1. PUBLISHABLE SUMMARY

1.1 Summary description of the project objectives. The aim of the Marie Curie Action-EGGALL was to investigate whether microbial transglutaminase (mTG)-mediated modification can be used to reduce the biological properties of the proteins ovomucoid and ovalbumin present in egg white.

This proposal involved studies that provided important information about how to manage egg proteins hypersensitivity.

Through improved understanding of this effect, we developed strategies for enzymatically modifying the major egg allergens such as ovalbumin and ovomucoid with mTG to reduce the immunoreactivity of these proteins. To achieve this we required a multidisciplinary approach, bringing together the following key skills and expertise:

- Biochemistry skills and expertise were required to modify ovalbumin and ovomucoid using mTG and to evaluate the antigenicity (ability of a protein or a peptide to bind an antibody) and the allergenicity of the modified forms.
- Physiological expertise was required to define and develop accurate models of protein digestion which are representative of the *in vivo* situation. In particular models that are relevant to the infant gut where egg allergy is developed.
- Immunological skill to use both polyclonal and monoclonal antibodies to study the persistence of potential epitopes through simulated digestion.

1.2 Description of the work performed since the beginning of the project. In order to develop a realistic scenario of the physicochemical processes occurring during the mTG-mediated modification of egg white proteins, the project has involved close collaboration within a large interdisciplinary team of researchers with the fellow Concetta Valeria L. Giosafatto playing a pivotal role. Novel methodology has been developed to define the effects of enzyme modification on both the rheological and digestion properties of ovomucoid and ovalbumin.

At the host institution (IFR) Concetta Valeria L. Giosafatto has, in particular, carried out experiments of *in vitro* digestion under physiological conditions following the established IFR protocol. In fact, mTG-modified samples and the corresponding controls, treated in the absence of the enzyme, were submitted to *in vitro* digestion. An *in vitro* gastric-followed by duodenal digestion was performed with/without the physiological surfactant phosphatidylcholine (PC). The results demonstrate feasible approaches for modifying the proteins by means of the crosslinking enzyme to control (reduce) the rate of proteolysis and hence potentially induce physiological responses that could moderate food consumption.

1.3 Description of the main results achieved so far.

The project work initially looked at the modification of the egg white proteins, ovalbumin and ovomucoid, by means of the enzyme mTG. Before the enzymatic modification, the proteins have been firstly purified and their secondary structure characterized by means of Circular Dichroism (CD) and Infrared spectroscopy (FTIR). These studies were then extended to investigate the effects of the enzymatic modification digestion on both gastric and duodenal conditions.

R1.CD spectra showed that there was little change in the ovomucoid molecule upon heating, but, conversely, ovalbumin was dramatically affected. Also FTIR demonstrated that ovalbumin is influenced by the heating, in fact the IR spectra showed pronounced shift in band position of two major bands, namely the side chain vibration at 1652 cm^{-1} corresponding to α-helices and β–type band at 1691 cm^{-1}. Conversely, no marked changes in the ovomucoid band intensities were found, confirming the stable structure of this protein.

R2. Ovalbumin and ovomucoid are both able to act as acyl-donor and acyl-acceptor for the enzyme mTG. It was not possible to modify these proteins in their native state because of their quite compact structure. In fact, ovalbumin could be modified following heat treatment (1 hour at 80°C). Ovomucoid was subjected to mTG-mediated modification following addition of the reducing agent DTT. In fact, ovomucoid consists of three structurally independent tandem domains, each domain possessing three intra-domain disulphide bonds.

R3. Heat-treated ovalbumin is both intra- and inter-molecularly crosslinked by mTG and gives rise to different polymers as well as to modified forms of the protein having similar molecular weight but lower Stokes radius if compared to unmodified ovalbumin. In fact, on SDS-PAGE it is possible to observe a protein band that migrates faster with respect to unmodified ovalbumin. This is probably due to the changes in the protein hydrodynamic size resulting from intra-molecular crosslinks. Ovomucoid is just able to form, upon addition of 10 mM of DTT, inter-molecular crosslinks probably because of the presence of only one glutamine residue on its primary structure acting as acyl donor substrate for the enzyme mTG.

R4. The influence of covalent crosslinks by mTG on the sequential in *vitro* gastric and duodenal digestion process of ovalbumin and ovomucoid molecule was also investigated. SDS-PAGE analysis indicated that the crosslinking mainly affected *in vitro* gastric digestion pattern, while the duodenal digestion pattern was less affected.

