentration of protein hydrolysate (expression in mM
237 equivalent glycine according to the OPA quantification). The K_D were determined at
238 equilibrium by fitting the experimental data with the 1:1 binding model. The affinity constant
239 (K_A , mM⁻¹ equivalent glycine) was calculated as the inverse of the dissociation constant.

240 **3. Results and Discussion**

241 **3.1. Efficiency of the different hydrolysis protocols**

242 Alcalase[®] and Protamex[®] are two serine endopeptidases obtained from *Bacillus*. Both comprise
243 the so-called “Subtilisin” enzyme, which has a broad hydrolysing specificity. Yet, according to
244 the literature, these two different commercialized crude enzymes led to different but efficient
245 DH when incubated with soy isolates (Zhang, Huang, and Jiang, 2014; Seo, Lee, and Baek
246 2008; Penta-Ramos and Xiong, 2002). The degree of hydrolysis (DH) of SPI hydrolysates
247 varies from 13 % (Protamex[®] 1h) to 32 % (Alcalase[®] 1h + Flavourzyme[®] 2h) (Figure A1)
248 depending on the enzyme used, the hydrolysis time, and individual or sequential enzymatic
249 hydrolysis. After 1 hour of hydrolysis, Alcalase[®] shows a higher DH than Protamex[®] (19.3 %
250 and 13.2 %, respectively). Raising up the time of hydrolysis by Alcalase[®] to 3 hours does not
251 reveal an improvement of the DH. However, 3 hours of Protamex[®] treatment increases the DH
252 to 19.7 %. So Alcalase[®] is more efficient than Protamex[®], resulting in a greater number of
253 peptides after 1 h of hydrolysis, but not after 3 hours.

254 Being an exopeptidase, Flavourzyme[®] is widely used as a second protease in sequential
255 hydrolysis studies with an endopeptidase being the first hydrolysing enzyme. After a sequential
256 enzymatic treatment, using either Alcalase[®] followed by Flavourzyme[®] or Protamex[®] followed
257 by Flavourzyme[®], the DH increased to 31.8 % and 29 % respectively. So, a sequential
258 enzymatic treatment gives a clear advantage in terms of quantities of generated peptides. Hence,
259 our results are in agreement with the literature, which reports that the primary use of an
260 endopeptidase facilitates the reaction of the exopeptidase in order to reach a better hydrolysis
261 (Vioque et al., 1999; Ugolini et al., 2015).

262 In our work, we demonstrated similar DH than certain other studies. For example, [Zhang et al.](#)
263 [\(2018\)](#) had a DH value of 20% after a 3-hour Alcalase[®] treatment of soy protein isolates (24%
264 in our study). After a 10-hours Protamex[®] treatment of soy protein isolate, [Xie et al. \(2012\)](#)
265 obtained a DH of 16.6 % (19% after 3 hours in our study). Concerning sequential hydrolysis
266 with Alcalase[®] followed by Flavourzyme[®], we have obtained a larger DH (31.8%) than [Ma et](#)
267 [al. \(2013\)](#) (24%). Among other factors, this difference may be due to the source of proteins
268 used, their purity but also the source of enzyme, the way the hydrolysis was carried out and its
269 monitoring.

270 According to the literature, the increased DH value could be correlated to an increased
271 antioxidant activity of hydrolysates, including metal-chelating property ([Zhang, Huang, and](#)
272 [Jiang, 2014; Theodore, Raghavan, and Kristinsson 2008; Raghavan and Kristinsson, 2008](#)).
273 Moreover, the hydrolysate fraction collected after ultrafiltration – thus, constituted of low
274 molecular weight peptides – could have better antioxidant activities than the one containing
275 high molecular weight peptides ([He et al., 2013; Ranamukhaarachchi, Meissner, and Moresoli,](#)
276 [2013; Tsou et al., 2010; Ren et al., 2010](#)). Thus, we focus our attention on examining the 1kDa-
277 ultrafiltrated samples in this study.

278 **3.2. Characterization of the produced hydrolysates**

279 The molecular weight distribution (%) of the 1 kDa-ultrafiltrated SPHs and PPHs was evaluated
280 according to the linear regression of Log (MW) and elution chromatograms obtained by SEC
281 (**Figure 1**). The results indicate that the molecular masses of the SPHs (except SPH by
282 Protamex[®] 1h) and PPHs are mostly distributed within 400-200 Da. The highest percentages
283 of peptides in this former range were collected for the SPH by Alcalase[®] 1h followed by
284 Flavourzyme[®] 2h (57%) and for SPH treated by Protamex 3h (65%).

285 The amino acid composition of four hydrolysates is shown in **Figure 2**. These compositions
286 are globally similar regardless of the protein source and the hydrolysis process, with some
287 differences. Indeed, regarding acidic amino acids (Asp and Glu), well known for their ability
288 to chelate metal ions through their carboxylic groups (Lv et al., 2013), the highest percentages
289 are observed in PPH and SPH, both sequentially hydrolysed by Alcalase[®] (1h) followed by
290 Flavourzyme[®] (2h) with 20% and 12.9%, for aspartic acid, respectively; both sequential PPH
291 and SPH show high percentages of glutamic acid as well (14.8 % and 7.9 %, respectively).
292 Besides, the SPH generated by Protamex[®] (1h) followed by Flavourzyme[®] (2h) contains 11.8%
293 of histidine, which is a well-known metal-chelating amino acid (Chen, Shen, and Xia, 2020;
294 Zhang et al., 2018; Sun et al., 2017; Torres-Fuentes, Alaiz, and Vioque, 2011). Finally, proline,
295 arginine and lysine, which are present at similar percentages in SPHs and PPHs, were
296 previously found in Ca²⁺-chelating peptides (Bredderman and Wasserman, 2002). Therefore,
297 those results suggest that the metal-chelating activity observed for the different hydrolysates
298 could reflect the presence of a great variety of MCPs.

