Induction of CCK and GLP-1 release in enteroendocrine cells by egg white peptides generated during gastrointestinal digestion

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Abstract

The effect of dietary protein on the induction of intestinal hormones is recognised. However, little is known about the nature of the digestion products involved in this intestinal signalling. Our aim was to characterise egg white protein digestion products and study their ability to induce CCK and GLP-1 release in enteroendocrine STC-1 cells. Intestinal digests triggered GLP-1 release at a higher rate than gastric digests. Peptides, but not free amino acids, showed a potent GLP-1 secretagogue effect, while proteins only had a modest effect. CCK was released in response to peptides and free amino acids but not proteins. Two hydrophobic negatively charged peptides triggered CCK release, while the highest GLP-1 response was found with a hydrophobic positively charged peptide, pointing to the involvement of different receptors or active sites. Identifying peptide sequences and receptors involved in hormonal secretion could open up new ways to control food intake and glucose metabolism.

Key words: protein digestion; enteroendocrine cells; CCK; GLP-1; intestinal signalling

1. Introduction

During gastrointestinal digestion of food, proteins are hydrolysed into a large variety of peptides and free amino acids. These protein digestion products can act, among other nutrients, as pre-absorptive signalling molecules by inducing the release of hormones relevant to satiety by gastrointestinal enteroendocrine cells (Fromentin, Darcel, Chaumontet, Marsset-Baglieri, Nadkarni & Tomé, 2012). Enteroendocrine cells are specialised cells capable of sensing luminal contents because of the existence of nutrient-specific receptors on their apical side. These cells reside scattered throughout the intestinal epithelium, produce and release a variety of hormonal regulators, such as, I-cells producing cholecystokinin (CCK) or L-cells secreting glucagon-like peptide-1 (GLP-1), that modulate a variety of physiological gastrointestinal and homeostatic functions. These two peripheral hormones inhibit gastric emptying and reduce gastric acid secretion and protein degradation products act as strong inducers in their release (Karhunen, Juvonen, Huotari, Purhonen & Herzig, 2008; Santos-Hernández, Miralles, Amigo & Recio, 2018a). However, it is not clear if the effect of these molecules is hormone-specific, and the nature of the protein-derived products with secretagogue activity is not elucidated. Cordier-Bussat et al. (1997) showed that protein hydrolysates from meat, casein, and soybean increased CCK and GLP-1 release in the enteroendocrine cell line STC-1, while a mixture of free amino acids or undigested proteins only behaved as weak stimulants of these hormones. Other authors have proposed that the protein source and the hydrolysis degree affect the release of CCK and GLP-1 in STC-1 cells. For instance, Geraedts, Troost, Fischer, Edens, and Saris (2011) showed that intact casein, pea, and wheat protein induced CCK secretion in STC-1 cell lines, while egg white protein and ovomucoid showed no significant effect. However, although these authors did not observe any effect with egg white protein, egg-hydrolysate exerted a significant effect on the secretion of CCK. In contrast, egg white intact protein and egg-hydrolysate had a significant response on GLP-1 release (Geraedts et al., 2011). Other studies have suggested that the length of peptide

fragments derived from proteins during enzymatic digestion is relevant to this activity, showing that only peptides composed of five or more amino acids were effective at stimulating CCK release from STC-1 cells (Tulipano, Faggi, Cacciamali & Caroli, 2017). Moreover, Caron et al. (2016a) found that peptides involved in CCK release shared certain occurrence of aromatic amino acids within their sequences. Komatsu et al. (2019) tested different synthetic peptides to evaluate GLP-1 secretion and found one β-casein-derived decapeptide with a significant response in GLUTag cells. In these studies, STC-1 enteroendocrine cell line was used to evaluate the release of CCK and GLP-1.

The performance of STC-1 cell line to assay protein gastrointestinal digests has been validated with digests obtained from human jejunum after oral administration of casein and whey protein in our previous study (Santos-Hernández, Tomé, Gaudichon & Recio, 2018b). Simulated gastrointestinal digests from whey proteins containing longer peptide fragments at the end of the intestinal digestion, induced higher CCK and GLP-1 levels than casein digests. Egg white protein is a high-quality protein source containing prominent levels of essential amino acids and comprises several proteins resistant to gastric pepsin (Benedé, López-Expósito, Molina & López-Fandiño, 2015). It is expected that, as with whey proteins, egg white gastric-resistant proteins will render longer peptide fragments after pancreatic digestion and this could influence hormonal release.

The aim of this work was to investigate the effects of egg white digests and peptide fractions, obtained by size-exclusion chromatography (SEC), on the secretion of CCK and GLP-1 in STC-1 cells. In addition, several peptides resistant to gastrointestinal digestion were chemically synthesised and assayed in this enteroendocrine cell culture. For this purpose, egg white proteins were digested following the internationally harmonized INFOGEST protocol (Brodkorb et al., 2019). Simulated gastrointestinal digests were characterised by their protein,

peptide, and free amino acid content, and their hormonal response is discussed with the composition of the digests.

2. Materials and methods

2.1. Samples

Egg white was manually separated from ecological fresh eggs obtained in a local supermarket. Purified proteins, lysozyme, ovalbumin, and ovomucoid were purchased from Sigma (Sigma-Aldrich, St Louis, MO, USA). Synthetic peptides derived from mucin 5B, ^{545}FRTATGAV^{552}, and from ovalbumin: ^{245}LLP^{247}, ^{364}PFL^{366}, ^{244}VLLPD^{248}, ^{360}RADHPFL^{366}, ^{244}VLLPDEVSGL^{253} and ^{219}RVASMASEKM^{228} were purchased from CSBio Ltd (Shanghai, China) and their protein content was determined by elemental analysis. The mixture of free amino acids was prepared using acid hydrolysis with excess of 6 N gas-HCl in a vacuum system at 110 °C for 20 - 24 h. The hydrolysed sample was dissolved in water, freeze-dried in a Telsar Lyobeta 15, and stored at -20 °C.

2.2. In vitro simulated gastrointestinal digestion

Egg white was digested according to the INFOGEST *in vitro* gastrointestinal protocol (Brodkorb et al., 2019; Minekus et al., 2014). Egg white was diluted in water to 60 mg of protein/mL, and then 1:1 (v:v) diluted with simulated salivary fluid without amylase for the absence of starch in the sample. The intestinal phase was conducted by adding pancreatin from porcine pancreas (100 U trypsin activity/mL of a final mixture, Sigma-Aldrich) and porcine bile extract (B8631-100G, Sigma-Aldrich) in simulated intestinal fluid. Because of the cytotoxic effects of 10 mM bile salts in the cell line STC-1, their concentration was reduced to 2.5 mM in the intestinal phase (Santos-Hernández et al., 2018b).

Gastric and intestinal digests were centrifuged for 15 min at 5,000 × *g* to separate soluble and insoluble material, followed by snap freezing in liquid nitrogen. The supernatants were freeze-dried, and the protein content was determined by elemental analysis.

2.3. Fractionation of intestinal digests by SEC

Intestinal digests were dissolved at 4 mg of protein/mL, in 0.15 M ammonium acetate. After centrifugation, at 11,000 × *g* for 10 min, samples were loaded onto a SuperdexTM Peptide 10/300 GL column (GE Healthcare Europe GmbH, Freiburg, Germany) connected to an AKTA explorer 100 FLPC (GE Health Life Sciences, Pittsburgh, PA, USA). The flow rate was fixed at 0.5 mL/min with ammonium acetate as running buffer; the absorbance was monitored at 215 nm. The procedure was performed several times to obtain enough material of each fraction for cell assays (ca. 5 mg). Three fractions were collected and the molecular weight of the peptides comprised in each fraction was calculated using peptide standards: an α_{s2} casein fragment ^{183}VYQHQKAMKPWIQPKTKVIPYVRYL^{207} (3,113.7 Da) and a β-casein fragment ^{60}YPF^{63} (425.5 Da). Fraction 1 included peptides with an MW > 3 kDa eluted between 13 to 25 min, Fraction 2 comprised peptides of MW 3 and 0.5 kDa eluted between 25 and 37 min, and Fraction 3 comprised small peptides of MW < 0.5 kDa and free amino acids with retention time above 37 min. The fractions were freeze-dried and kept at -20°C until analysis. Protein content of intestinal SEC-fractions was determined by total amino acid analysis after acid hydrolysis with HCl 6 N at 110 °C for 24 h. The analysis was performed in a Biochrom 30 series Amino Acid Analyser (Biochrom Ltd, Cambridge, UK) as previously described (Santos-Hernández et al., 2018b).

