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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<sup>®</sup> and Protamex<sup>®</sup>, used alone or followed by the exopeptidase Flavourzyme<sup>®</sup>. 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.

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

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<sup>1</sup>*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 ( $\text{Fe}^{2+}$ ) and copper II ( $\text{Cu}^{2+}$ ) 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  $\alpha$ -amino group, carboxyl group, and R side  
50 chain of amino acids. The carboxylate group ( $\text{COO}^-$ ) 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<sup>®</sup>, Protamex<sup>®</sup>, Flavourzyme<sup>®</sup>, *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<sup>®</sup> 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<sup>®</sup> (Protease from *Bacillus licheniformis*, ≥ 2.4  
105 U/g, Sigma-Aldrich; **Alc**) and Protamex<sup>®</sup> (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<sup>®</sup> treatment and pH = 7 for the Protamex<sup>®</sup>  
108 treatment. Two sequential hydrolysis were also performed on SPI and PPI solutions using in  
109 each case an additional enzymatic treatment with Flavourzyme<sup>®</sup> (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<sup>®</sup> was applied for 1  
112 hour (pH = 8) followed by Flavourzyme<sup>®</sup> treatment for 2 hours (pH = 7; **Alc+Flv**). Similarly,

113 Protamex<sup>®</sup> was applied for 1 hour followed by Flavourzyme<sup>®</sup> 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<sup>-1</sup>)  
126 <sup>1</sup>) and fractionated under stirring, using consecutively 10 kDa and 1 kDa Ultracel<sup>®</sup> 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  $\mu$ L, 1 mg/mL) prepared in Borax buffer was mixed  
144 with the OPA solution (200  $\mu$ 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  $\mu$ 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<sup>®</sup> followed by Flavourzyme<sup>®</sup> or by Protamex<sup>®</sup> followed by Flavourzyme<sup>®</sup>) were  
185 separated on C18 Alltima reverse phase column (150 x 2.1mm, 5 $\mu$ m – Grace/Alltech,  
186 Darmstadt, Germany) equipped with a C18 Alltima pre-column (7.5 x2.1mm, 5 $\mu$ 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<sup>+</sup>) 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 Cu<sup>2+</sup> 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. NH<sub>2</sub>. 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 CuSO<sub>4</sub> 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 (A<sub>485</sub>/A<sub>520</sub>)  
214 was considered proportional to the free copper ion (Cu<sup>2+</sup>) 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 (A<sub>485</sub>/A<sub>520</sub>)<sub>0</sub> = ratio of absorbances measured in the absence of sample, and (A<sub>485</sub>/A<sub>520</sub>)<sub>s</sub> =  
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 Ni<sup>2+</sup> was analysed by  
222 SPR at 25°C using a Biacore X100 instrument (GE Healthcare, Uppsala, Sweden) equipped  
223 with Ni<sup>2+</sup>-NTA sensor chips. All binding experiments were performed at a flow rate of 20  
224 μL.min<sup>-1</sup>.

225 First, Ni<sup>2+</sup> was injected for 1 min onto the NTA chip using a NiCl<sub>2</sub> solution (500 mM, Biacore  
226 kit) and followed by a 1-min stabilization. The NTA flow channel uncharged in Ni<sup>2+</sup> 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<sup>-1</sup> for 1  
231 min and SDS (0.5 % v/v) at a flow rate of 40 μL.min<sup>-1</sup> 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<sup>-1</sup> 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<sup>®</sup> and Protamex<sup>®</sup> 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<sup>®</sup> 1h) to 32 % (Alcalase<sup>®</sup> 1h + Flavourzyme<sup>®</sup> 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<sup>®</sup> shows a higher DH than Protamex<sup>®</sup> (19.3 %  
250 and 13.2 %, respectively). Raising up the time of hydrolysis by Alcalase<sup>®</sup> to 3 hours does not  
251 reveal an improvement of the DH. However, 3 hours of Protamex<sup>®</sup> treatment increases the DH  
252 to 19.7 %. So Alcalase<sup>®</sup> is more efficient than Protamex<sup>®</sup>, resulting in a greater number of  
253 peptides after 1 h of hydrolysis, but not after 3 hours.

254 Being an exopeptidase, Flavourzyme<sup>®</sup> 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<sup>®</sup> followed by Flavourzyme<sup>®</sup> or Protamex<sup>®</sup> followed  
257 by Flavourzyme<sup>®</sup>, 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<sup>®</sup> (1h) followed by  
290 Flavourzyme<sup>®</sup> (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<sup>®</sup> (1h) followed by Flavourzyme<sup>®</sup> (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 Ca<sup>2+</sup>-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 CuSO<sub>4</sub>) (**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 Ni<sup>2+</sup> was used due to its  
320 similarities with Cu<sup>2+</sup> (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 Ni<sup>2+</sup> 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 Ni<sup>2+</sup> 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  $\text{Ni}^{2+}$  ion varied from 0.12 to 0.38  $\text{mM}^{-1}$  equiv Gly. Hydrolysates produced by  
340 Alcalase<sup>®</sup> treatment present better apparent affinity compared to those produced by Protamex<sup>®</sup>  
341 treatment, and the best apparent affinity is observed for only 1 hour of Alcalase<sup>®</sup> treatment.  
342 This shows that there is no direct correlation between DH and apparent affinity for  $\text{Ni}^{2+}$ .  
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  $\text{mM}^{-1}$  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  $\text{mM}^{-1}$  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<sup>®</sup> or Protamex<sup>®</sup>) followed by an exopeptidase (Flavourzyme<sup>®</sup>) 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  $\text{Ni}^{2+}$ . 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>)