299 **3.3. Determination of the metal-chelation properties**

300 *3.3.1. By UV-spectrophotometry*

301 The copper-chelation capacities of the 1kDa-ultrafiltrated SPHs and PPHs were evaluated by
302 spectrophotometry and expressed as a function of the molar ratio (mM equiv Glycine for
303 hydrolysate / mM CuSO₄) (**Figure 3**). Whatever the hydrolysate treatment, we observed a
304 Langmuir-shaped graph and a saturation plateau, indicating the presence of peptides able to
305 bind copper ions in all the 1kDa-ultrafiltrated hydrolysates, with similar capacities. From these
306 presented graphs, we determined various indices similarly as in Canabady-Rochelle et al., 2015
307 and 2018. **Table 1** shows the EDTA and carnosine equivalent chelating capacities (EECC and

308 CECC, no unit) calculated for all the samples. The EECC and CECC values ranged between
309 [1.54 – 2.52] and [2.19 – 3.58] respectively, without significant differences between each
310 hydrolysate according to SEM. This implies that the ultrafiltrated soy and pea hydrolysates
311 contain copper-chelating peptides, but the spectrophotometry method has a low sensitivity in
312 differentiating between the chelation power of the different hydrolysates. In fact, screening of
313 bioactive peptides by UV-spectrophotometry often depends on their relative concentration in
314 the hydrolysates.

315 3.3.2. *By Surface Plasmon Resonance*

316 SPR is a sensitive and selective method for MCPs screening (Canabady-Rochelle et al., 2018;
317 Maalouli et al., 2011; Knecht et al., 2009). The SPR signal is detected when the peptide is
318 interacting and surrounding the metal-ion in its favourable geometry, producing accurate and
319 comparable information about the hydrolysates. The immobilized Ni²⁺ was used due to its
320 similarities with Cu²⁺ (Grenács, et al., 2013; Grenács, et al., 2014; Rajković et al., 2003) in regards
321 to its coordination properties with amino acids according to the HSAB theory and since Ni²⁺ is
322 commonly used for immobilization on NTA for IMAC purification procedures than can come
323 after screening (Sóvágó and Ósz, 2006).

324 The SPR response (RU) is plotted as a function of the hydrolysate concentrations (mM equiv.
325 Glycine) (**Figure 4**). Note that for each investigated hydrolysate, the hydrolysate concentration
326 had to be adapted in SPR in order to get sorption isotherms with a plateau value. These so-
327 called sorption isotherms indicate the binding response of investigated hydrolysates on the
328 immobilized Ni²⁺ at equilibrium. They are presented either as a function of the enzymatic
329 treatment applied to soy protein (panel A) or as a function of the source of protein used for
330 proteolysis (soy or pea), using the same enzymatic treatment (panel B). Whatever the enzymatic

331 treatment or protein source used, the isotherms show hyperbolic profiles with saturation or a
332 tendency for saturation. The differences are more visible in the saturation plateau of the metal-
333 binding response, unlike UV-spectrophotometry data. The maximum response, R_{\max} (R.U.) are
334 presented in **Table A.1**. Dissociation constants (K_D) obtained from the fitting of these binding
335 isotherms and the calculated association constants (K_A) are reported in **Table 2**. Note that the
336 K_D value for each investigated sample was determined from the whole fitting by the
337 BIAevaluate software, which is more accurate compared to a graphical determination.

338 For single protease hydrolysis of SPI, and considering the K_A values, apparent affinity of
339 peptides for Ni^{2+} ion varied from 0.12 to 0.38 mM^{-1} equiv Gly. Hydrolysates produced by
340 Alcalase[®] treatment present better apparent affinity compared to those produced by Protamex[®]
341 treatment, and the best apparent affinity is observed for only 1 hour of Alcalase[®] treatment.
342 This shows that there is no direct correlation between DH and apparent affinity for Ni^{2+} .
343 Moreover, addition of exopeptidase (Flv treatment for 2 h) in the hydrolysis procedure give rise
344 to an increase of the apparent affinity (from 0.38 to 0.42 and 0.12 to 0.23 mM^{-1} equiv Gly, for
345 Alc 1h and Prt 1h, respectively). Finally, the results are independent from the protein source,
346 since similar affinities are observed for PPH after sequential hydrolysis with Flv (0.45 and 0.27
347 mM^{-1} equiv Gly for Alc 1h and Prt 1h, respectively). This could be probably due to the fact that
348 both soy and pea are legumes. Therefore, the hydrolysis treatments had an effect on the metal-
349 chelating activity of the ultrafiltrated produced hydrolysates. A sequential treatment with an
350 endopeptidase (Alcalase[®] or Protamex[®]) followed by an exopeptidase (Flavourzyme[®]) is more
351 efficient than individual hydrolysis by an endopeptidase and the small-sized peptides ($\leq 1\text{kDa}$)
352 produced have better metal-chelating affinity *i.e.*, these hydrolysates globally contain peptides
353 with better affinity for Ni^{2+} . This difference could only be detected by a sensitive screening
354 method like SPR.

355 **4. Conclusion**

356 In conclusion, soy and peas, which are the two most produced legumes in France, constitute
357 rich sources of metal-chelating peptides. The fact that both soy and pea are from the legume
358 family and that both proteins are complete might reflect the global similarities observed in the
359 molecular weight distributions and amino acid compositions regardless of the hydrolysis
360 condition employed.

361 As perspective of this work, the MCPs present in hydrolysates could be identified by MS
362 experiments (Paris et al. 2021). In addition, Immobilized Metal ion Affinity Chromatography
363 (IMAC) coupled to mass spectrometry experiments is currently under development in order to
364 isolate and determine some MCPs present in these hydrolysates. Then, these MCPs will be
365 purified in order to increase their concentration for further applications related to human oral
366 ingestion.

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374 of Lorraine; <https://umsibslor.univ-lorraine.fr/en/facility/biophysics-structural-biology-b2s>)