2.4. Fractionation of gastric digests by ultrafiltration

Egg white gastric digests were ultrafiltered using Minimate^{TM} Tangential Flow Filtration (Pall Life Science, Ann Arbor, MI, USA) with a 5 kDa Omega membrane (Pall Life Science) according to the manufacturer's instructions. Permeate and retentate fractions were freeze-

dried and protein content of both fractions was determined by elemental analysis. The permeate fraction from the ultrafiltration process from the gastric digests could not be assayed because of its low protein concentration.

2.5. SDS-PAGE

SDS-PAGE was performed as previously described by (Sanchón et al., 2018). Gastric and intestinal digests were dissolved at 0.5 mg of protein/mL in sample buffer and intestinal SEC-fractions were dissolved at 0.7 mg of protein/mL.

2.7. MALDI-TOF/TOF analyses of the peptide bands

Electrophoretic bands were identified by MALDI-TOF/TOF analysis of tryptic digests. Bands were manually excised from gels, and in-gel digestion was carried out as previously described (Shevchenko, Tomas, Havlis, Olsen & Mann, 2006). After digestion, the supernatant was dried in speed vacuo prior to reconstitution in trifluoroacetic acid 0.1%. Samples were cleaned with C_{18} Zip Tip pipette tips (Millipore, Billerica, MA, USA) prior to being spotted into a MALDI target plate with a 2,5-dihydroxybenzoic acid matrix. Analyses were performed on an Autoflex SpeedTM (Bruker Daltonic, Bremen, Germany). Mass spectra were acquired in positive reflection mode and were collected from the sum of 100 - 600 lasers shots. Monoisotopic peaks were generated using FlexAnalysis software. Using the lift method for MS/MS analysis, the laser pulses accumulated were 1,000. For peptide identification the MASCOT Server 2.1 and Biotools version 2.1 (Bruker) were used.

2.8. Peptide identification by mass spectrometry analysis

The identification of resistant peptides to intestinal digestion was performed by HPLC-tandem mass spectrometry (HPLC-MS/MS). Freeze-dried intestinal SEC-fractions were reconstituted in solvent A (water:formic acid, 100:0.1, v:v); prior to analysis fractions were centrifuged at 11,000 × *g* for 10 min. Freeze-dried egg white digests were reduced with 70 mM

1,4-dithiothreitol (Sigma-Aldrich) for 1 h at 37 °C and pH 7, to improve the identification of the disulphide-linked fragments. Samples were analysed using HPLC-MS/MS in duplicate as previously described (Santos-Hernández et al., 2018b). The spectra were recorded over the mass/charge (*m/z*) ranges of 100 - 600, 100 - 1,700, and 100 - 2,000, selecting 500, 750, and 1,200 and as target mass, respectively. A homemade database of egg white proteins was used for peptide sequencing in MASCOT v2.4 software (Matrix Science). Biotools version 3.2 was used for interpreting the matched MS/MS spectra.

2.9. Cell culture conditions

STC-1 cells, supplied by ATCC (ATCC CRL3254), were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/L of glucose and 5 mM L-glutamine (DMEM, Life Technologies, Paisley, UK) supplemented with 100 U/mL penicillin, 100 mg/L streptomycin, amphotericin, and 10% foetal bovine serum. STC-1 cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere and when they reached 80% confluence, they were trypsinized and seeded according to each cell study requirement. The cells were used between passage numbers 25 - 40.

2.10. Hormone secretion studies

Cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere for 48 h into 24-well plates at a density of 3 x 10^5 cells per well. Cells were washed twice with HEPES buffer (20 mM HEPES 1 M, 10 mM glucose, 140 mM NaCl, 4.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, pH 7.4) and were incubated for 1 h in HEPES buffer prior to adding buffer (control) or buffer supplemented with protein digests, protein fractions, purified undigested proteins or synthetic peptides. After 2 h incubation, supernatants were collected and stored at -80 °C with Halt Protease and phosphatase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA). Measurement of the CCK and GLP-1 concentration was performed using a commercial enzyme immunoassay CCK 26-33, non-sulphated EIA Kit (Phoenix Pharmaceuticals Inc., Burlingame, CA,

177 USA) and Glucagon-Like Peptide-1 Active ELISA (EMD Millipore, Billerica, MA, USA),
178 respectively. To detect cross-linked reactions, all samples were directly tested at the highest
179 assayed concentration using CCK and GLP-1 ELISA kits prior to assay cell supernatants. All
180 experiments were conducted at least twice using three biological replicates; ELISA
181 measurements were performed in duplicate.

182 2.11. Cell viability

183 A concentration of 5 x 10^4 cells per well were seeded into 96-well plates and cultured
184 for 24 h at 37 °C in a 5% CO_2 humidified atmosphere. After 2.5 h incubation with the samples
185 (purified proteins, egg white protein digests, intestinal SEC-fractions and synthetic peptides) in
186 HEPES buffer, the medium was removed and the plate was further incubated (37 °C for 1 h)
187 with Alamar Blue Cell Viability Reagent (Thermo Fisher Scientific) (100 µL, diluted 1:10 v:v),
188 prior to a fluorescence reading (excitation wavelength of 580 nm and fluorescence emission at
189 610 nm) in a microplate reader.

190 2.12. Statistical analysis

191 ELISA data were compared using one-way ANOVA with Tukey's post hoc test for
192 pairwise comparisons. The results were significant if $p < 0.5$. GraphPad Prism version 6.01 for
193 Windows (La Jolla, CA, USA) was used for graphics and calculations.

194 3. Results

195 3.1. CCK and GLP-1 secretion in STC-1 cells

196 Prior to the secretion assays, cell viability was evaluated after incubation for 2.5 h with
197 the samples at the highest concentration used (2 mg/mL). The digests, their fractions or
198 purified commercial egg white proteins did not affect cell viability (Figure 1A in Supplementary
199 material). In addition, all samples were also assayed against the antibodies used for the

quantitative determination of CCK and GLP-1 to discard those samples that show cross-reactivity in the immunoassays. No assayed samples were recognised by the GLP-1 antibody but the egg white gastric digest, and its UF-retentate gave a high signal in the CCK-ELISA assay, and therefore, were not further considered for CCK-releasing assays (Figure 2 in Supplementary material). The sample of purified ovalbumin at 2 mg/ml gave a signal in the CCK-ELISA kit equivalent to 110 pM of CCK.

