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The extent of ovalbumin *in vitro* digestion and the nature of generated peptides are modulated by the morphology of protein aggregates

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ABSTRACT

The impact of heat-induced aggregation on the extent of ovalbumin digestion and the nature of peptides released was investigated using an *in vitro* digestion model. The extent of hydrolysis, estimated by the disappearance of intact ovalbumin and the appearance of soluble peptides, was greater for the linear aggregates as compared to the spherical aggregates. The latter result may be due to differences in the surface area to volume ratio of the aggregates, or the degree of unfolding of the proteins during aggregate preparation. Peptide identification using LC–MS/MS highlighted that ovalbumin aggregation rendered a number of peptide bonds accessible to digestive proteases which were not accessible in native ovalbumin. Moreover, the peptide bonds that were cleaved appeared to be specific depending on the morphology of the aggregates. This work illustrates the links existing between food structure and their breakdown during the digestive process. Such quantitative and qualitative differences may have important nutritional consequences.

1. Introduction

The desire to improve the nutritional quality of food products has, over recent years, led the food industry and public health policy authorities to reconsider the approaches used for nutritional evaluation of food (Turgeon & Rioux, 2011). Although nutrient composition has long been the main criteria for assessing the nutritional quality of foods, it is now recognized that the food matrix, and in particular the food microstructure, must also be an important consideration (Parada & Aguilera, 2007). Foods often undergo processing and storage prior to their consumption which can lead to changes in their physicochemical properties, which in turn can have consequences to the food structure overall and the subsequent digestion of foods within gastrointestinal tract. Such changes, whether they be changes in the extent of digestion or the rate of digestion, may impact the overall nutritional properties of food (Jiménez-Saiz, Ruiz-Henestrosa, López-Fandiño, & Molina, 2012). With respect to dietary protein, studies on meat and wheat proteins have shown that heat-induced aggregation can decrease protein digestibility, at least when tested using *in vitro* digestion models (Bax et al., 2012; Petitot et al., 2009).

Conformational changes triggered by heat can affect the folded structure of proteins (Weijers, Barneveld, Cohen Stuart, & Visschers, 2003). Moreover, once globular proteins are unfolded, various types of aggregate structures can form depending on the balance of attractive and repulsive interactions (Bryant & McClements, 1998). Moreover, the final aggregate morphology is a function not only of heat, but also a range of other physicochemical conditions such as pH, the type and quantity of added salt and the protein concentration (Nicolai & Durand, 2013). Heat-induced aggregation commonly occurs during the processing of globular food proteins.

Egg products, especially egg white proteins (EWP), are used extensively in food systems due to their unique functional (foaming, gelling, emulsifying) and nutritional properties (Mine, 1995; Mine & Zhang, 2013, chap. 5). The behavior of EWP is strongly influenced by ovalbumin (OVA) which is its major constituent. This globular protein (45 kDa) represents 54% of EWP by weight (Lechevalier, Croguennec, Nau, & Guérin-Dubiard, 2007). There has been a growing interest in the impact of processing on the gastrointestinal digestion of OVA, largely with respect to overall digestibility (Jimenez-Saiz, Pineda-Vadillo, Lopez-Fandino, & Molina, 2012;

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Joo & Kato, 2006; Oliveira et al., 2007; Takagi, Teshima, Okunuki, & Sawada, 2003). However, investigating protein aggregation as a means of modulating the course of the digestion has not yet been fully explored. Consequently, the present study aimed to investigate the impact of OVA aggregate structure on the extent of digestion and nature of the peptides generated after digestion. Purified OVA, rather than EWP per se, was used as a model protein system in order to simplify the interpretation of the results.

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 (OVA) 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, with detection by absorbance at 214 nm. Small amounts of several other egg white proteins, particularly ovotransferrin, were also observed in the purified OVA fraction.

2.2. Heat-induced aggregation of OVA

OVA (1 g) was dissolved in 50 ml of ultrapure water with 0.05% NaN_3 to prevent bacterial growth. The OVA was either non-aggregated (control), or was heated using four different combinations of pH and ionic strength to form a range of different protein aggregate morphologies (test). The four combinations used were pH 9/ionic strength (IS) 0.03 M NaCl, pH 7/IS 0.03 M NaCl, pH 7/IS 0.3 M NaCl and pH 5/IS 0.8 M NaCl. NaOH or HCl were used to adjust the pH. Each OVA solution was stirred at room temperature until complete protein dissolution was achieved. The test OVA solutions (25 ml) were then heated for 6 h at 80 °C in Pyrex test tubes (20 mm inner diameter) in a temperature-controlled water bath. After heating, the solutions were rapidly cooled by placing the tubes in ice-water. A 6 h heating time was used to ensure that OVA aggregation was virtually complete, since the presence of non-aggregated OVA in the aggregates sample would have been a confounding factor. The concentration of soluble native OVA before heat treatment was determined by measuring the absorbance at 280 nm and using a molar extinction coefficient for protein of 32,050 L mol^{-1} cm^{-1} (Hatta, Kitabatake, & Doi, 1986). The absence of soluble native OVA after heat treatment was tested by measuring the absorbance at 280 nm of the supernatant after centrifugation at 16,000*g* for 1 h (Beckman J2-21, Brea, CA, USA).