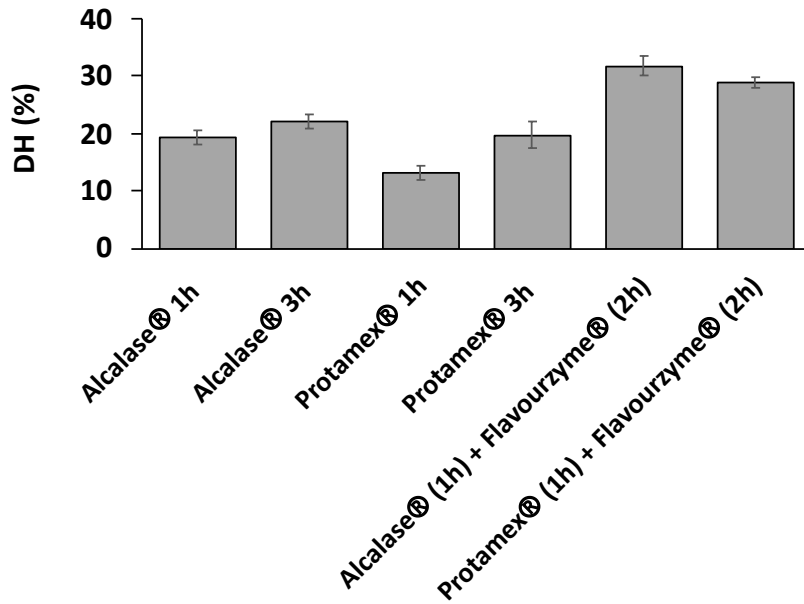
375 **Conflicts of Interest**

376 The authors declare no conflict of interest

377

378 **Appendix A. Supplementary data**

379 **Figure. A.1.**



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383

384 **Table A.1.**

Sample	R_{max} (RU)	STD (R_{max})
SPH by Alc (1h)	109.32	4.9
SPH by Alc (3h)	157.01	21
SPH by Prt (1h)	270.34	24
SPH by Prt (3h)	166.49	11
SPH by Alc (1h) + Flv (2h)	192.24	10
SPH by Prt (1h) + Flv (2h)	84.43	16
PPH by Alc (1h) + Flv (2h)	67.84	3
PPH by Prt (1h) + Flv (2h)	27.56	2

385 SPH: Soy Protein Hydrolysate

386 PPH: Pea Protein Hydrolysate

387 Alc 1h: Alcalase[®] treatment for 1h

388 Alc 3h: Alcalase[®] treatment for 3h

389 Prt 1h: Protamex[®] treatment for 1h

390 Prt 3h: Protamex[®] treatment for 3h

391 Alc (1h) + Flv (2h): Alcalase[®] treatment for 1h followed by Flavourzyme[®] treatment for 2h

392 Prt (1h) + Flv (2h): Protamex[®] treatment for 1h followed by Flavourzyme[®] treatment for 2h

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588 Captions to Figures

589 **Figure A.1.** Degree of hydrolysis (DH%) of soy protein isolates treated with different enzymes
590 for different durations. Non-filtrated soy hydrolysates are analysed for DH. Results
591 are presented as mean \pm standard deviation from 5 replicates.

592 **Figure 1.** Molecular weight distribution of the 1kDa-ultrafiltrated SPHs and PPHs after (A)
593 Alcalase[®] treatment for 1 h or 3 h or 1 h followed by 2 h of Flavourzyme[®]
594 treatment, and (B) Protamex[®] treatment for 1 h or 3 h or 1 h followed by 2 h of
595 Flavourzyme[®] treatment. PPHs were produced only by Alcalase[®] 1 h (or
596 Protamex[®] 1 h) followed by 2 h of Flavourzyme[®]. SPH: Soy Protein Hydrolysate.
597 PPH: Pea Protein Hydrolysate. Alc (1h): Alcalase treatment for 1 h; Alc (3h):
598 Alcalase treatment for 3 h; Prt (1h): Protamex treatment for 1 h; Prt (3h): Protamex
599 treatment for 3 h; Alc (1h) + Flv (2h): Alcalase treatment for 1 h followed by
600 Flavourzyme treatment for 2 h; Prt (1h) + Flv (2h): Protamex treatment for 1 h
601 followed by Flavourzyme treatment for 2 h.

602 **Figure 2.** Amino acid composition of 1kDa-ultrafiltrated SPH and PPH produced by sequential
603 hydrolysis treatment, *i.e.*, Alcalase[®] or Protamex[®] followed by Flavourzyme[®].
604 SPH: Soy Protein Hydrolysate. PPH: Pea Protein Hydrolysate. Alc (1h): Alcalase
605 treatment for 1 h; Alc (3h): Alcalase treatment for 3 h; Prt (1h): Protamex treatment
606 for 1 h; Prt (3h): Protamex treatment for 3 h; Alc (1h) + Flv (2h): Alcalase treatment
607 for 1 h followed by Flavourzyme treatment for 2 h; Prt (1h) + Flv (2h): Protamex
608 treatment for 1 h followed by Flavourzyme treatment for 2 h.

609 **Figure 3.** Copper chelating activity (%) of the different 1-kDa ultrafiltrated (A) SPHs and (B)
610 PPHs as a function of molar ratio (mM eq Glycine of hydrolysate / mM CuSO₄).
611 SPH: Soy Protein Hydrolysate. PPH: Pea Protein Hydrolysate. Alc (1h): Alcalase
612 treatment for 1 h; Alc (3h): Alcalase treatment for 3 h; Prt (1h): Protamex treatment
613 for 1 h; Prt (3h): Protamex treatment for 3 h; Alc (1h) + Flv (2h): Alcalase treatment
614 for 1 h followed by Flavourzyme treatment for 2 h; Prt (1h) + Flv (2h): Protamex
615 treatment for 1 h followed by Flavourzyme treatment for 2 h. Results are presented
616 as average ± SEM. 5 replicates.

617 **Figure 4.** SPR response signal (R.U.) of the different 1-kDa ultrafiltrated (A) SPHs and (B)
618 PPHs as a function of different molar concentrations (mM eq Glycine of
619 hydrolysate). SPH: Soy Protein Hydrolysate. PPH: Pea Protein Hydrolysate. Alc
620 (1h): Alcalase treatment for 1 h; Alc (3h): Alcalase treatment for 3 h; Prt (1h):
621 Protamex treatment for 1 h; Prt (3h): Protamex treatment for 3 h; Alc (1h) + Flv
622 (2h): Alcalase treatment for 1 h followed by Flavourzyme treatment for 2 h; Prt (1h)
623 + Flv (2h): Protamex treatment for 1 h followed by Flavourzyme treatment for 2 h.

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632 **Tables**

633 **Table 1.** EDTA equivalent chelating capacity (EECC) and carnosine equivalent chelating
 634 capacity (CECC) values determined for 1kDa-ultrafiltrated SPHs and PPHs.

	EECC		CECC	
	Mean	± SEM	Mean	± SEM
SPH Alc (1h)	2.52	0.06	3.58	0.08
SPH Alc (3h)	1.94	0.02	2.76	0.03
SPH Alc (1h) + Flv (2h)	2.10	0.01	2.98	0.02
SPH Prt (1h)	2.33	0.05	3.31	0.07
SPH Prt (3h)	1.95	0.04	2.77	0.06
SPH Prt (1h) + Flv (2h)	2.08	0.01	2.96	0.02
PPH Alc (1h) +Flv (2h)	1.81	0.15	2.57	0.21
PPH Prt (1h) +Flv (2h)	1.54	0.02	2.19	0.03

635 Results are presented as mean ± standard error mean from 5 replicates.

636 SPH: Soy Protein Hydrolysate

637 PPH: Pea Protein Hydrolysate

638 Alc (1h): Alcalase® treatment for 1 h

639 Alc (3h): Alcalase® treatment for 3 h

640 Prt (1h: Protamex® treatment for 1 h

641 Prt (3h): Protamex® treatment for 3 h

642 Alc (1h) + Flv (2h): Alcalase® treatment for 1 h followed by Flavourzyme® treatment for 2 h

643 Prt (1h) + Flv (2h): Protamex® treatment for 1 h followed by Flavourzyme® treatment for 2 h

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650 **Table 2.** Peptide concentration (mM equivalent glycine) determined by OPA dosage (n=5), Dissociation constant (K_D , mM) and, Affinity constant
 651 (K_A , mM⁻¹) determined by Surface Plasmon Resonance \pm standard deviation for the 1kDa-ultrafiltrated SPHs and PPHs.