After incubation for 2 h with STC-1 cells, gastric and intestinal egg white digests induced significant secretion of GLP-1 in a dose-dependent manner (Figure 1A). GLP-1 secretion was maximised with the egg white intestinal digest, reaching GLP-1 concentrations ca. 200 pM in the cell medium. This response was higher than that found with the gastric digest (77 pM). Because hormonal secretion could be influenced by the peptide size, egg white gastric digest was subjected to ultrafiltration through a 5 kDa membrane, while the intestinal digest was fractionated by SEC into three different fractions: F1, F2, and F3 (Figure 3 in Supplementary material). In addition, purified egg white proteins: ovalbumin; ovomucoid; and lysozyme, were also assayed under the same experimental conditions. The retentate fraction from the gastric phase reproduced the moderate effect observed with the total gastric digest, suggesting that GLP-1 induction is caused by proteins and large peptides contained in this fraction. In agreement with this effect, purified ovalbumin, assayed at 2 mg/ml, stimulated GLP-1 release in a similar manner than the retentate (Figure 1B). The UF-permeate fraction could not be tested due to its low protein content. The GLP-1 levels induced by purified lysozyme or ovomucoid were not statistically different from the control (Figure 1B). Intestinal fractions containing large (F1 > 3k Da) or medium-size peptides (F2, 3 - 0.5 kDa) stimulated GLP-1 secretion, but not the fraction containing small peptides and free amino acids (F3 < 0.5 kDa), nor the mixture of free amino acids (Figure 1C). Intestinal SEC-fractions were assayed at a lower concentration range than digests or purified proteins, from 0.5 to 0.0075 mg/mL, as

225 determined by total amino acid analysis. A dose dependant response was observed for all
226 samples with a significant secretagogue activity compared to the untreated cell control.

227 The intestinal egg white digest also induced a significant CCK release, reaching levels
228 ca. 472 pM in the cellular medium (Figure 2A). However, purified ovomucoid and lysozyme,
229 tested at the same concentrations, did not cause a significant CCK release from the cells
230 (Figure 2B), suggesting that intact proteins are not CCK inducers in this cell line. Because of
231 cross-reactivity in the CCK immunoassay, the release of this hormone could not be measured
232 for purified ovalbumin, the egg white gastric digest or the 5 kDa UF-retentate (Figure 2 in
233 Supplementary material). All SEC-fractions from the egg white intestinal digest showed a
234 similar CCK release, significantly higher that the control, including the mixture of free amino
235 acids obtained by acid hydrolysis of egg white (Figure 2C).

236 Peptides contained in the intestinal egg white digest and the SEC-fractions were
237 characterised and identified by tandem MS (section 3.2). Seven peptides belonging to different
238 ovalbumin domains and mucin 5B were selected based on different structural characteristics
239 regarding peptide length, hydrophobicity, charge, and isoelectric point. Cell viability and cross-
240 reactivity with the antibodies of chemically synthesised peptides was also verified (Figure 1B in
241 Supplementary material). The ovalbumin-derived decapeptide ^{244}VLLPDEVSGL^{253} and the
242 related pentapeptide ^{244}VLLPD^{248}, both with a net negative charge, provoked a significant CCK
243 secretion in STC-1 cells, while a tripeptide belonging to the same region, ^{245}LLP^{247}, did not
244 induce any effect on CCK release. Similarly, three other ovalbumin-derived peptides,
245 ^{360}RADHPFL^{366}, ^{219}RVASMASEKM^{228}, and ^{364}PFL^{366} along with the mucin 5B-derived peptide,
246 ^{545}FRTATGAV^{552}, did not stimulate CCK release (Figure 3B). Remarkably, two different peptides
247 from the selected sequences, ^{219}RVASMASEK^{227} and ^{364}PFL^{366}, stimulated the release of GLP-1,
248 reaching levels ca. 63 pM and 74 pM, respectively (Figure 3A).

249 *3.2. Characterisation of egg white digests and fractions*

To characterise those molecules inducing hormonal release, the analysis of the egg white digests and SEC-fractions regarding their protein, peptide and amino acid content was assessed. At the end of the gastric phase, 1% of the total nitrogen content was in the form of free amino acids but increased to 27% by the end of the intestinal phase. Total amino acids were also determined and the difference between total and free amino acids in the soluble part of the digests corresponded to proteins and peptides, which reached 91% at the end of the gastric phase and 72% at the end of the intestinal phase.

Protein degradation during simulated gastrointestinal digestion was followed by SDS-PAGE. Identification of the electrophoretic bands was confirmed by excision of the band from the gel, reduction, alkylation, in-gel trypsin digestion, and MALDI-TOF/TOF analysis of the tryptic peptides. At the end of the gastric phase, besides ovalbumin, ovalbumin-related protein Y, ovomucoid, and lysozyme, several electrophoretic bands with MW < 10 kDa were detected (Figure 4A). The protein profile of the retentate from the gastric phase was like the total gastric digest, however, lysozyme disappeared from the gel and was retained in the UF membrane, as confirmed by SDS-PAGE analysis of the membrane washing solution (2 M NaCl) (data not shown). The gastric digest and its retentate fraction were analysed by MALDI-TOF where the presence of large peptides between 3,500 and 8,550 Da was confirmed. At the end of the intestinal phase, electrophoretic bands corresponding to ovalbumin and ovalbumin-related protein Y were still detected, revealing their high resistance to gastrointestinal enzymes. In addition, other bands corresponding to pancreatic enzymes were identified. The SEC-fractions from the egg white intestinal digest were also characterised. F1 contained proteins and large MW peptides between 3,900 and 6,500 Da (Figure 4B) and showed a similar protein profile to the total intestinal digest, by SDS-PAGE (Figure 4A). As expected, no protein bands were detected by SDS-PAGE in SEC-fractions F2 and F3, which comprised peptides < 3 kDa. Peptides at the end of the intestinal phase and those included in the different SEC-fractions were identified by LC-MS and were represented using Peptigram Bioware tool (Figure

4C). Peptides in F2 comprised peptides up to 15 amino acids. Because of the abundance of negatively charged residues, 80% of the identified peptides in this fraction had a pI ≤ 7. F3 contained low MW peptides (< 0.5 kDa) and free amino acids. Under our LC-MS conditions, peptides under 0.5 kDa could not be identified by peptide fragmentation. Free amino acids were quantitatively determined, and the most abundant free amino acids found in F3 were Leu and Ser followed by Phe, Tyr, Ala and Arg (Figure 4 in Supplementary material).

4. Discussion

The analysis of the digestome, defined as a portrait of protein-derived digestion products, is possible through the development of the proteomic and peptidomic tools (De Cicco, Mamone, Di Stasio, Ferranti, Addeo & Picariello, 2019). This knowledge can help to clarify how protein signalling occurs at the intestinal lumen, and concretely with enteroendocrine cells which, in response, produce a variety of intestinal hormones (Santos-Hernández et al., 2018a). These hormones control the progress of the gastrointestinal digestion, nutrient metabolism, and food intake. Especially, the incretin GLP-1 is triggered by nutrients (glucose, fatty acids, and protein digestion products) after food ingestion. This hormone stimulates insulin secretion by the pancreas, reduces gastric emptying, and induces satiety (Hira, Pinyo & Hara, 2020). Peptides and free amino acids were proposed as inducing molecules for GLP-1 secretion by interaction with different G-protein-coupled receptors, like the Ca-sensing receptor (CaSR) and transporters, such as PEP-T1 (Diakogiannaki et al., 2013). Our results showed a higher secretion of this incretin in response to intestinal than gastric egg white digests. Because of the high resistance of egg white proteins to gastrointestinal digestion, the intestinal phase of digestion contained traces of undigested proteins, peptides, and free amino acids. Results showed that the fraction of free amino acids accounted for a 27% of the total nitrogen content at the end of the digestion, while the remaining 72% corresponded mainly to peptides. To elucidate main factors in the GLP-1 secretagogue effect,

