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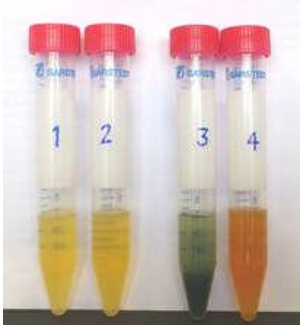
Pre-commercial scale-up of biologically active milk protein hydrolysates (FHI Project WP3)



Iron-binding (1, 2) by an *in vitro* gastric-stable peptide enriched fraction isolated from whey protein

Key external stakeholders:

This Industry-led, EI-funded Food for Health Ireland (FHI) project was co-funded by 4 major Irish dairy manufacturers Glanbia, Kerry, Carbery and Dairygold. The FHI project was governed by a consortium agreement drawn-up in conjunction with all participants which set out protocols for the uptake of results.

Practical implications for stakeholders:

Successful precommercial scale-up work at Moorepark retained bioactivity of FHI lead functional compounds (LFCs) i.e. enzymatically-produced milk protein hydrolysates and their sub-fractions in line with their original laboratory-based protocols, and also satisfied the microbiological specification necessary for formulation of the active ingredients in human clinical trial diets (undertaken by UCD).

- Pre-commercial scale-up contributed substantively towards the compilation of technological data which will be incorporated in scientific dossiers setting out health claims for individual LFCs to be submitted to the European Food Safety Authority (EFSA).
- In addition to the protocols and LFC's assigned by FHI, the pre-commercial scale-up team generated a novel casein-based hydrolysate and sub-fractions which was biologically active against multiple physiological functions (anti-inflammatory; endothelial and satiety-ghrelin)
- Technological developments employed to enrich biological activity during scale-up included advances in membrane separation technology e.g. charged- and electro-membrane based processes

Main results:

The following is a list of outputs accomplished by the FHI pre-commercial scale-up team:

No. protocols validated (laboratory):	150
No. plant scale-up trials:	50 (small) and 35 (large)
LFC's (Lead Functional Compounds)**:**	6 based on the MF025 hydrolysate series
ACR (Available Centre Result)**:**	1 (Hypoallergenic Infant Dessert)
NPD (Novel Product Development)**:**	3 (Family Milk & HA Infant Dessert)

Complementary research highlighted the benefits of protein aggregation-enhanced enzymatic hydrolysis.

Opportunity / Benefit:

Ground rules laid down in the FHI consortium agreement set out conditions for priority right of access by its Industry Partners to project outputs with commercial potential. Otherwise, expressions of interest in the scale-up and characterisation of FHI milk protein hydrolysates and their fractions will be entertained by the technology transfer officer. An FHI 'available centre result' (ACR) based on the novel formulation of a hypoallergenic infant food (desert-format) is currently licensed out for evaluation.

Collaborating Institutions:

University of Limerick, University College Dublin, University College Cork, Dublin City University

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Teagasc project team: Dr. Phil Kelly (PI)
Dr. Andre Brodkorb
Dr. Brian Murray
Mr. Ian O'Loughlin
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External collaborators: Prof. Dick Fitzgerald (UL)
Prof. Ted Dinan and Ms. Harriet Schellekens (UCC)
Prof. Torres Sweeney, Dr. Ashling Robinson and Dr. James Lyng (UCD)

1. Project background:

The Food for Health Ireland (FHI) project represents an alliance of academic and industry partners with the single goal of creating a critical scientific mass to test scientific hypotheses surrounding the preparation of bioactive peptides from milk, investigate them *in vitro*, assess the mechanism by which their effects are exerted and undertake clinical studies using animals and humans to confirm their efficacy.

FHI featured an intelligent milk mining programme and an extensive bioassay analytical platform to screen over 1000 peptides in order to identify those with biological activity. Out of this, a total of 75 lead functional compounds (LFCs) or bioactives with a potential health benefit were qualified. A select number of these LFCs were scaled-up up in Moorepark's pilot plant facilities under good manufacturing practice (GMP) in order to generate key ingredients for evaluation in clinical trials (UCD) in the course of completing scientific dossiers in accordance with the European Food Safety Authority's (EFSA) procedures for establishment of health claims.

FHI industry partners were actively engaged in the research throughout by providing guidance and ingredient substrates for mining and scale-up studies. The industry partners through their own market interactions were able to set out mission critical information concerning end-product specifications and techno-economic constraints to guide the technology transfer process.

2. Questions by the project:

Pre-commercial scale-up (WP3) was required to take charge of upscaling laboratory protocols for the preparation of milk protein hydrolysates (and where relevant, their sub-fractions) generated by FHI's milk mining laboratories. The principle obligation on the WP3 precommercial scale-up team was to transfer protocols faithfully in accordance with their original biological characterization while at the same time adapting technology to accomplish more sustainable processes without loss of activity. In terms of the overall project, the team's contribution in preparing LFC ingredients for clinical trial created another critical knowledge input in the chain of event that complete the framing of individual health claims.