Sample	Peptide concentration (mM equiv Gly for 1g/L SPH)	K_D (mM equiv Gly)	STD (K_D)	K_A (mM ⁻¹ equiv Gly)
SPH by Alc (1h)	1.91	2.59	0.59	0.38
SPH by Alc (3h)	2.39	5.35	1.80	0.19
SPH by Prt (1h)	1.92	8.18	1.50	0.12
SPH by Prt (3h)	2.41	7.43	1.00	0.13
SPH by Alc (1h) + Flv (2h)	1.29	2.43	0.64	0.42
SPH by Prt (1h) + Flv (2h)	3.06	4.44	2.60	0.23
PPH by Alc (1h) + Flv (2h)	2.54	2.23	0.44	0.45
PPH by Prt (1h) + Flv (2h)	3.54	3.76	0.96	0.27

652 SPH: Soy Protein Hydrolysate

653 PPH: Pea Protein Hydrolysate

654 Alc (1h): Alcalase[®] treatment for 1 h

655 Alc (3h): Alcalase[®] treatment for 3 h

656 Prt (1h): Protamex[®] treatment for 1 h

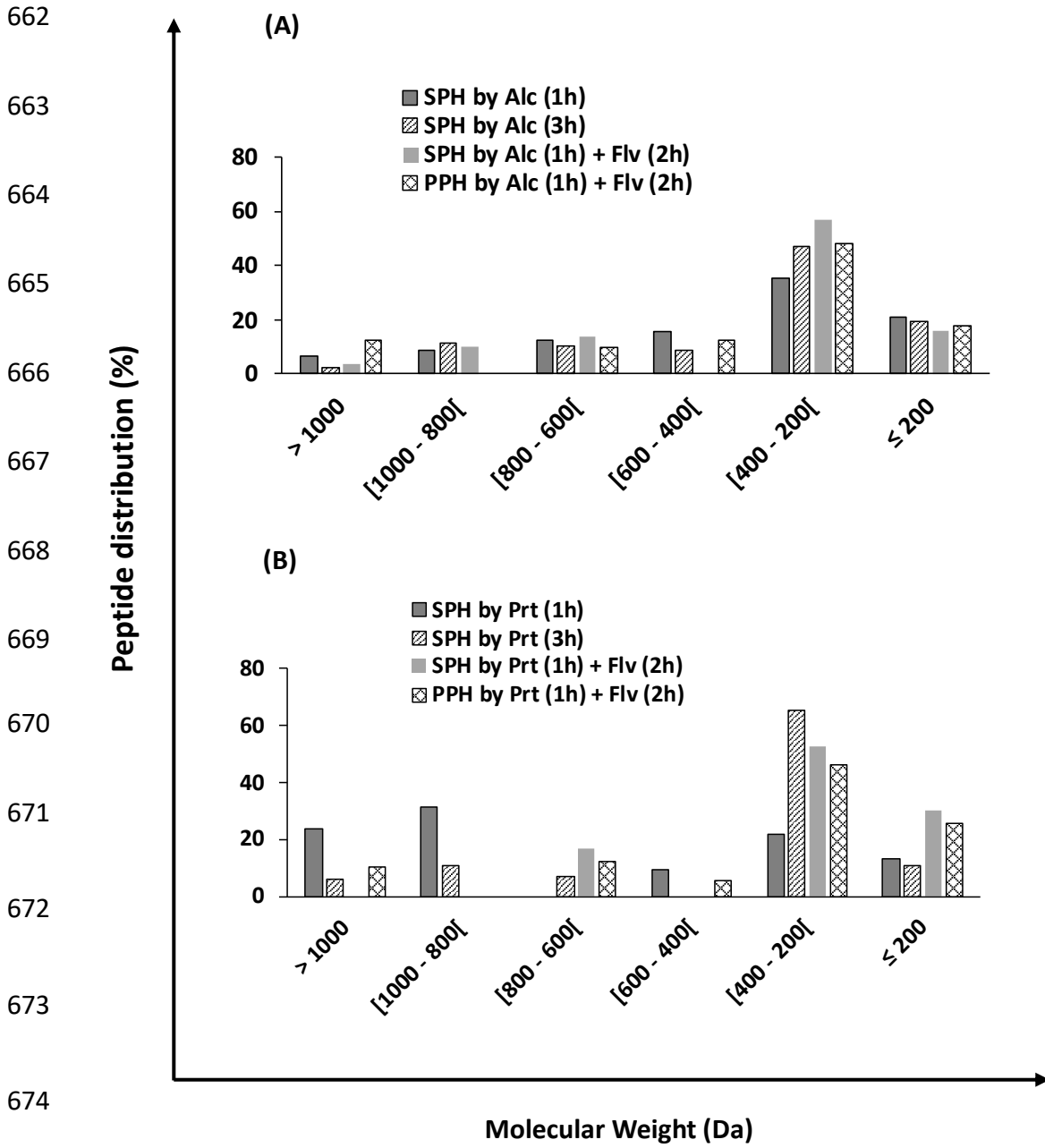
657 Prt (3h): Protamex[®] treatment for 3 h