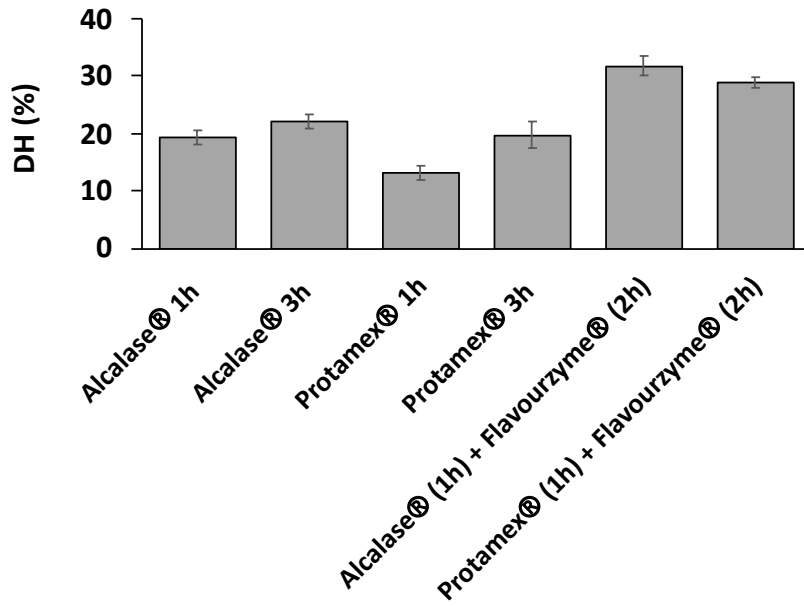
## 375 **Conflicts of Interest**

376 The authors declare no conflict of interest

377

378 **Appendix A. Supplementary data**

379 **Figure. A.1.**



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384 **Table A.1.**

<b>Sample</b>	<b>R<sub>max</sub> (RU)</b>	<b>STD (R<sub>max</sub>)</b>
<b>SPH by Alc (1h)</b>	109.32	4.9
<b>SPH by Alc (3h)</b>	157.01	21
<b>SPH by Prt (1h)</b>	270.34	24
<b>SPH by Prt (3h)</b>	166.49	11
<b>SPH by Alc (1h) + Flv (2h)</b>	192.24	10
<b>SPH by Prt (1h) + Flv (2h)</b>	84.43	16
<b>PPH by Alc (1h) + Flv (2h)</b>	67.84	3
<b>PPH by Prt (1h) + Flv (2h)</b>	27.56	2

385 SPH: Soy Protein Hydrolysate

386 PPH: Pea Protein Hydrolysate

387 Alc 1h: Alcalase<sup>®</sup> treatment for 1h

388 Alc 3h: Alcalase<sup>®</sup> treatment for 3h

389 Prt 1h: Protamex<sup>®</sup> treatment for 1h

390 Prt 3h: Protamex<sup>®</sup> treatment for 3h

391 Alc (1h) + Flv (2h): Alcalase<sup>®</sup> treatment for 1h followed by Flavourzyme<sup>®</sup> treatment for 2h

392 Prt (1h) + Flv (2h): Protamex<sup>®</sup> treatment for 1h followed by Flavourzyme<sup>®</sup> 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<sup>®</sup> treatment for 1 h or 3 h or 1 h followed by 2 h of Flavourzyme<sup>®</sup>  
594 treatment, and (B) Protamex<sup>®</sup> treatment for 1 h or 3 h or 1 h followed by 2 h of  
595 Flavourzyme<sup>®</sup> treatment. PPHs were produced only by Alcalase<sup>®</sup> 1 h (or  
596 Protamex<sup>®</sup> 1 h) followed by 2 h of Flavourzyme<sup>®</sup>. 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<sup>®</sup> or Protamex<sup>®</sup> followed by Flavourzyme<sup>®</sup>.  
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 CuSO<sub>4</sub>).  
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
<b>SPH Alc (1h)</b>	2.52	0.06	3.58	0.08
<b>SPH Alc (3h)</b>	1.94	0.02	2.76	0.03
<b>SPH Alc (1h) + Flv (2h)</b>	2.10	0.01	2.98	0.02
<b>SPH Prt (1h)</b>	2.33	0.05	3.31	0.07
<b>SPH Prt (3h)</b>	1.95	0.04	2.77	0.06
<b>SPH Prt (1h) + Flv (2h)</b>	2.08	0.01	2.96	0.02
<b>PPH Alc (1h) +Flv (2h)</b>	1.81	0.15	2.57	0.21
<b>PPH Prt (1h) +Flv (2h)</b>	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<sup>-1</sup>) 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 <sup>-1</sup> equiv Gly)
<b>SPH by Alc (1h)</b>	1.91	2.59	0.59	0.38
<b>SPH by Alc (3h)</b>	2.39	5.35	1.80	0.19
<b>SPH by Prt (1h)</b>	1.92	8.18	1.50	0.12
<b>SPH by Prt (3h)</b>	2.41	7.43	1.00	0.13
<b>SPH by Alc (1h) + Flv (2h)</b>	1.29	2.43	0.64	0.42
<b>SPH by Prt (1h) + Flv (2h)</b>	3.06	4.44	2.60	0.23
<b>PPH by Alc (1h) + Flv (2h)</b>	2.54	2.23	0.44	0.45
<b>PPH by Prt (1h) + Flv (2h)</b>	3.54	3.76	0.96	0.27