commercial proteins, SEC-fractions, and a mixture of free amino acids from egg white proteins were assayed in the enteroendocrine cell culture, using the same conditions. It was shown that neither free amino acids or small peptides (< 0.5 kDa) triggered GLP-1 secretion, but SEC-fractions containing peptides > 0.5 kDa increased GLP-1 secretion significantly in this cell culture. These results agree with previous reports describing the lack of activity of free amino acids under similar conditions (Cordier-Bussat et al., 1997; Cudennec, Fouchereau-Peron, Ferry, Duclos & Ravallec, 2012). However, STC-1 cells have shown a low expression of PEPT transporters (Liou, Chavez, Espero, Hao, Wank & Raybould, 2011). Therefore, our results would need validation via *in vivo* trials or *ex vivo* preparations of intestinal tissue. Interestingly, the secretion of GLP-1 was maximised with SEC-fraction F2, comprising peptides between 0.5 and 3 kDa, and F1 which contained longer peptides and traces of undigested proteins. However, the GLP-1 secretagogue effect could be attributed to peptides, since undigested purified proteins (ovalbumin, ovomucoid, and lysozyme) gave a similar or lower GLP-1 response than SEC-fractions when tested at 10-times higher concentrations (2 mg/ml *vs* 0.2 mg/ml). Geraedts et al. (2011) showed strong release of GLP-1 with intact egg protein, although the sample used was not characterised and derived peptides in a commercial sample cannot be excluded. Since the peptide fraction exerted a potent GLP-1 secretagogue effect on this cell culture, several peptides identified in the gastrointestinal digest were chemically synthesised and assayed at a final concentration of 2 mM. Peptides of different lengths (from 3 to 10 amino acids), isoelectric points (pI from 3 to 11) and hydrophobicities were selected. Only two peptides gave a significant GLP-1 release, ^{364}PFL^{366} and ^{219}RVASMASEKM^{228}, with this latter peptide characterised by a high pI (10.1) and a hydrophobicity index of 18.42. As of writing this report, only few food-derived peptides have demonstrated a GLP-1 secretagogue effect, like haemoglobin fragments (Caron et al., 2016a) and the 11-amino acid peptide at the C-terminus of β-casein (Komatsu et al., 2019). This β-casein fragment shares a high net positive charge (pI 11.1) and a high hydrophobicity (33.69) with the egg white peptide described in this

study. However, other selected peptides in our study, with similar characteristics, ^{545}FRTATGAV^{552} (eight amino acids long, pI 11.1, and a hydrophobicity 14.20) did not elicit any effect on GLP-1. Therefore, not only peptide size and characteristics are important but the sequence of these peptides is crucial to exert this secretagogue effect. Initially, CaSR and PEP-T1 were proposed as sensors of peptones, di-, and tri-peptides to induce GLP-1 (Diakogiannaki et al., 2013) but recently the peptide receptor GPR93 was also suggested as a target. This receptor was found to co-localize with GLP-1 secreting cells by double staining immunolabelling (Symonds et al., 2015).

The secretion of CCK was stimulated by the gastrointestinal digests of egg white proteins. Gastric digests, their fractions or undigested ovalbumin could not be assayed because of cross-reaction with the CCK antibody. This highlights the importance of evaluating all samples with ELISA assays used for hormone quantification to avoid false-positive results. Our results show that CCK secretion was induced by peptides and amino acids but not by intact proteins. This agrees with previous studies of gastrointestinal digests of haemoglobin (Caron et al., 2016b) or soybean (Bin Sufian, Hira, Nakamori, Furuta, Asano & Hara, 2011). Similarly, Geraedts et al. (2011) did not find a significant response on CCK secretion with intact egg white protein. Still, the secretion of this hormone induced by Phe, Leu, and Glu had been previously described in STC-1 cells and mouse proximal intestinal tissue (Daly, Al-Rammahi, Moran, Marcello, Ninomiya & Shirazi-Beechey, 2013). In porcine jejunum tissue, Leu, Ile, or a mixture of both amino acids significantly increased secretion of CCK (Tian et al., 2019). In our gastrointestinal egg white digests, Phe and Leu are among the most abundant free amino acids, reaching values of 7 µmol/ml explaining the CCK response observed with the SEC-fraction F3. Secretion of this hormone was also stimulated with the SEC-fractions containing peptides (F1 and F2). From the selected peptides, ^{244}VLLPDEVSGL^{253} and a derived fragment, ^{244}VLLPD^{248}, induced CCK release and were inactive for GLP-1 secretion. These two peptides had a net negative charge (pI around 3) and a marked hydrophobic character. Other authors

have reported peptides with one or two glutamic residues Glu-Glu-Phe/-Met/-Val able to stimulate CCK by activation of CaSR (Yang, Bai, Zeng & Cui, 2019). This agrees with egg white peptides surviving gastrointestinal digestion, which contain a high proportion of negatively charged residues, accounting 9% Glu and 7% Asp of the total amino acids comprised within peptide sequences; and 80% of the gastrointestinal digestion resistant peptides had a pI < 7. While CaSR has been hypothesised as the receptor involved in CCK secretion, activated by free amino acids (Feng, Kang, Wang, Ding, Zhu & Hang, 2019; Wang et al., 2018), the intestinal signalling of peptides may also be mediated through the peptide receptor GPR93 (also known as LPAR5) (Zhan, Weng, Hunt, Davidson, Liu & Le, 2018).

In conclusion, egg white gastrointestinal digests induce the secretion of GLP-1 and CCK in a dose-dependent manner in STC-1 enteroendocrine cells. Egg white peptides and, to a lesser extent, intact proteins act as inducers of GLP-1; while free amino acids have no effect in this cell culture on the secretion of this incretin. In contrast, peptides and free amino acids, but not intact proteins, can induce CCK secretion in the cell model used in this study. Therefore, gastrointestinal released peptides were identified as the most potent secretagogue molecules on the secretion of both anorexigenic hormones. The experiments with synthetic peptides demonstrated that sequences inducing the secretion of each hormone had different structural characteristics, pointing to interaction with different receptors at intestinal level or interaction with different active sites of the receptors. Experiments with receptor inhibitors are already in progress to confirm this. Still, this study highlights the relevance of the peptide fraction in the intestinal lumen as inducers of the hormonal secretion at this level.

CRediT authorship contribution statement

M.S.-H. and I.R. conceived of the present idea. M.S.-H. carried out the experimental part. I.R. wrote the manuscript with support from M.S.-H. and L.A. I. R. supervised the project.

Conflict of Competing Interest

The authors declare no competing financial interest.

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Figure captions

Figure 1. GLP-1 secretion after 2 h incubation of STC-1 cells with (A) egg white gastric (120G) and intestinal (120I) digests, (B) gastric retentate (120G Ret) and purified proteins ovalbumin (OVA), ovomucoid (OM) and lysozyme (LYS), at different protein concentrations (2, 0.5 and 0.125 mg/mL). GLP-1 secretion from STC-1 cells after incubation with (C) SEC-intestinal fractions (120I F1, 120I F2, 120I F3) and a mixture of free amino acids (Free AA) of egg white tested at different concentrations (mg/mL). GLP-1 secretion was determined by ELISA. Error bars indicate SEM (n=3). Statistical significance compared with control (one way ANOVA with Tukey's post hoc test) is indicated by *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$. Different letters denote statistically significant differences ($p < 0.05$) between different time points and protein concentrations.

Figure 2. CCK secretion after 2 h incubation of STC-1 cells with (A) egg white intestinal (120I) digest, (B) purified proteins ovomucoid (OM) and lysozyme (LYS), at different protein concentrations (2, 0.5 and 0.125 mg/mL). CCK secretion from STC-1 cells after incubation with (C) SEC-intestinal fractions (120I F1, 120I F2, 120I F3) and a mixture of free amino acids (Free AA) of egg white tested at different concentrations (mg/mL). CCK secretion was determined by ELISA. Error bars indicate SEM (n=3). Statistical significance compared with control (one-way ANOVA with Tukey's post hoc test) is indicated by * $p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and

498 ****p < 0.0001. Different letters denote statistically significant differences (p < 0.05) between
499 different time points and protein concentrations.

500500

501 **Figure 3**: GLP-1 (A) and CCK (B) secretion after 2 h incubation of STC-1 cells with synthetic
502 peptides identified at the end of gastrointestinal digestion of egg white protein at 2 mM. CCK
503 and GLP-1 secretion was determined by ELISA. Error bars indicate SEM (n=3). Statistical
504 significance compared with control (one-way ANOVA with Tukey's post hoc test) is indicated
505 by *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. Different letters denote statistically
506 significant differences (p < 0.05) between different time points and protein concentrations.