R5. Rheological measurements have shown that mTG leads to the formation of egg protein gel with enhanced elasticity. The rate of development of the gel rigidity increases with increasing incubation time with the microbial enzyme. This observation suggests that the development of more solidlike three-dimensional networks during the incubation treatment resulted from the formation of ε-(γ-glutaminyl)lysine crosslinks in the proteins.

An other aspect that was studied in this project is the deglycosylation of ovomucoid molecule. In fact, ovomucoid possesses prominent carbohydrate domains, containing as much as 25% carbohydrates, present as oligosaccharides, each joined to the polypeptide chain by an asparagyl residue. In this part of the project Dr Giosafatto examines the enzymatic *in vitro* deglycosylation of ovomucoid using the enzyme Peptide-N4-(acetyl-β-glucosaminyl) asparagines amidase (PNGase F). Such enzyme is one of the most widely used enzymes for the removal of the N-linked glycans from glycoproteins (Xuan et al., 1998; Kuhn et al., 1995; Tarentino et al., 1974). This enzyme has a broader substrate specificity and hydrolyzes at the glycosylamine linkage and generates a carbohydrate-free peptide and an intact oligosaccharide with the di-N-acetylchitobiose unit at the reducing agent (Tarentino, 1986). This enzymatic release of N-glycans is also accompanied by the deamidation of asparagine residue to aspartic acid which provides an indirect indication of N-glycosylation sites of a glycoprotein (O'Neill, 1996). The efficiency of both heat and reducing agent on the extent of ovomucoid deglycosylation was evaluated. Deglycosylation of the protein was assessed on the basis of the shift in molecular weight associated with the sugar removal by SDS-PAGE and Western blot. The absence of carbohydrates from ovomucoid was also established by different analytical techniques such as Infrared Spectroscopy (FTIR), Gel Filtration (GF), Matrix Assisted Laser Desorption Ionisation Time-Of-Flight Mass Spectrometry (Maldi Tof-MS) and by Liquid Chromatography coupled with tandem mass spectrometry (LC-MS/MS). All these techniques have shown the capability of ovomucoid to be deglycosylated by PNGase F following the protein denaturation by both heat and reducing agent treatment.

References

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1.4 Expected final results, potential impact and use:

EGGALL has shown that not only is there a need for, but also real value in a fundamental biochemical and physical approach to studying this type of complex biological problem. Adopting this rigorous approach to elucidate generic questions has provided a more comprehensive understanding of these complex systems. The improved understanding will enable the rational design of new food types with different digestion and viscoelastic profiles. In fact, it has been demonstrated that the microbial enzyme affects both the digestion and rheological properties of egg white protein gels.

EGGALL has enabled the use of rheological tools to investigate the effect of mTG on the viscoelastic properties of protein gels. Secondly simulated digestion experiments under gastric and duodenal conditions were carried out on unmodified and mTG-modified samples. Furthermore, by using an enzymatic approach, based on the enzyme Peptide-N4-(acetyl-β-glucosaminyl) asparagines amidase (PNGase F), dr Giosafatto was able to full deglycosylate the ovomucoid protein. The study has shown that the heating treatment (100 °C for 1 h), the reduction with a sulfhydryl reagent, such as β-mercaptoethanol or dithiothreitol, and denaturation with a detergent greatly improves oligosaccharide release. The denaturation of the protein was followed by means of CD and FTIR. The important finding of a synergism between sulfhydryl reagent, surfactants and heating suggests that similar approaches could also be applied for the deglycosylation of different glycoproteins, of which the glycans are responsible of their immunological properties. This is generating renewed scientific interest due to the growing social and economic consequences of the allergy crisis in the developed world. The design of healthier foods to control the allergy is a top priority in the EU: it is estimated that treating allergy and related conditions takes up to 7% of the total healthcare budget within the EU. EGGALL has shown that fundamental science can reveal that an enzymatic approach, based on the enzyme microbial mTG can change the digestibility and rheological properties of proteins. Furthermore, EGGALL also demonstrated that an enzymatic approach by using PNGase F can remove glycans from proteins. The carbohydrate-depleted ovomucoid form was, in fact, characterized with different analytical techniques Applying physical and biochemical science principles to understanding fundamental processes such as protein deglycosylation and to modulate digestion and viscoelastic properties of complex food structures is a novel, emerging area of research.