652 SPH: Soy Protein Hydrolysate

653 PPH: Pea Protein Hydrolysate

654 Alc (1h): Alcalase<sup>®</sup> treatment for 1 h

655 Alc (3h): Alcalase<sup>®</sup> treatment for 3 h

656 Prt (1h): Protamex<sup>®</sup> treatment for 1 h

657 Prt (3h): Protamex<sup>®</sup> treatment for 3 h

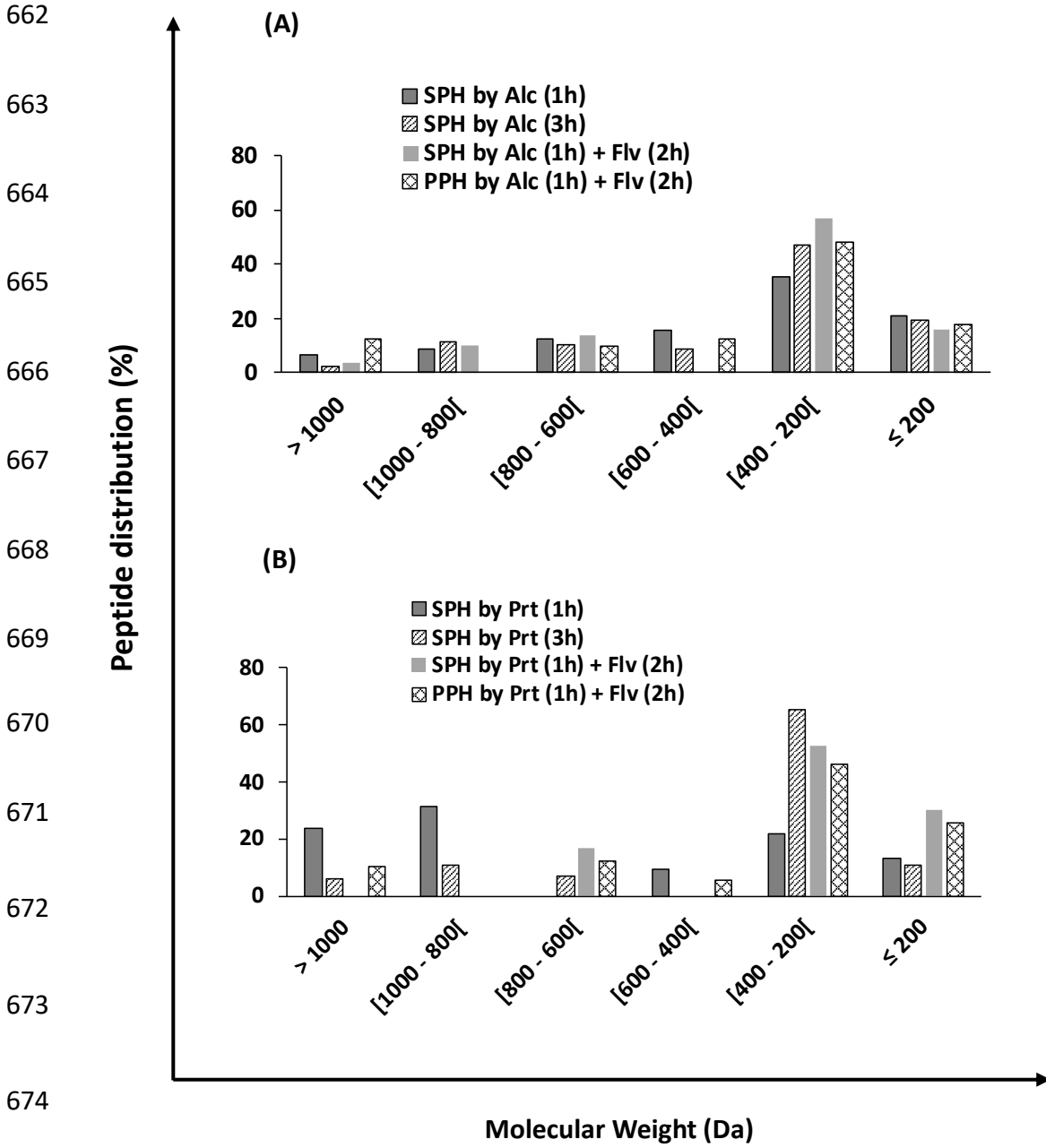
658 Alc (1h) + Flv (2h): Alcalase<sup>®</sup> treatment for 1 h followed by Flavourzyme<sup>®</sup> treatment for 2 h

