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Investigating the impact of ovalbumin aggregate morphology on *in vitro* ovalbumin digestion using label-free quantitative peptidomics and multivariate data analysis

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ABSTRACT

This study aimed to investigate how food structure, in the form of different ovalbumin aggregate morphologies, impacted the proteolysis of ovalbumin using an *in vitro* model that simulated digestion in the adult gastrointestinal tract. Four different aggregate morphologies were prepared by heating ovalbumin solution using different combinations of pH and ionic strength. Quantitative peptidomics (label-free) and multivariate data analysis of the resulting *in vitro* digests were performed. The 593 identified peptides were distributed in 6 homogeneous clusters based on the relative amount of peptide release from the different aggregate morphologies. Each cluster gathered peptides with common physicochemical characteristics. The results suggest that peptic and chymotryptic cleavages were favored by aggregation regardless of the aggregate morphology, while tryptic cleavages were favored when ovalbumin aggregates were spherical-agglomerated. It is notable that even after extensive digestion, the initial aggregate morphology influenced the amount of each peptide released.

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1. Introduction

The digestion behavior of foods is greatly influenced by the food matrix and in particular the food microstructure (Parada & Aguilera, 2007). With respect to dietary proteins, studies on meat, wheat and milk casein proteins have shown that heat-induced aggregation can decrease protein digestibility, at least when tested using *in vitro* digestion models (Bax et al., 2012; Dupont et al., 2010; Petitot et al., 2009). In contrast, other *in vitro* studies focusing on ovalbumin (OVA), β-lactoglobulin and concanavalin A have shown that heat-treatment can enhance protein digestibility (Peram, Loveday, Ye, & Singh, 2013; Takagi, Teshima, Okunuki, & Sawada, 2003). A recent study investigated the gastric digestion of aggregated whey proteins, prepared by heating at different pHs, using an *in vitro* model that simulated gastric digestion and reported that the protein's susceptibility to digestion by gastric enzymes differed according to the physicochemical properties of the heat-induced aggregates (Zhang & Vardhanabhuti, 2014).

Heat-induced aggregation commonly occurs during the processing of globular food proteins. Thermal processing triggers conformational changes which affect the folded structure of proteins (Weijers, Barneveld, Cohen Stuart, & Visschers, 2003). Once globular proteins are unfolded, various types of aggregates can form depending on the balance of attractive and repulsive interactions (Bryant & McClements, 1998). The final aggregate structure is a function of physicochemical conditions such as pH, the type and quantity of added salt, and the protein concentration (Nicolai & Durand, 2013). Under conditions where the protein net charge is minimized, i.e. pH close to the isoelectric point (pI) and at high ionic strength, the formation of spherical particles predominates (Nicolai & Durand, 2013). In contrast, under conditions where electrostatic repulsions are high, i.e. at pH far from pI and at low ionic strength, the formation of linear aggregates is favored (Nicolai & Durand, 2013).

Improving the nutritional properties of a food can be achieved through a fundamental understanding of food structures and how that impacts digestion (Kaufmann & Palzer, 2011). Within the context of food proteins, mass spectrometry (MS) can be used for the quantification and characterization of peptides derived from the digestion of food proteins (Mamone, Picariello, Caira, Addeo, & Ferranti, 2009). Consequently, peptidomics, based on MS analysis, can be a powerful tool for providing information about how food proteins are digested in the gastrointestinal tract. Improvements in the sensitivity, mass accuracy and resolution of modern mass spectrometers have greatly increased the popularity of proteomics as an approach to describe protein digestion

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(Herrero, Simo, Garcia-Canas, Ibanez, & Cifuentes, 2012). However, the large volume of data generated by the MS analysis of protein digests makes the data analysis challenging and requires the use of sophisticated statistical approaches in order to interpret the results in a meaningful way (Bantscheff, Schirle, Sweetman, Rick, & Kuster, 2007).

Consequently, the present study aimed to explore the impact of the morphology of protein aggregates on the nature and relative concentration of the different generated peptides after digestion. This was done by using an *in vitro* digestion model that simulated digestion in the gastrointestinal tract and by using label-free quantitative peptidomics analysis and statistical multivariate data analysis. OVA was used as a model food protein.

2. Materials and methods

2.1. Chemicals and materials

Unless otherwise stated, chemicals were purchased from Sigma (St Louis, MO, USA). Ultrapure water was purified using a Milli-Q system (Millipore, Molsheim, France). Ovalbumin was purified from egg white using the method of Croguennec, Nau, Pezennec, and Brule (2000). The Q-Sepharose anion exchanger resin used for the latter purification was obtained from GE Healthcare Bio-sciences (Uppsala, Sweden). The purity of the purified OVA fraction was 87%, as determined by reversed-phase high-performance liquid chromatography (RP-HPLC) analysis and using detection by absorbance at 214 nm.

2.2. Heat-induced aggregation of OVA

The heat-induced aggregation of OVA was performed at 80 °C for 6 h using the method of Nyemb et al. (2014). Briefly, OVA was either unheated (non-aggregated control) or heated using four different combinations of pH and ionic strength to form a range of different protein aggregate morphologies. The aggregate morphologies obtained were linear (pH 9/ionic strength (IS) 0.03 M NaCl), linear-branched (pH 7/IS 0.03 M NaCl), spherical (pH 7/IS 0.3 M NaCl), or spherical-agglomerated (pH 5/IS 0.8 M NaCl).