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508 **Figure 4.** Characterization of egg white gastrointestinal digests and their fractions thereof by
509 SDS-PAGE (A), MALDI-TOF (B) and HPLC tandem mass spectrometry (C). (A) SDS-PAGE protein
510 profiles of egg white protein at different times of simulated gastrointestinal digestion and their
511 fractions thereof. MW, molecular weight marker; EW corresponds to egg white undigested
512 protein; 120G corresponds to 120 min of gastric digestion; 120G Ret corresponds to the
513 retentate fraction of the gastric digest; 120G Perm corresponds to the permeate fraction of
514 the gastric digest; 120I corresponds to 120 min of intestinal digestion; 120I F1 corresponds to a
515 SEC-intestinal fraction composed by peptides with a MW > 3kDa; 120I F2 corresponds to a SEC-
516 intestinal fractions composed by peptides with a MW (3 – 0.5 kDa); 120I F3 corresponds to a
517 SEC-intestinal fraction composed by peptides with MW < 0.5 kDa and free amino acids. (B)
518 MALDI-TOF mass spectra of 120I F1. (C) Peptides from ovalbumin identified in egg white
519 intestinal digests and their SEC-intestinal fractions were represented by Peptigram Bioware
520 tool. Each vertical bar corresponds to an amino acid identified as part of a peptide sequence.
521 The height of the bar is proportional to the number of the peptides overlapping this position
522 and the colour intensity is proportional to the sum of the intensities of the peptides

overlapping a given position. Each line corresponds to a different time point: 120I, 120I F1, 120I F2, 120I F3.

Figure 1

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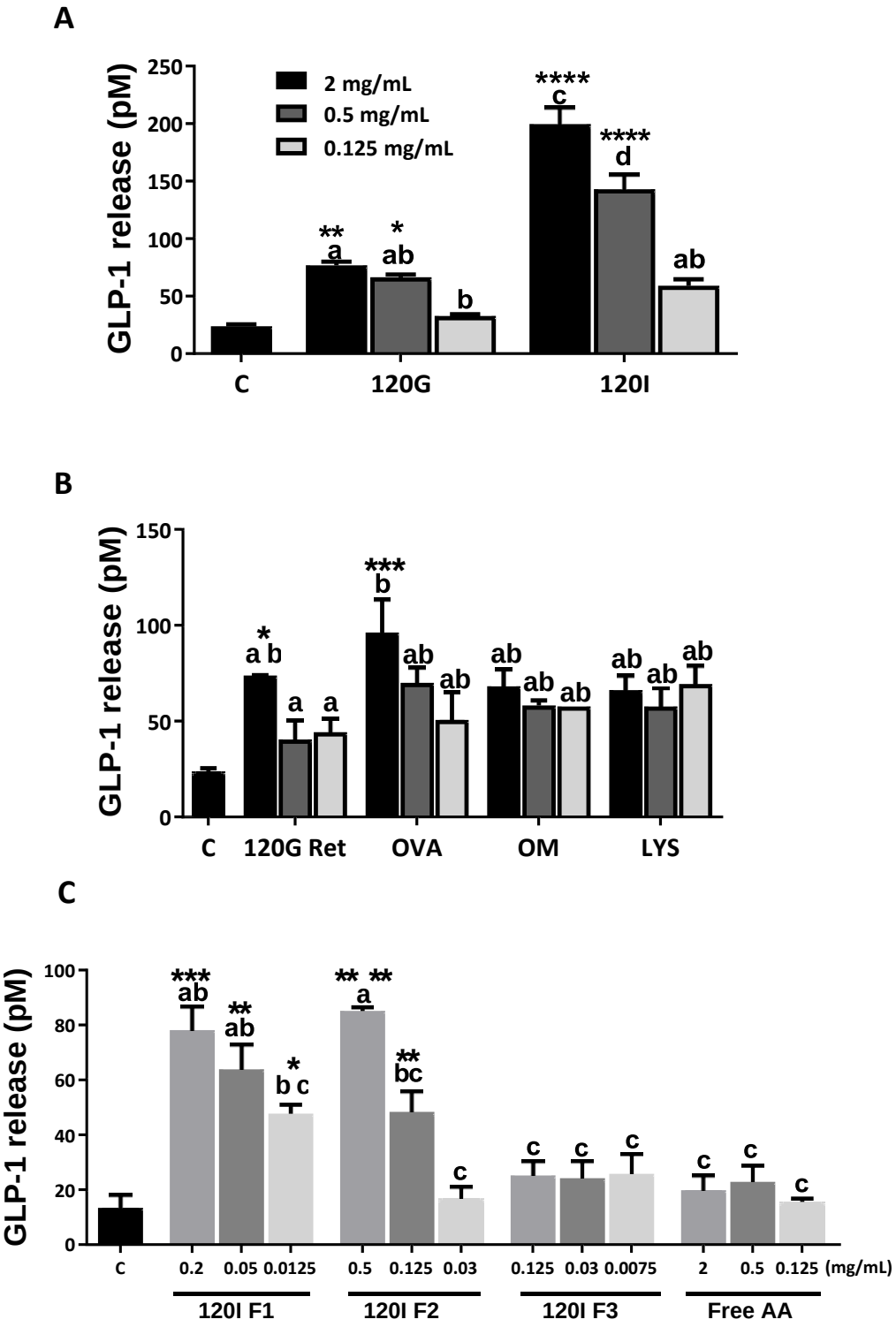

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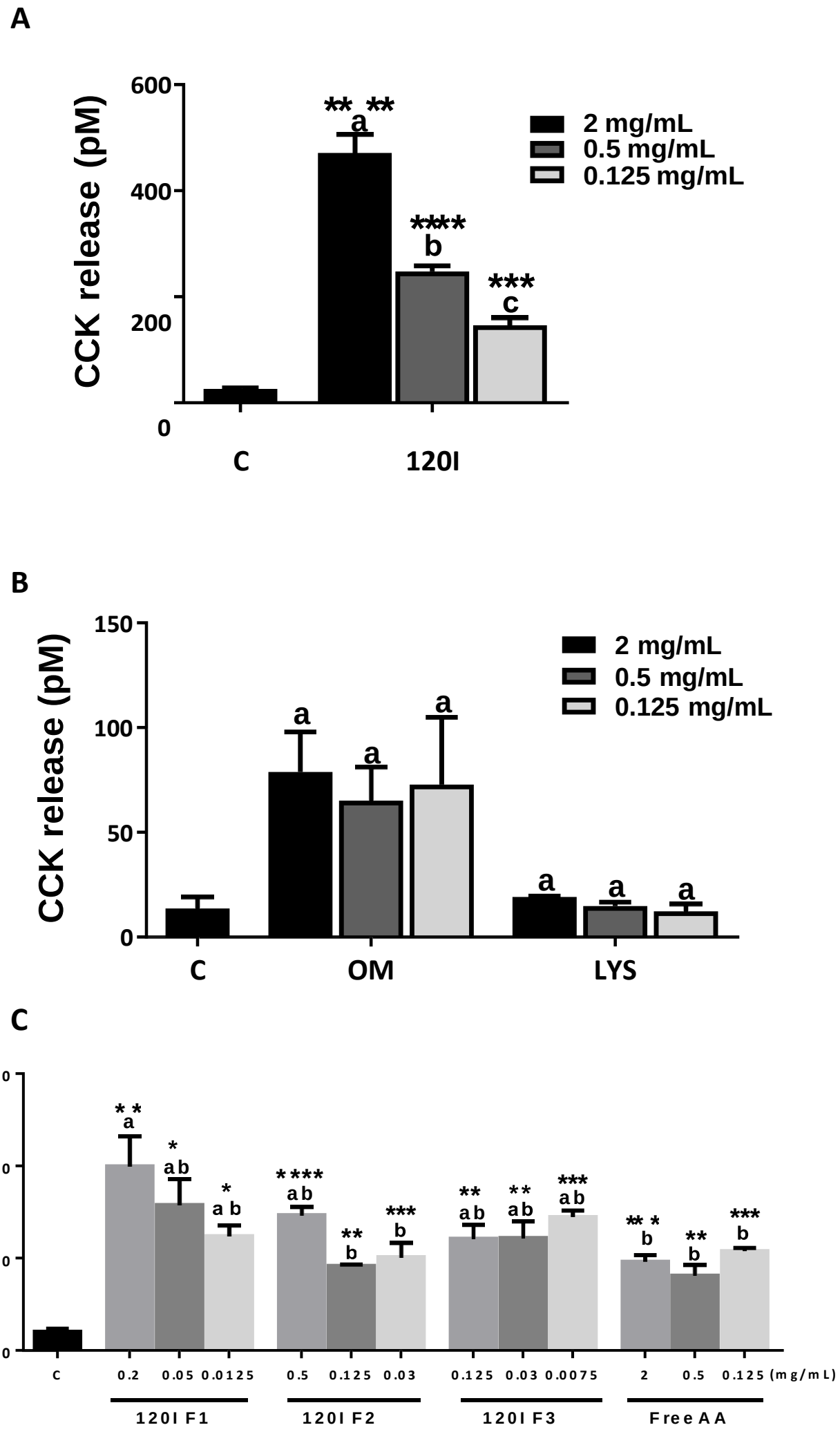