658 Alc (1h) + Flv (2h): Alcalase[®] treatment for 1 h followed by Flavourzyme[®] treatment for 2 h

659 Prt (1h) + Flv (2h): Protamex[®] treatment for 1 h followed by Flavourzyme[®] treatment for 2 h

660 **Figures**

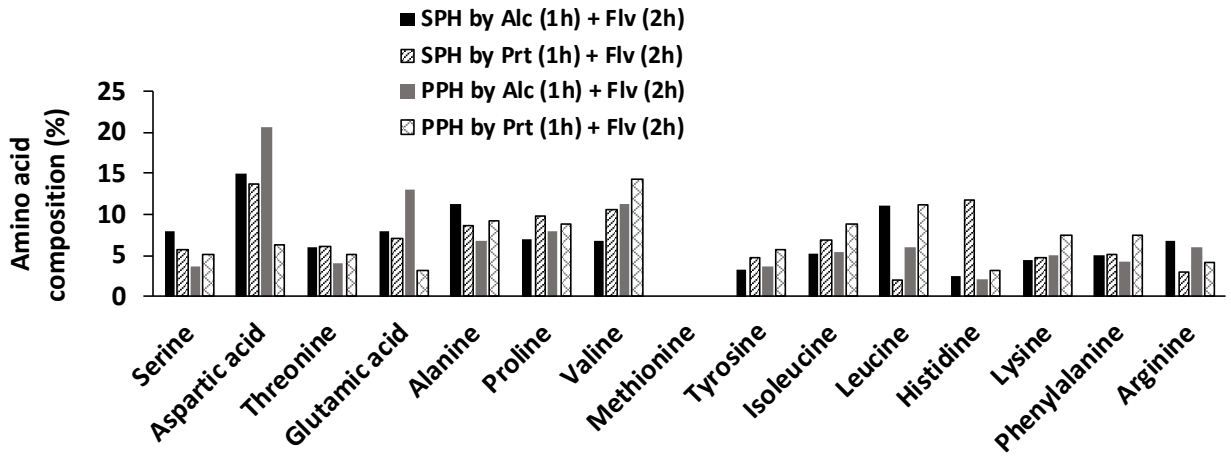
661 **Figure 1.**



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676 **Figure 2.**

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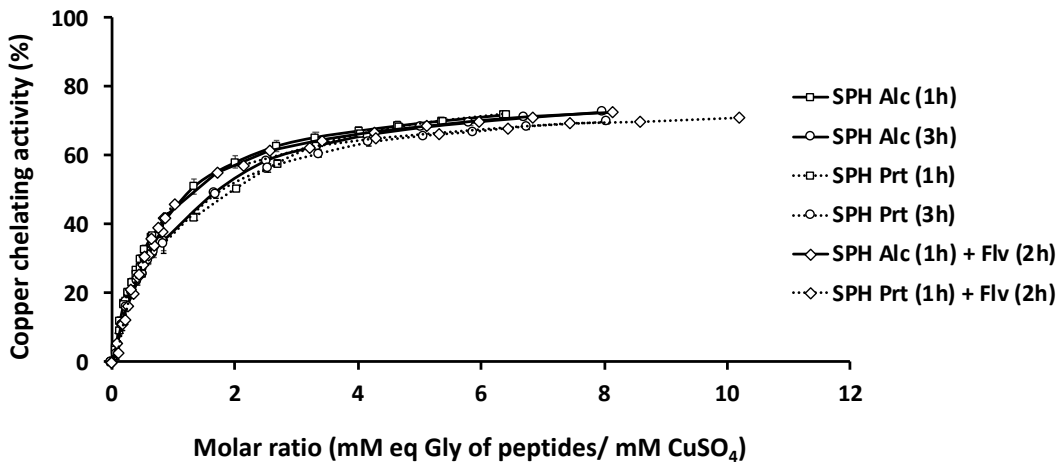
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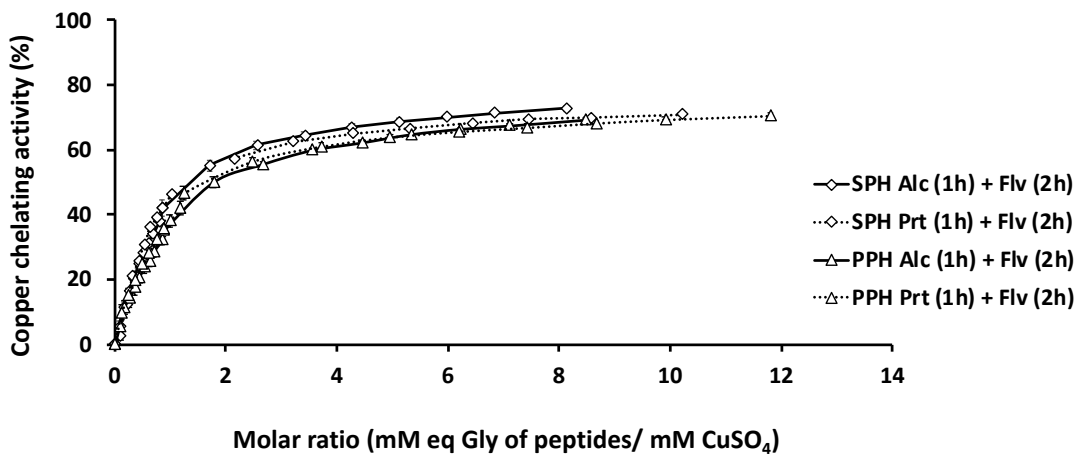
(A)