659 Prt (1h) + Flv (2h): Protamex<sup>®</sup> treatment for 1 h followed by Flavourzyme<sup>®</sup> treatment for 2 h



660 **Figures**

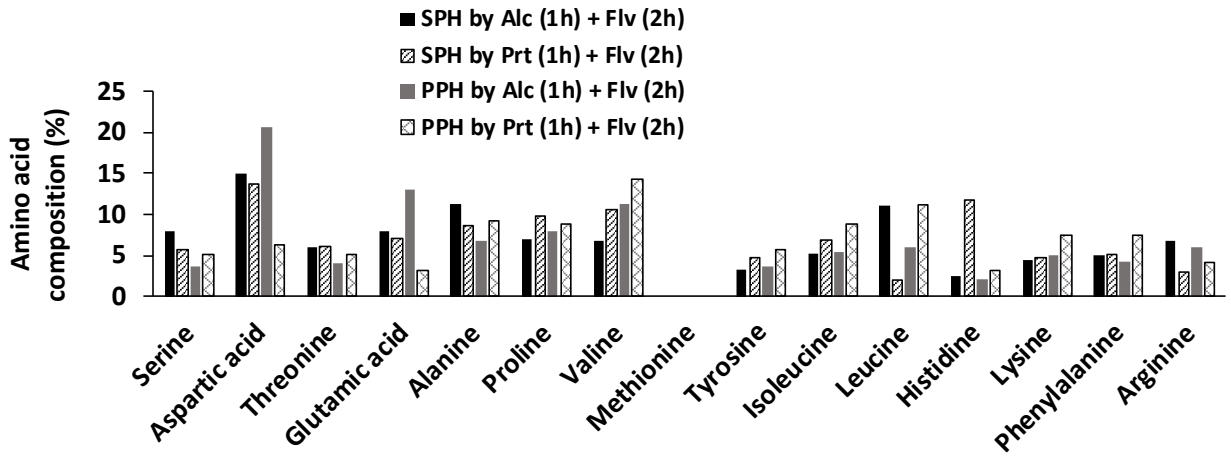
661 **Figure 1.**



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676 **Figure 2.**

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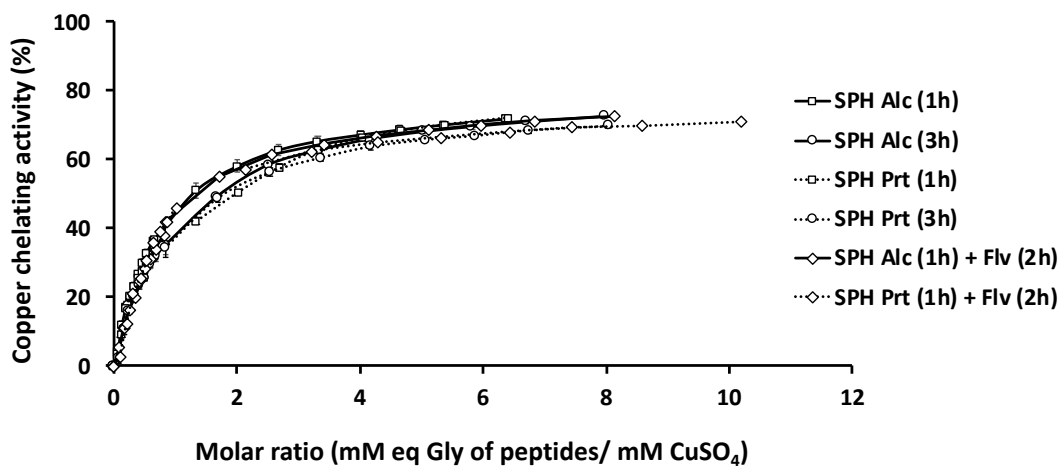