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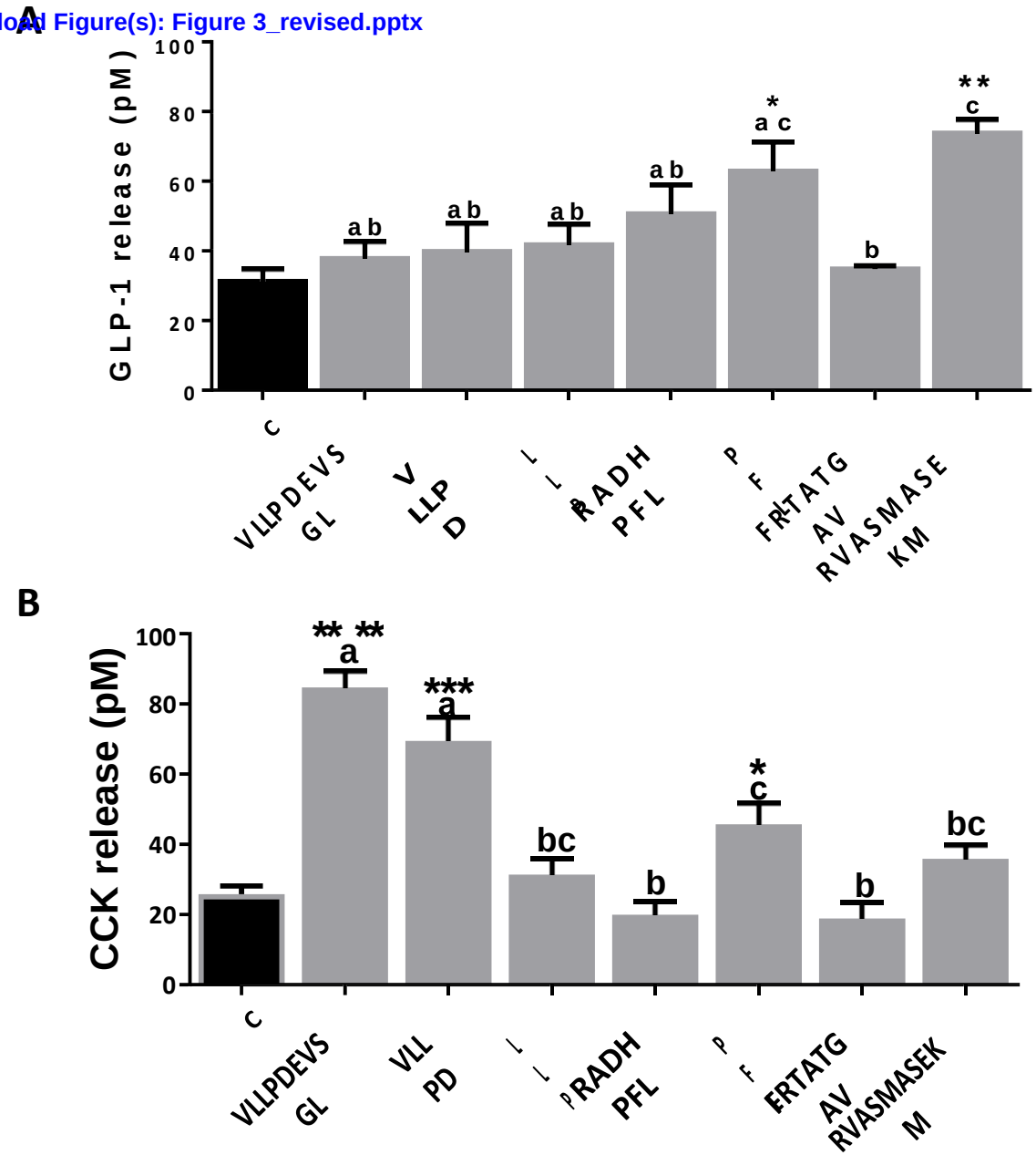

Figure 4

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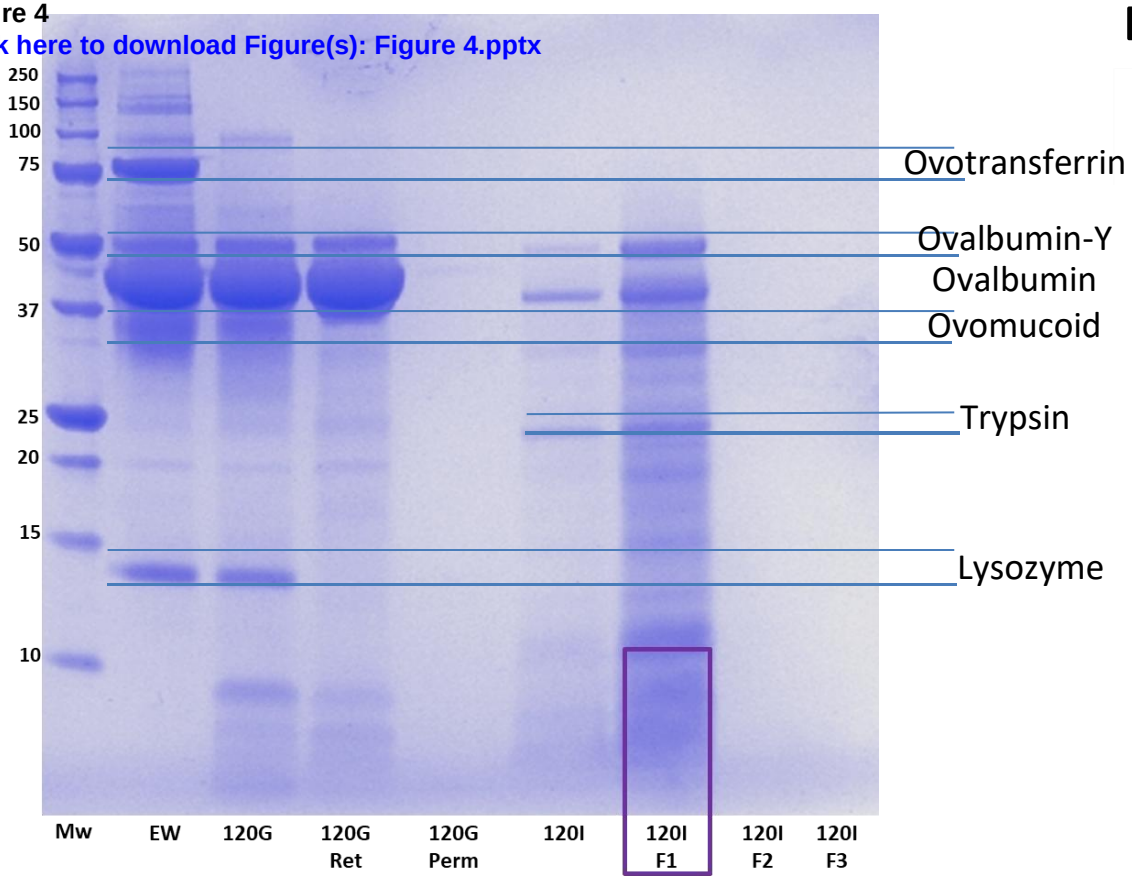