2.3. In vitro digestion

The *in vitro* digestion protocol was previously described by Nyemb et al. (2014), based on an *in vitro* digestion model described by Dupont et al. (2010). The model was used to simulate gastric and intestinal digestion in the adult human. Briefly, simulated gastric digestion was performed by adding porcine pepsin (182 U per mg OVA) to simulated gastric fluid (SGF) at pH 2.5 containing 10 $mg \cdot ml^{-1}$ of OVA. Aliquots (1 ml) were taken over the 60 min gastric digestion period. Pepsinolysis was immediately stopped after sampling by raising the pH to 7.0 using 0.5 M ammonium bicarbonate. For simulated gastric plus small intestinal digestion, the gastric digestion phase was conducted as described above. After the gastric digestion phase, the pH of the reaction mixture was raised to 8.0 to irreversibly inactivate pepsin, and then adjusted to 6.5. After pH adjustment, bile salts and pancreatic enzymes were added to give final concentrations as follows: 0.0625 M sodium taurocholate, 0.065 M sodium glycodeoxycholate, 0.4 U bovine α-chymotrypsin per mg OVA and 34.4 U porcine trypsin per mg of OVA. Aliquots (1 ml) were taken from the reaction mixture over the 30 min small intestinal digestion period. Proteolysis was stopped by adding 0.06 mM soybean Bowmann–Birk trypsin–chymotrypsin inhibitor.

All digestions were carried out in duplicate. The digests were centrifuged at 10,000 *g* for 30 min (Microcentrifuge 5415C, Eppendorf, Hamburg, Germany), and the resulting supernatants filtered through a 0.2 μm membrane (Sartorius stedim biotech, Goettingen, Germany) to remove any particle matter.

2.4. Native OVA removal from the non-aggregated OVA digests

A RP-HPLC C4 214TP Vydac column (4.6 mm i.d. × 50 mm length, 3 μm particle size, 300 Å pore size; Grace, Lokeren, Belgium) was used to remove any undigested OVA remaining in the digests of the non-aggregated OVA as described by Nyemb et al. (2014). Peptides were separated using a gradient of 4.9 to 49% acetonitrile (ACN) over 17 min with a flow rate of 0.8 $ml \cdot min^{-1}$; peptides were detected using absorbance at 214 nm and 280 nm with a UV–Vis detector (Spectra Physics UV 100). A single fraction containing all the peptides present was collected prior to the elution of the undigested OVA. ACN was removed by evaporation using a SpeedVac Concentrator SVC100H centrifugal evaporator (Savant Instruments, Farmingdale, NY, USA). The collected peptide fraction was then diluted with deionized water to its original volume prior to further analysis by mass spectrometry.

2.5. Mass spectrometry

2.5.1. Identification of peptides

MS experiments were performed using a nanoRSLC Dionex U3000 system fitted to a Q Exactive mass spectrometer (Thermo Scientific, San Jose, USA) equipped with a nanoelectrospray ion source. A preliminary sample concentration step was performed on a μ-precolumn C18 pepMap100 (C18 column, 300 μm i.d. × 5 mm length, 5 μm particle size, 100 Å pore size; Dionex, Amsterdam, Netherlands). Separation was performed on a reversed-phase column (Easy Spray PepMap RSLC C18 75 μm i.d. × 150 mm length, 3 μm particle size, 100 Å pore size; Dionex) with a column temperature of 35 °C, using solvent A (2% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA in deionized water) and solvent B (95% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA in deionized water). Peptides were separated using a gradient of 4–60% solvent B over 44 min followed by 60–80% solvent B over 1 min and at a flow rate of 0.3 $\mu l \cdot min^{-1}$. Eluted peptides were directly electrosprayed into the mass spectrometer operating in positive ion mode with a voltage of 1.8 kV. The mass spectra were recorded in full MS mode using the range m/z 300–3000. The resolution of the mass analyzer for a m/z of 200 a.m.u. (atomic mass unit) was set to 70,000 in the acquisition method. For each scan, the ten most intense ions were selected for fragmentation. MS/MS spectra were recorded with a resolution of 17,500 at m/z of 200 Th and the parent ion was subsequently excluded from MS/MS fragmentation during 20 s. The instrument was externally calibrated according to the supplier's instructions. All samples were analyzed in triplicate.

Peptides were identified from MS/MS spectra using X!Tandem pipeline software (Plateforme d'Analyse Protéomique de Paris Sud-Ouest (PAPPSO), INRA, Jouy-en-Josas, France, http://pappso.inra.fr). The peptide identification database was an in-house database composed of major milk and egg proteins derived from www.uniprot.org (180 proteins in total). Database search parameters were specified as follows: a non-specific enzyme cleavage was selected; a 0.05 Da mass error was allowed on fragment ions while 10 ppm mass error was allowed for parent ions; phosphorylation of serine was selected as a variable modification; for each peptide identified, a minimum score corresponding to an e-value below 0.05 was considered to be a prerequisite for valid peptide identification.

2.5.2. Quantification of peptides

SIEVE 2.0 (ThermoFischer Scientific, San Jose, USA), a label-free mass spectrometry-based protein quantification software, was used to perform a semi-quantitative non-differential single class analysis of the identified peptides. This quantification method enables the comparison of the intensity values of a given peptide in different digests (relative concentration), but not the comparison of different intensity values of different peptides in the same digests. The chromatograms were time-aligned before the intensities of all identified peptides were measured. The framing parameters were set at 2 min for the retention time

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