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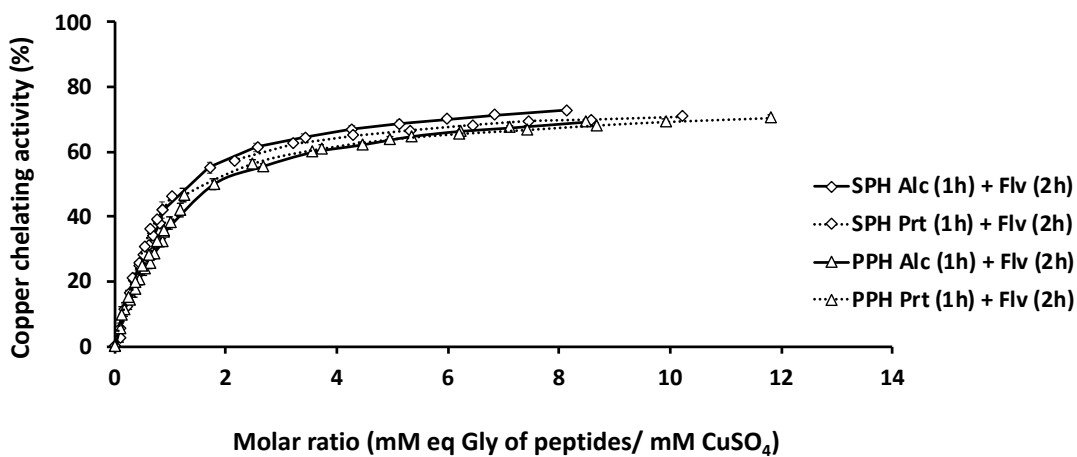
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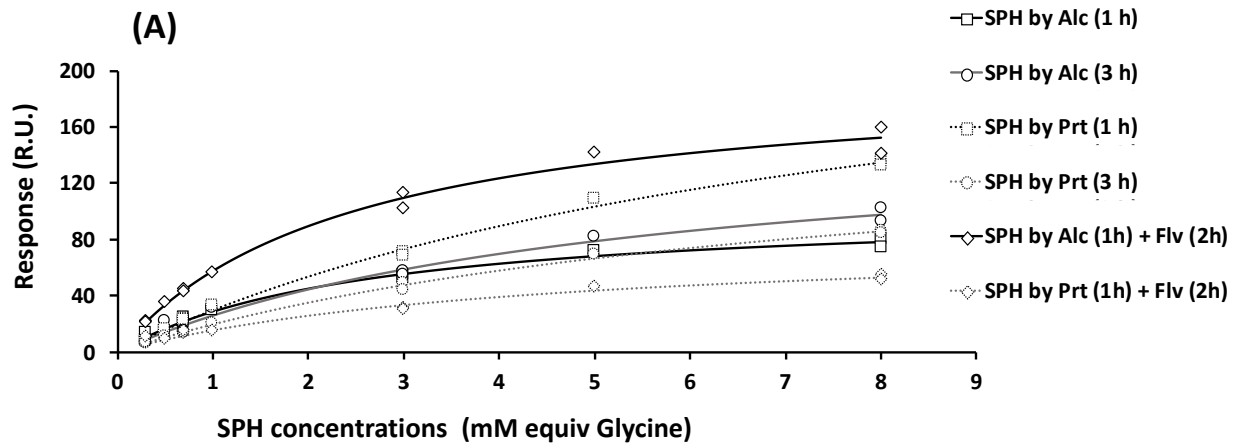
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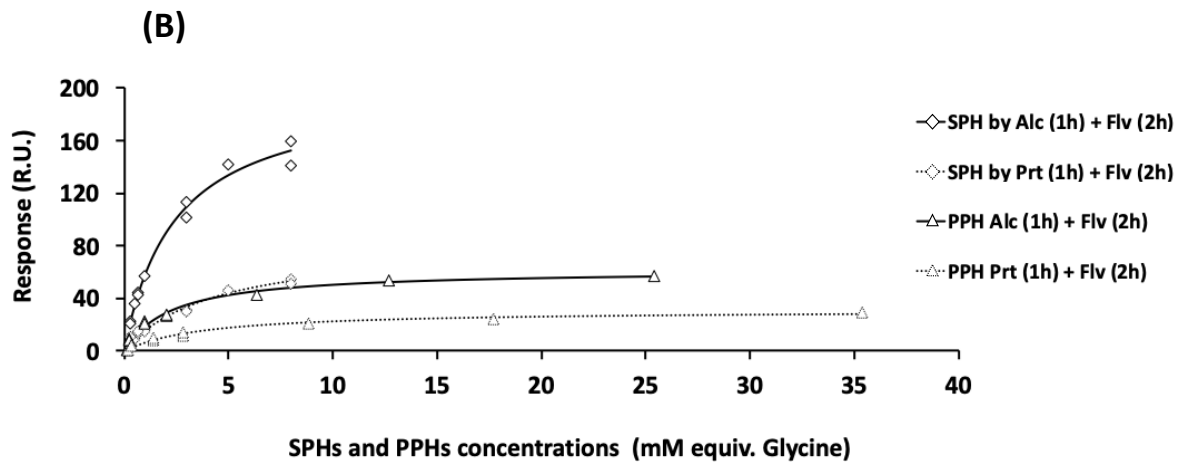
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702 Figure 4.