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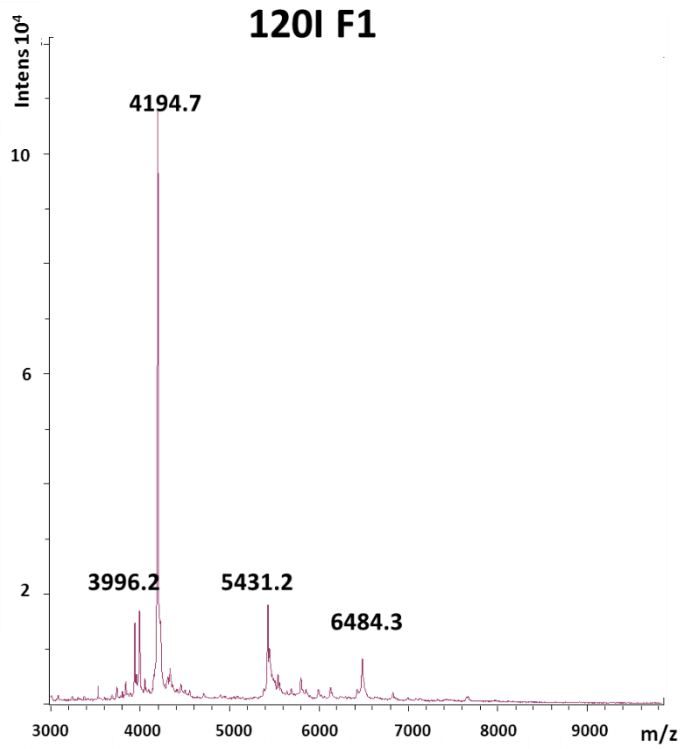

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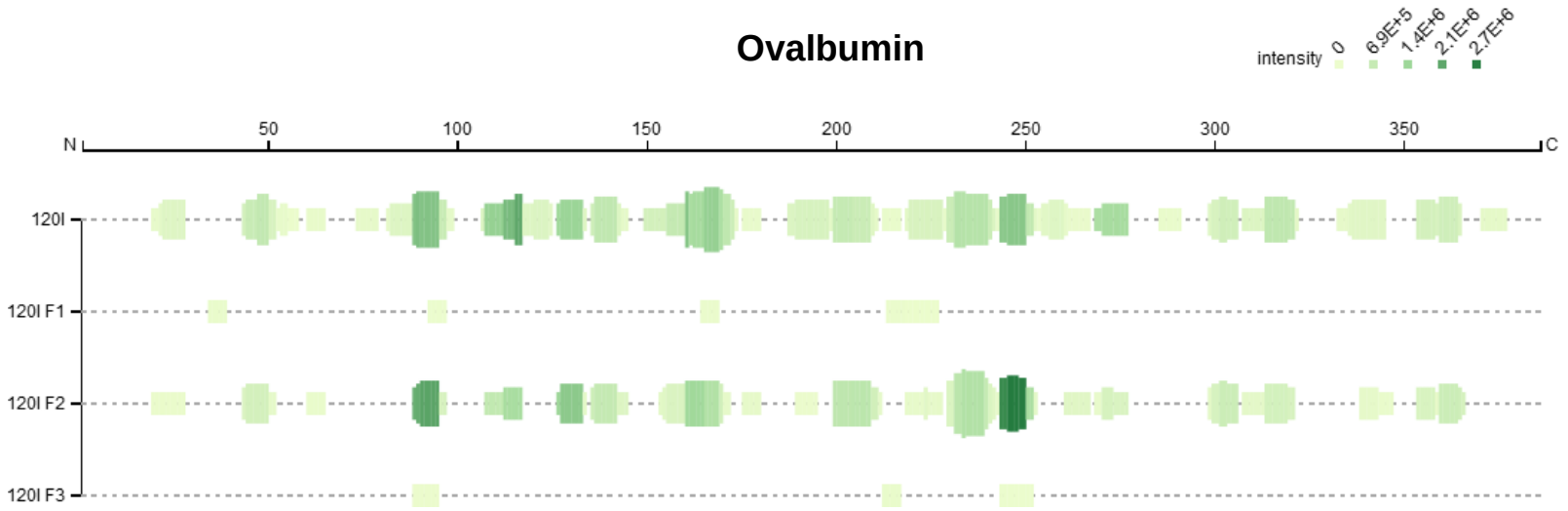

The effect of egg white protein digests in the secretion of CCK and GLP-1 in STC-1 cells

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1. Cell viability

The methodology is described in the manuscript.