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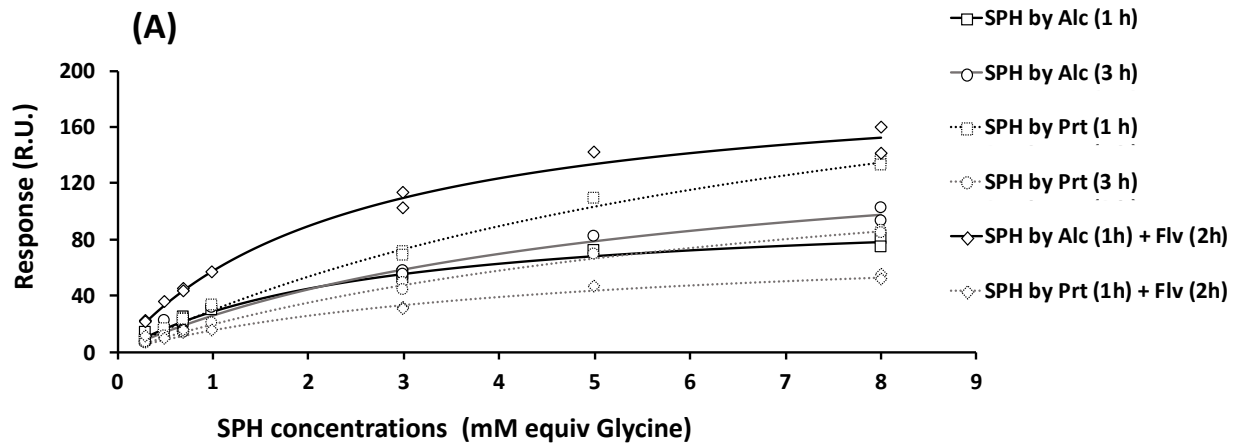
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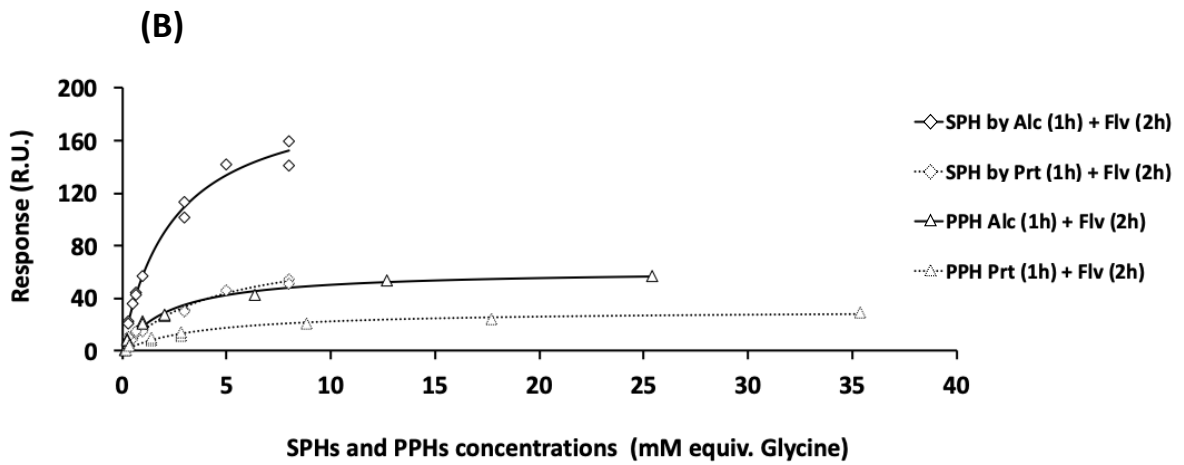
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702 Figure 4.



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Figure 1.

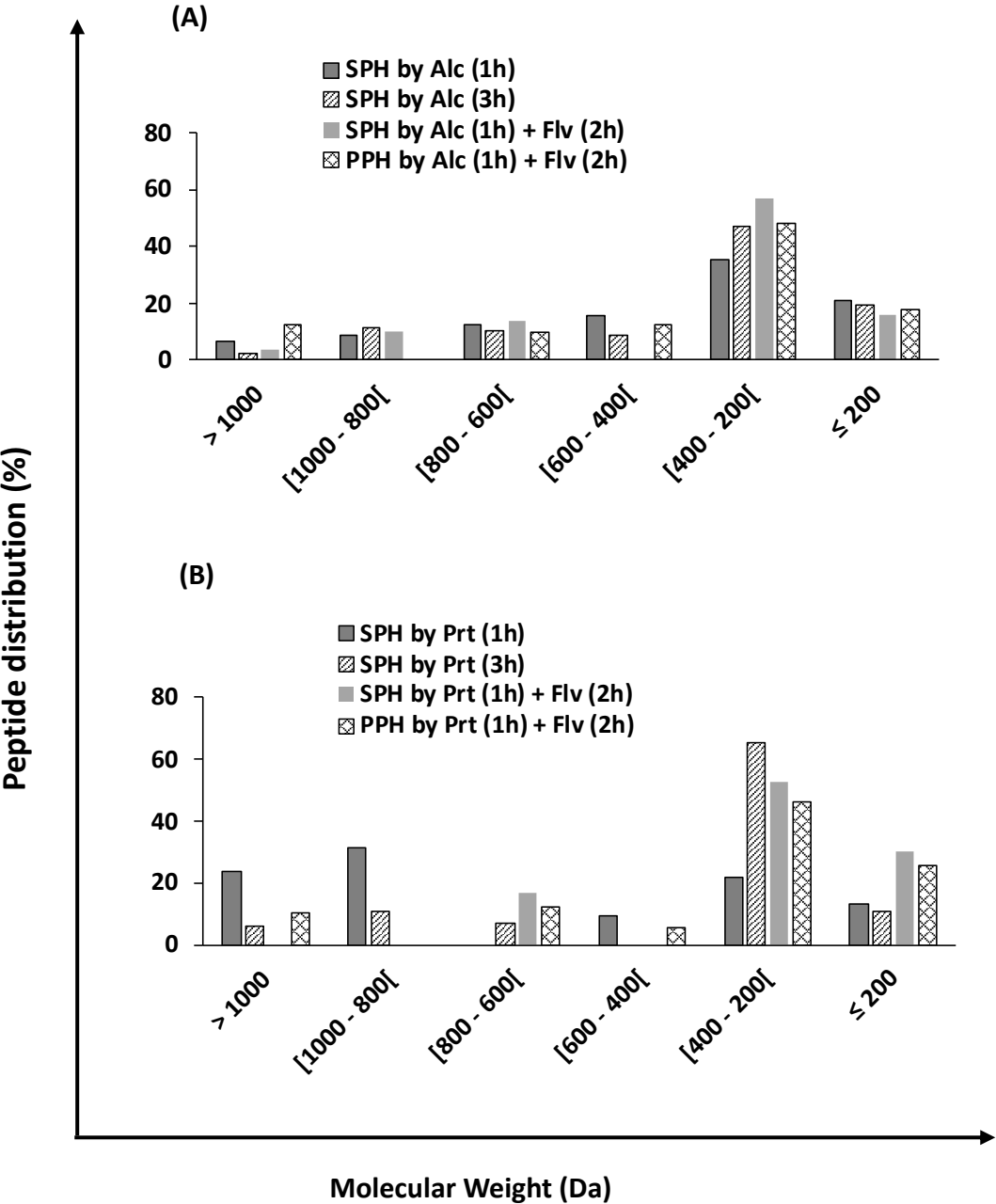


Figure 2.