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Figure 1.

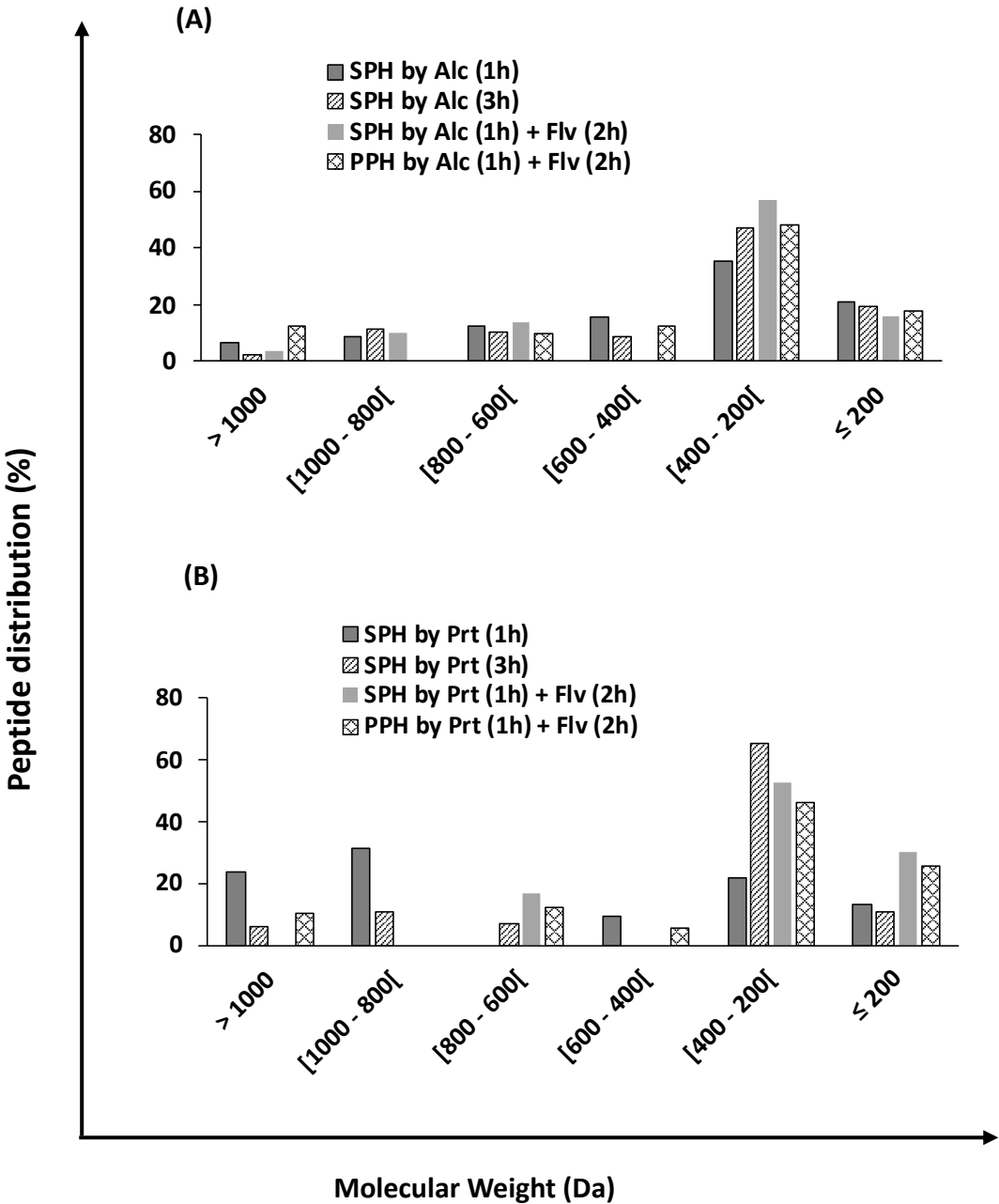


Figure 2.

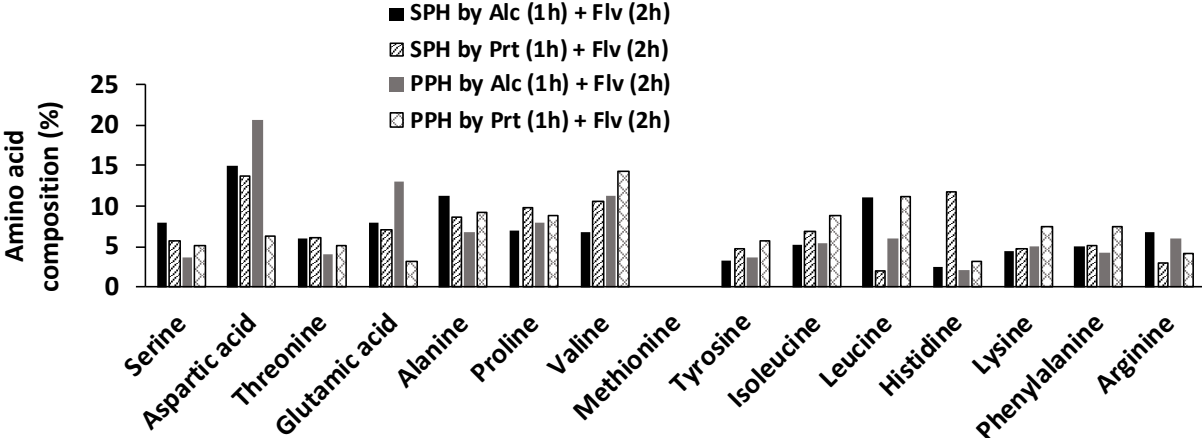


Figure 3.