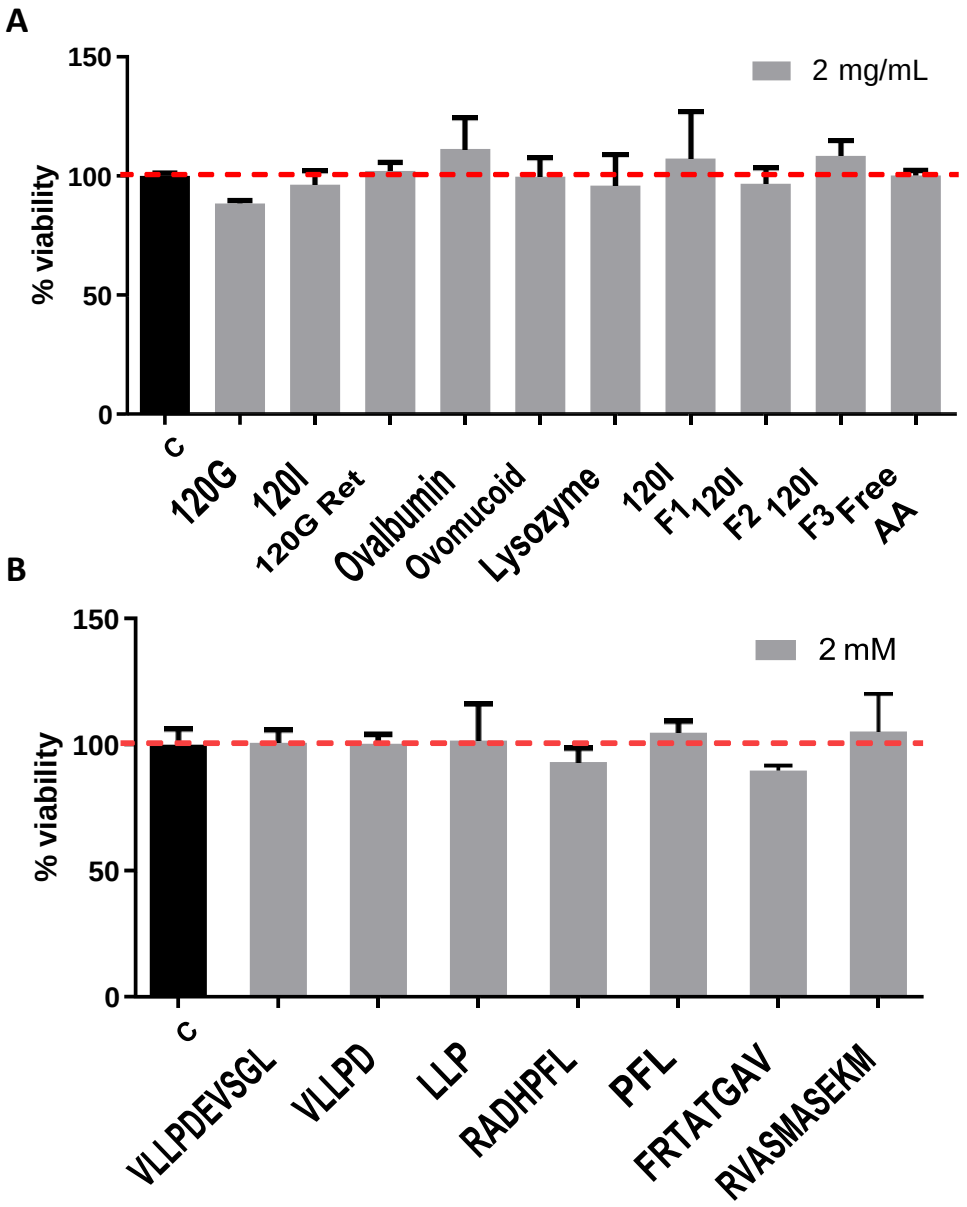

Figure 1: Cell viability in STC-1 cells (mean ± SEM, n=3) following 2 h and 15 min of incubation with (A) 120G *in vitro* egg white protein gastric digests, 120I *in vitro* egg white protein gastrointestinal digests, gastric retentate (120G Ret), SEC-intestinal fractions (120I F1, 120I F2, 120I F3), a mixture of free amino acids (Free AA) and purified proteins, ovalbumin, ovomucoid and lysozyme, at 2 mg of protein per mL, (B) synthetic peptides at 2 mM of concentration. There is no statistical significance in comparison with control (C) (one-way ANOVA with Tukey's post hoc test).

2. Cross-linked reactivity with CCK ELISA kit

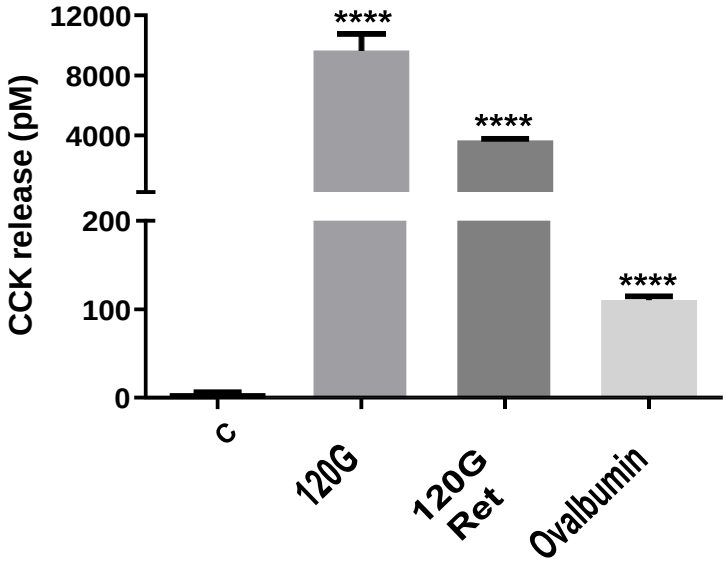

Figure 2: Study of the cross-linked reactivity between 120G egg white *in vitro* gastric digest, retentate thereof (120G Ret) and ovalbumin with CCK antibody of ELISA kit. Samples were incubated directly in the ELISA kit at 2 mg of protein per mL. Statistical significance compared with control (C) (one-way ANOVA with Tukey's post hoc test) is indicated by ****$p < 0.0001$.

3. Size – exclusion chromatogram of egg white intestinal *in vitro* digestion

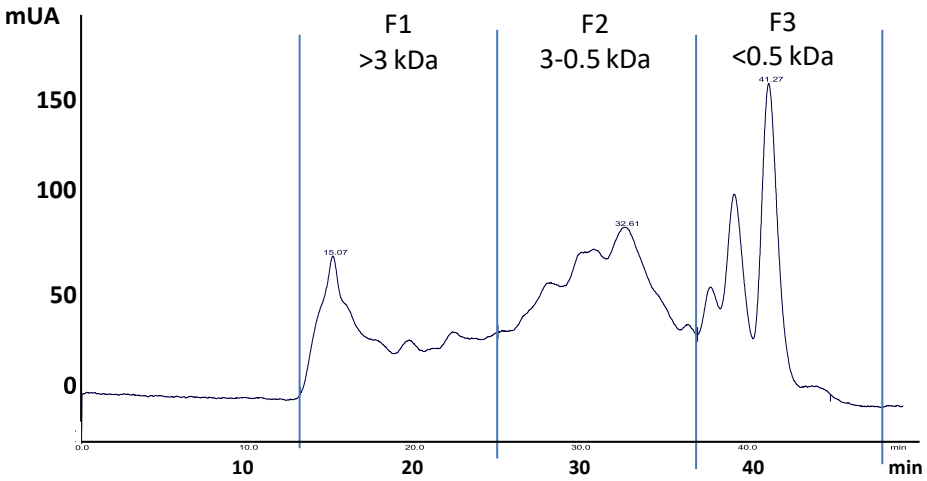

Figure 3: Chromatogram of egg white *in vitro* intestinal digest obtained by size-exclusion chromatography. The digest was fractionated into three different fractions: F1 which contains proteins and peptides > 3 kDa, F2 with peptides between 3 and 0.5 kDa, and F3 composed by peptides < 0.5 kDa and free amino acids. The molecular weight of the peptides comprised in each fraction was calculated with the use of standard proteins and peptides: an α_{s2}-casein fragment ^{183}VYQHQKAMKPWIQPKTKVIPYVRYL^{207} and a β-casein fragment ^{60}YPF^{63}.

4. Free amino acidic profile of the third SEC-intestinal fraction of egg white digest

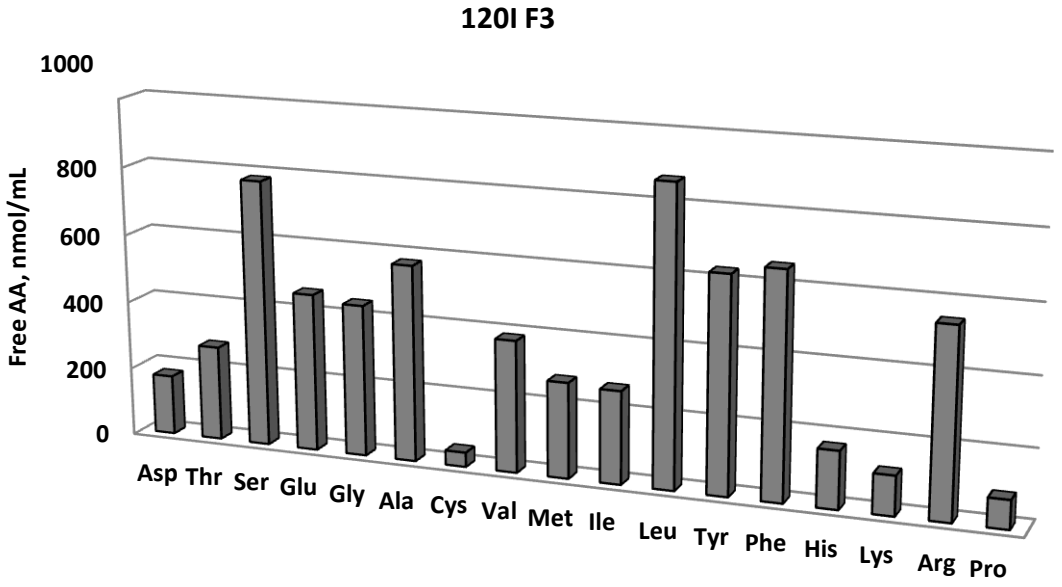

Figure 4: Free amino acid content (nmol/mL) in the third SEC-intestinal fraction (120I F3) of egg white protein. Free amino acids were analyzed by cation exchange-HPLC and ninhydrin derivatization.