3. The experimental studies:

Replication of laboratory protocols at Moorepark required different scales of reactors for the initial hydrolysis step in order to confirm hydrolysis conditions and rates in collaboration with the bioassay testing platform. HPLC analyses provided a profile of the hydrolysate peptide mixture. Fractionation of hydrolysates was undertaken by means of membrane filtration technologies with membranes ranging in molecular weight cut-off (MWCO) from 50 kDa down to 1 kDa. These membrane separation steps were also scalable from laboratory to large pilot-plant.

Selective heat-treatments of enriched fractions of individual whey proteins and whey protein isolate(s) (WPI) were undertaken to evaluate their effects on aggregation behaviour and subsequent susceptibility to hydrolysis. This was examined at both a sub-molecular and macro-molecular level in line with varying degrees of hydrolysis (DH).

Selected hydrolysis processes of heat denatured/aggregate proteins were successfully scaled-up in the pilot plant, and incorporated successive membrane filtration steps (cascade membrane fractionation) to produce a range of spray dried hydrolysate fractions with altered molecular weight (Mw) distributions and bio-functional characteristics. This enabled partition of both the iron chelating and the angiotensin-I-converting enzyme (ACE) inhibitory properties in both control and heat-treated systems.

4. Main results:

Of 150 validation tests undertaken at laboratory level on protocols received, 50 were progressed to small

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pilot scale processing (<5 kg dried hydrolysate ingredient output) from which 35 were taken to large pilot scale preparation and spray drying (<100 kg).

Developments in separation technologies e.g. use of electrofocussing, and charged membranes were key to the characterisation of bioactive peptides at approx. 1,000 Da. FTIR (Fourier transformed infra-red) technology provided some insights into the conformation changes taking place in whey protein structure. Aggregation-enhanced hydrolysis (Figure 1) not alone improved kinetics but also shaped final outcomes e.g. hydrolysis of denatured WPI favoured the generation of higher levels of free essential amino acids; lysine, phenylalanine and arginine compared to the unheated substrate. Distinct peptides release from the heat-treated system were mapped to parent molecules and theoretically attributed to certain endo-protease activities. The heat pre-treated substrates, which exhibited increased viscosity and surface hydrophobicity, demonstrated significantly increased ($P < 0.001$) hydrolysis rates with the enzymatic preparation Corolase® PP. The proteinaceous components were hydrolysed in the order: CMP > β-Lg A > β-Lg B > α-La. Hydrolysates (5 %DH) revealed an increase in soluble molecular weight (Mw) material greater than 30 kDa which was discerned to be material derived mainly from 1Leu-Arg40, 70Lys-Phe82 and 140Leu-Met145 regions of β-Lg and the 1Met-Ile20 region of CMP.

Strong iron-binding peptides were also identified which were found to be *in vitro* gastric stable. A positive correlation ($P < 0.01$) was established between the average Mw of fractions and ferrous (Fe^{2+}) chelating capability. Upon solid phase extraction these fractions possessed high total concentrations of the basic amino acids and possessed ferrous chelation equivalent to 84.4 µM EDTA. The strongest ACE inhibitory fractions were the 1 kDa permeates of both control and prior heat-treated WPI process streams (activity as IC50 = 0.17 g L-1). Isoelectric focussing (IEF) of the hydrolysate fraction further increased ACE-inhibition in fractions collected within the pH range 6.1 – 6.6.