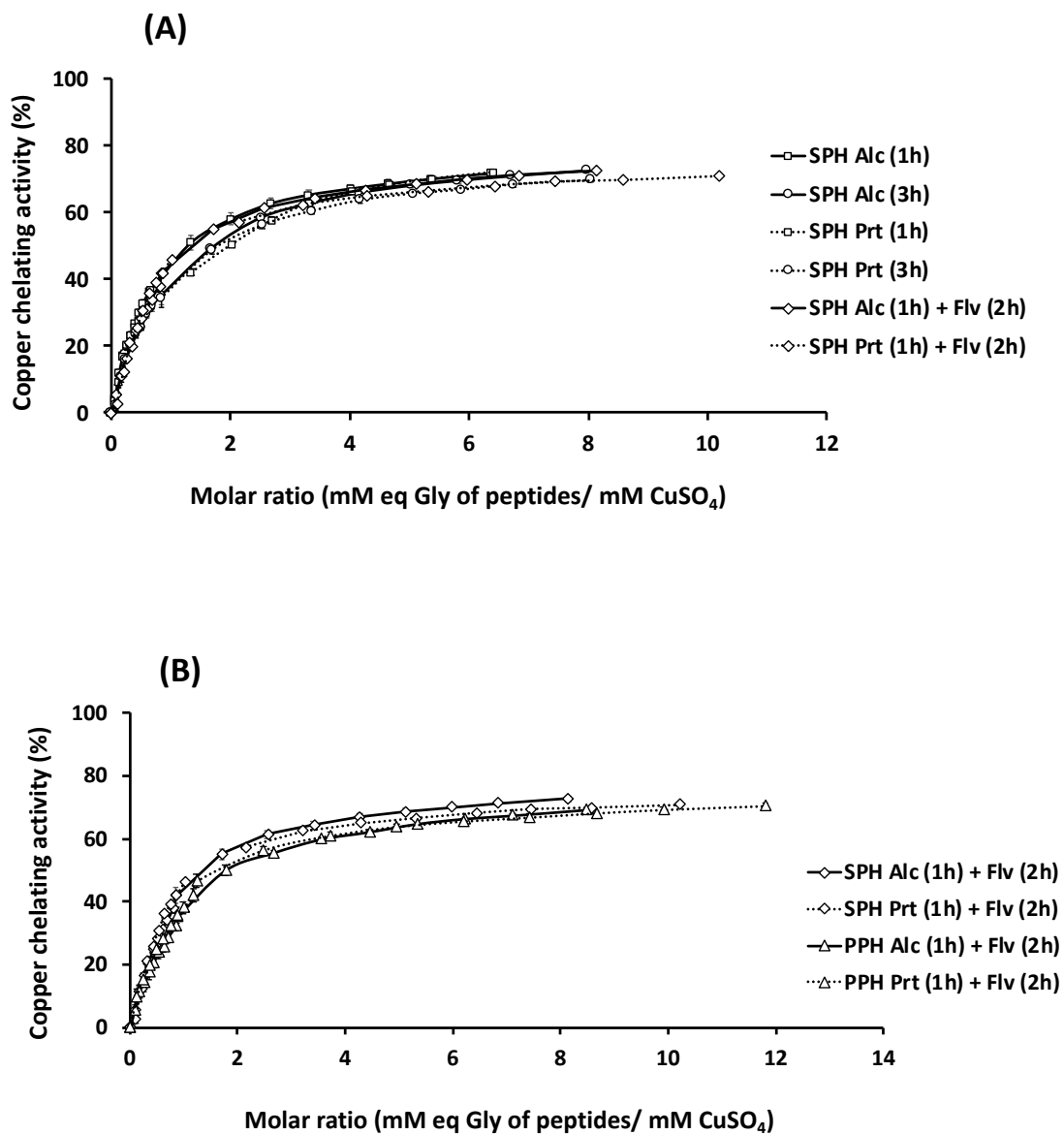
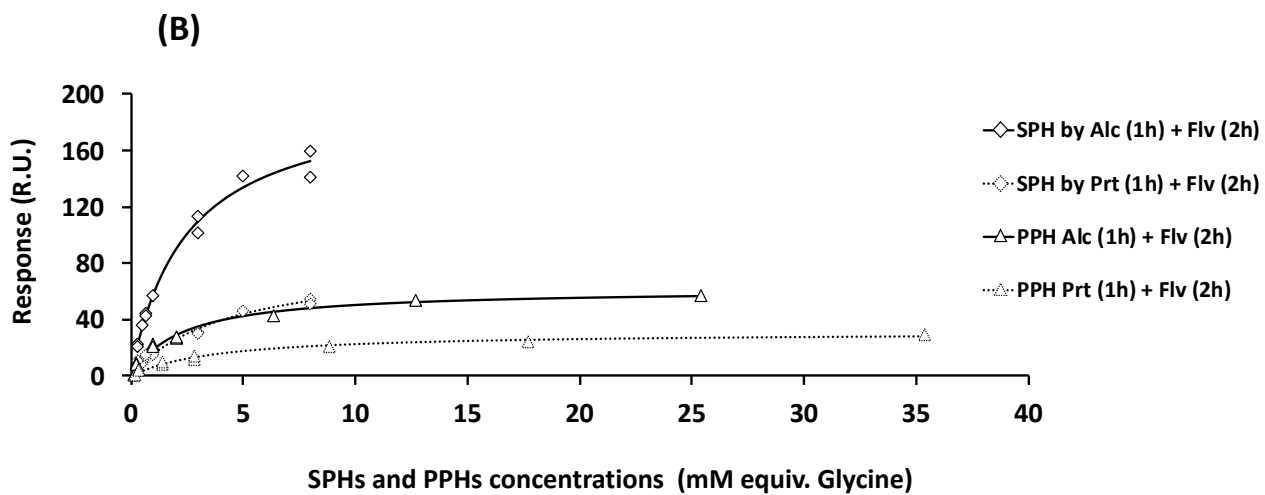
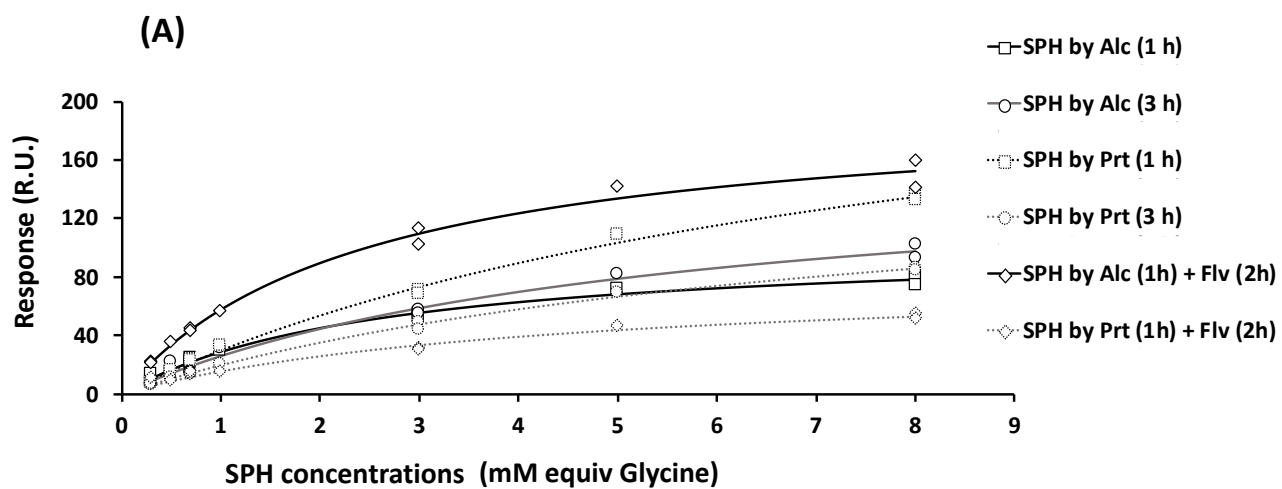