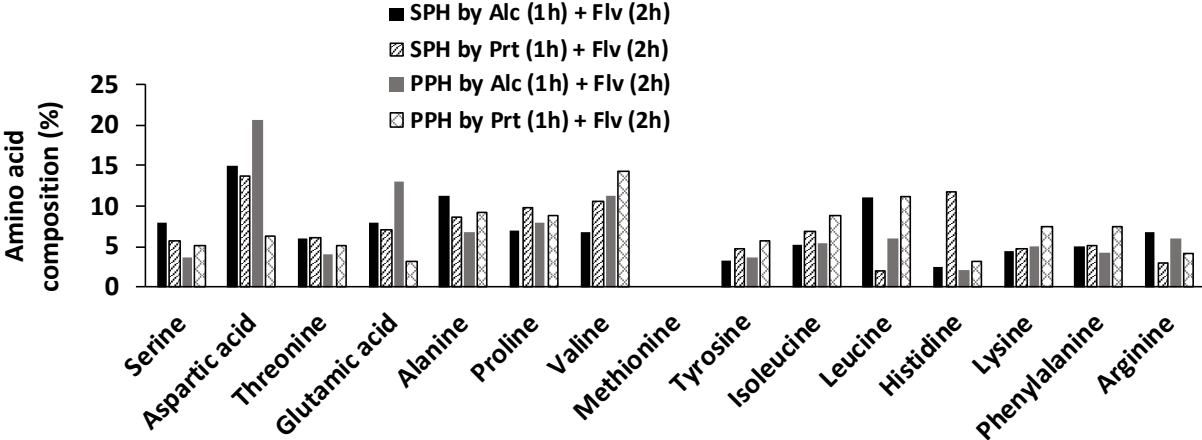


Figure 3.

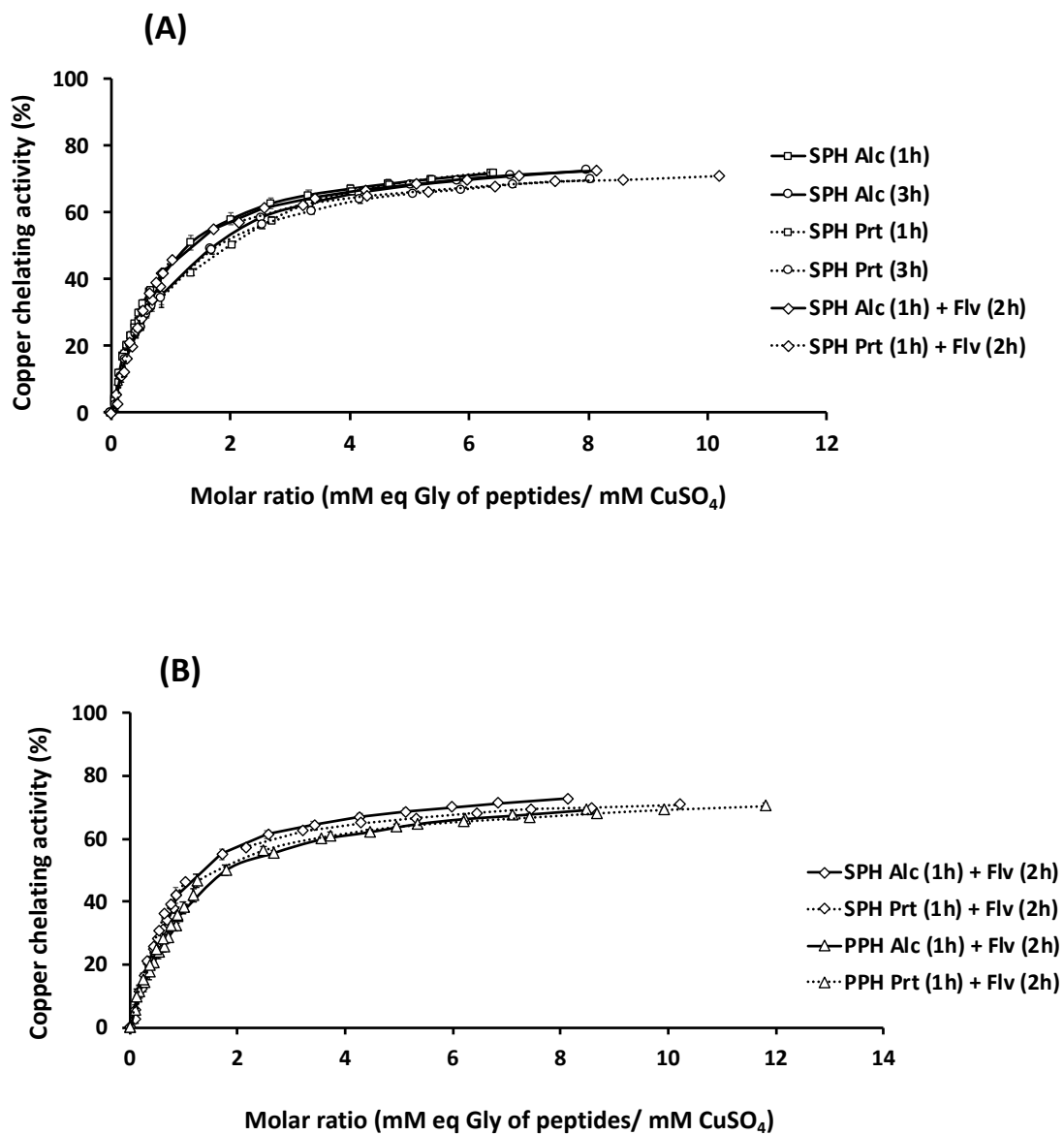


Figure 4.