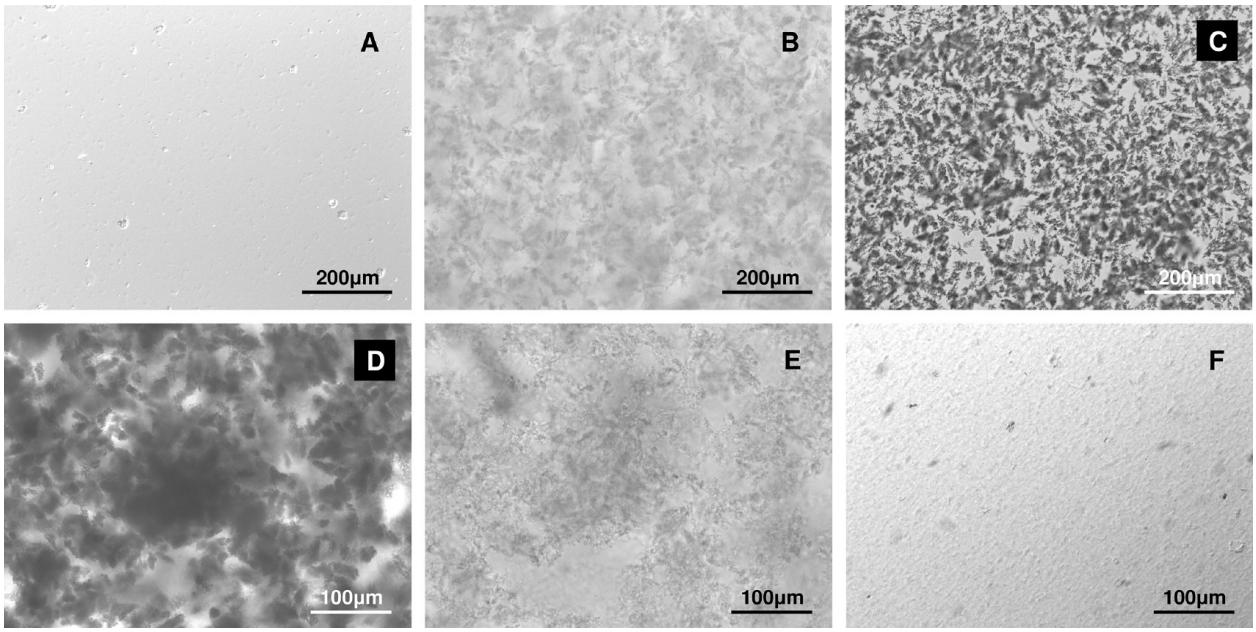

Figure. 1: Confocal laser micrographs of heat-induced aggregation of whey protein isolate with increasing intensity (frames A, B, C) and aggregate disintegration during the course of enzymatic hydrolysis (frames D, E, F) with accompanying reduction in mean particle size. Source: National Food Imaging Centre (Dr. Mark Auty)

5. Opportunity/Benefit:

A hydrolysate preparation (MF 025) developed by the Moorepark WP3 team generated multiple biological hits i.e. anti-inflammatory, endothelial function and satiety / ghrelin activation. Six LFC's were produced in the course of sub-fractionating the parent hydrolysate – further study is underway to underpin IP claims (Teagasc/UCC collaboration) which are in the course of being established for the fraction responsible for ghrelin activation. The process for enrichment of iron-binding peptides may be a possible contender for commercial evaluation. The IP offer of this know-how would need to be firstly made to the FHI industry partner before being put on general release.

6. Dissemination:

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A commercialisation protocol within the FHI consortium agreement sets out rules whereby the industry partners have first opportunity to express interest in evaluating project outputs in conjunction with their business interests for a limited time period.

Main publications:

Gaudel C, Nongonierma AB, Maher S, Flynn S, Krause M, Murray BA, Kelly PM, Baird AW, FitzGerald RJ, Newsholme P. (2013) A whey protein hydrolysate promotes insulinotropic activity in a clonal pancreatic β-cell line and enhances glycemic function in ob/ob mice. *J Nutr.*143:1109-14. doi: 10.3945/jn.113.174912

O'Loughlin, I.B., Murray, B.A., Kelly, P.M., FitzGerald, R.J. and Brodkorb, A. (2012). Enzymatic hydrolysis of heat-induced aggregates of whey protein isolate. *Journal of Agricultural and Food Chemistry* 60: 4895 - 4904.

O'Loughlin, I.B., Murray, B.A., Brodkorb, A, FitzGerald, R.J. and Kelly, P.M. (2014a). Pilot-scale production of hydrolysates with altered bio-functionalities. *International Dairy Journal* 34:146 - 152.

O'Loughlin, I.B., Murray, B.A., FitzGerald, R.J., A. Brodkorb, Robinson, A.A, Holton, T.A. and Kelly, P.M. (2013). Whey protein isolate polydispersity affects enzymatic hydrolysis outcomes. *Food Chemistry* 141: 2334 - 2342.

O'Loughlin, I.B., Murray, B.A., Brodkorb, A., FitzGerald, R.J. & Kelly, P.M. (2014b). Production of whey protein isolate hydrolysate fractions with enriched ACE-inhibitory activity. *International Dairy Journal* 38, 101 – 103.

O'Loughlin, I.B., (2014) Enzymatic hydrolysis of heat-denatured whey proteins. PhD thesis. University of Limerick

Popular publications:

Ian O'Loughlin, PhD Walsh Fellow, attached to the project was the recipient of the following awards during the period 2010-13 for his research achievements:

2010: Young Researcher winner of ***Travel Grant for attendance at the European PhD Conference*** on Food Science & Technology, Berlin
2011: Annual Food Science & Technology Conference, UCC – Best Student Presentation
2012: University of Limerick, Department of Life Sciences Annual Student Conference – Best Student Presentation
2012: Winner of the Eamonn P. McCormick medal for Best Student Poster presented at the **Society of Dairy Technology Conference** UCC, September 2013
2013: Shortlisted for Young Scientist Award at the 8th NIZO Dairy Conference

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