Figure 4.





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

	EECC		CECC	
	Mean	± SEM	Mean	± SEM
<b>SPH Alc (1h)</b>	2.52	0.06	3.58	0.08
<b>SPH Alc (3h)</b>	1.94	0.02	2.76	0.03
<b>SPH Alc (1h) + Flv (2h)</b>	2.10	0.01	2.98	0.02
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Results are presented as mean ± standard error mean from 5 replicates.

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

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

<b>Sample</b>	<b>Peptide concentration (mM equiv Gly for 1g/L SPH)</b>	<b><math>K_D</math> (mM equiv Gly)</b>	<b>STD (<math>K_D</math>)</b>	<b><math>K_A</math> (mM<sup>-1</sup> equiv Gly)</b>
<b>SPH by Alc (1h)</b>	1.91	2.59	0.59	0.38
<b>SPH by Alc (3h)</b>	2.39	5.35	1.80	0.19
<b>SPH by Prt (1h)</b>	1.92	8.18	1.50	0.12
<b>SPH by Prt (3h)</b>	2.41	7.43	1.00	0.13
<b>SPH by Alc (1h) + Flv (2h)</b>	1.29	2.43	0.64	0.42
<b>SPH by Prt (1h) + Flv (2h)</b>	3.06	4.44	2.60	0.23
<b>PPH by Alc (1h) + Flv (2h)</b>	2.54	2.23	0.44	0.45
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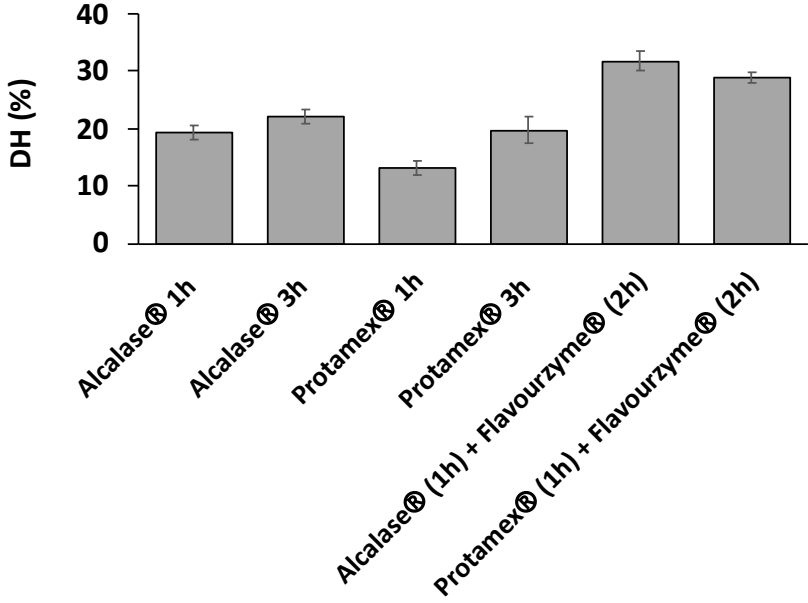
Prt 1h: Protamex<sup>®</sup> treatment for 1 h

Prt 3h: Protamex<sup>®</sup> treatment for 3 h

Alc (1h) + Flv (2h): Alcalase<sup>®</sup> treatment for 1 h followed by Flavourzyme<sup>®</sup> treatment for 2 h

Prt (1h) + Flv (2h): Protamex<sup>®</sup> treatment for 1 h followed by Flavourzyme<sup>®</sup> 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.

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

SPH: Soy Protein Hydrolysate

PPH: Pea Protein Hydrolysate

Alc 1h: Alcalase<sup>®</sup> treatment for 1 h

Alc 3h: Alcalase<sup>®</sup> treatment for 3 h

Prt 1h: Protamex<sup>®</sup> treatment for 1 h

Prt 3h: Protamex<sup>®</sup> treatment for 3 h

Alc (1h) + Flv (2h): Alcalase<sup>®</sup> treatment for 1 h followed by Flavourzyme<sup>®</sup> treatment for 2 h

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