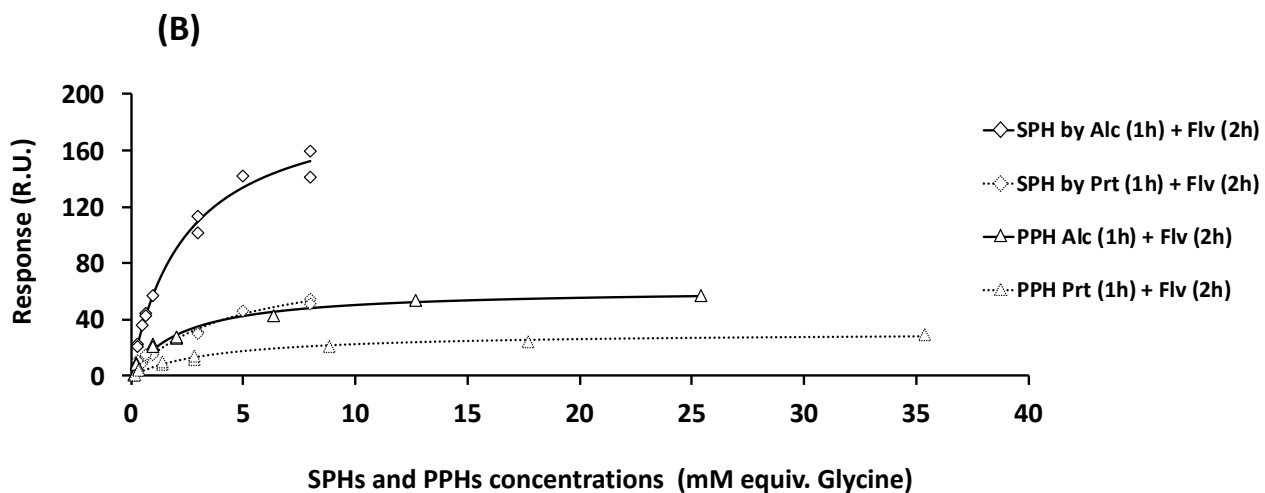
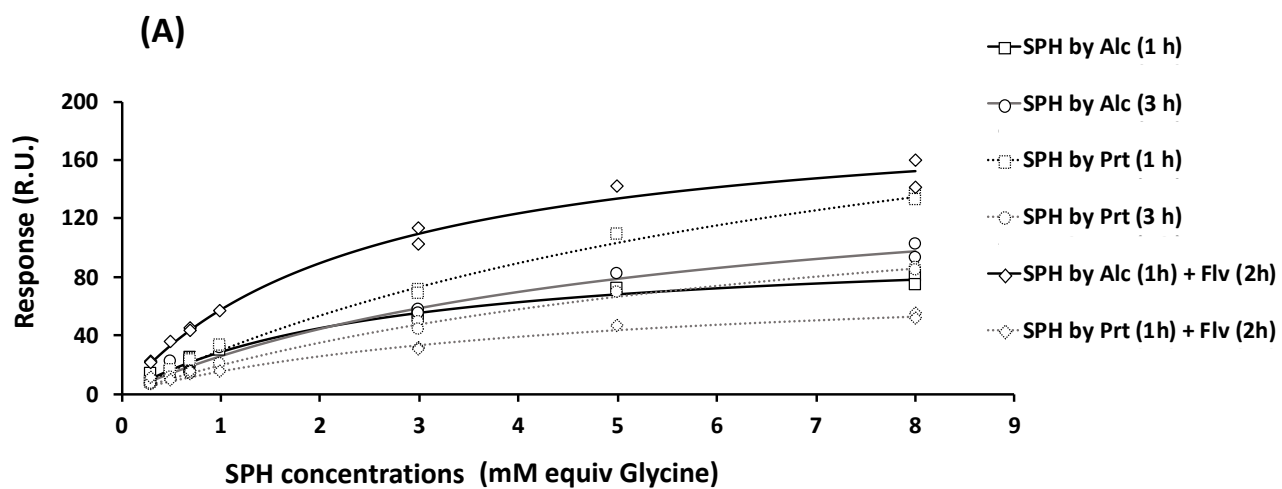


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Prt (1h) + Flv (2h): Protamex® treatment for 1 h followed by Flavourzyme® treatment for 2 h

Table 2. Peptide concentration (mM equivalent glycine) determined by OPA dosage (n=5), Dissociation (K_D , mM) and, Affinity constants (K_A , mM⁻¹) determined by Surface Plasmon Resonance \pm standard deviation for the 1kDa-ultrafiltrated SPHs and PPHs.

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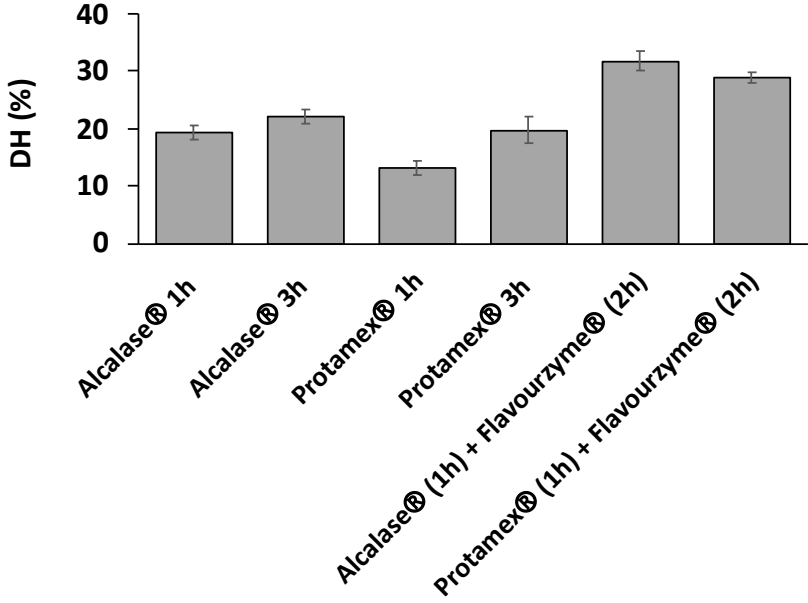
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Prt 3h: Protamex[®] treatment for 3 h

Alc (1h) + Flv (2h): Alcalase[®] treatment for 1 h followed by Flavourzyme[®] treatment for 2 h

Prt (1h) + Flv (2h): Protamex[®] treatment for 1 h followed by Flavourzyme[®] treatment for 2 h

Figure A.1. Degree of hydrolysis (DH%) of soy protein isolates treated with different enzymes for different durations. Non-filtrated soy hydrolysates are analysed for DH. Results are presented as mean \pm standard deviation from 5 replicates.



Results are presented as mean \pm standard error mean from 5 replicates.

Table A.1. R_{\max} (R.U) determined by surface plasmon resonance \pm standard deviation for the 1kDa-ultrafiltrated SPHs and PPHs.

Sample	R_{\max} (RU)	STD (R_{\max})
SPH by Alc (1h)	109.32	4.9
SPH by Alc (3h)	157.01	21
SPH by Prt (1h)	270.34	24
SPH by Prt (3h)	166.49	11
SPH by Alc (1h) + Flv (2h)	192.24	10
SPH by Prt (1h) + Flv (2h)	84.43	16
PPH by Alc (1h) + Flv (2h)	67.84	3
PPH by Prt (1h) + Flv (2h)	27.56	2

SPH: Soy Protein Hydrolysate

PPH: Pea Protein Hydrolysate

Alc 1h: Alcalase[®] treatment for 1 h

Alc 3h: Alcalase[®] treatment for 3 h

Prt 1h: Protamex[®] treatment for 1 h

Prt 3h: Protamex[®] treatment for 3 h

Alc (1h) + Flv (2h): Alcalase[®] treatment for 1 h followed by Flavourzyme[®] treatment for 2 h

Prt (1h) + Flv (2h): Protamex[®] treatment for 1 h followed by Flavourzyme[®] treatment for 2 h