2.3. In vitro digestion model

The *in vitro* digestion model used in the present study simulated gastric and intestinal digestion in the adult human and was based on that of Dupont et al. (2010). Briefly, OVA samples (aggregated and non-aggregated) were dissolved in simulated gastric fluid (SGF; 0.15 M solution of NaCl adjusted at pH 2.5 with 1 M HCl) to obtain 21 ml of a solution containing 10 mg of protein per ml, after which the pH was adjusted to 2.5 with 1 M HCl. Porcine gastric mucosa pepsin (EC 3.4.23.1, Sigma, with a determined activity of 3294 U/mg of protein using haemoglobin as a substrate) was added at a concentration of 182 U of pepsin per mg OVA. Aliquots (1 ml) were taken over the 60 min gastric digestion period. For the *in vitro* digests simulating gastric digestion, pepsinolysis was stopped by raising the pH to 7.0 using 0.5 M ammonium bicarbonate. For the *in vitro* digests simulating gastric plus small intestinal digestion, the pH was raised to 8.0 after the gastric digestion phase by adding 1 M NaOH to irreversibly inactivate pepsin; the pH was then adjusted to 6.5 by adding 1 M HCl and 0.5 M bis-Tris pH 6.5. For the small intestinal digestion phase, several compounds were added to give final concentrations as follows: 0.0125 M bile salt mixture (0.0625 M sodium taurocholate, 0.065 M sodium glycodeoxycholate), 0.4 U bovine α-chymotrypsin per mg OVA (with a determined enzyme activity of 53 U/mg of protein using benzoyltyrosine ethyl esther as substrate) and 34.4 U porcine trypsin per mg of OVA (with a determined enzyme activity of 14,886 U/mg of protein using benzoylarginine ethyl esther as substrate). 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 the OVA samples were digested in duplicate.

2.4. Light scattering methods

Two different instruments were used to measure the particle size of the samples, due to the wide range of particle size across the different treatments.

Size distribution of pH 7/IS 0.3 M NaCl and pH 5/IS 0.8 M NaCl was determined by laser light scattering (LLS) using a Mastersizer 2000 (Malvern instruments, Malvern, UK) at 25 °C. The obscuration rate for measurements was similar between samples and was fixed at 9%. Samples were dispersed in 100 ml deionized water. The latter method assumes that the particles being measured are spherical. Since the aggregates examined in the presently reported study were not spherical, the particle size data presented in this report should be viewed as indicative only.

The particle size of non-aggregated OVA, pH 9/IS 0.03 M NaCl and pH 7/IS 0.03 M NaCl was measured using dynamic light scattering (DLS) at a set angle of 90° at 25 °C on a Zetasizer 3000HS (Malvern Instruments, Orsay, France), using an He–Ne laser, with a 633 nm wavelength. All samples were filtered prior to analysis to remove any insoluble material (0.8 μm, Acrodisc Syringe filter, Pall Life Science, MI, USA). Measurements were performed using 1.33 as the refractive index for water and 1.554 as the refractive index for the protein particles (Arakawa, Tuminello, Khare, & Milham, 2001). Each measurement was performed either in duplicate or in triplicate.

2.5. Transmission electronic microscopy (TEM)

The Transmission Electronic Microscopy (TEM) samples were prepared by negative staining. One drop of the diluted sample and another drop of 2% filtered (0.2 μm, Millipore, VWR International) uranyl acetate (Panreac, Barcelona, Spain) were deposited onto a plastic paraffin film. A glow-discharged copper grid was placed on the diluted sample drop and allowed to stand for 1 min, after which the excess sample aliquot was removed using filter paper. The copper grid was then placed onto the uranyl acetate drop, allowed to stand for 1 min, and any excess uranyl acetate was removed as described above. Electron micrographs were made using a JEM-1400 transmission electron microscope operating at 120 kV (JEOL, Tokyo, Japan).

2.6. SDS–PAGE analysis

SDS–PAGE analysis of the OVA aggregates and non-aggregated OVA was performed on precast 4-15% gradient polyacrylamide tris/HCl gel (Biorad, St Louis Mo., USA) according to the manufacturer's instructions. Protein samples were analysed in the presence