Gastrointestinal Endogenous Proteins as a Source of Bioactive Peptides

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Abstract

Gastrointestinal endogenous proteins (GEP) were investigated as a source of bioactive peptides. *In silico* and *in vitro* methods were used singly or in combination to study GEP-derived peptides after simulated digestion. The presence of bioactive peptides after *in vivo* digestion was determined using a porcine model. Bioactivity of the peptides was assessed using selected *in vitro* bioactivity assays, and peptides were characterised using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry.

In the *in silico* study, twenty six different GEP and seven dietary proteins were subjected to simulated *in silico* gastrointestinal (SIGIT) digestion. The predicted resultant peptides possessing amino acid sequences identical to those of known bioactive peptides were identified by screening them against an online database of bioactive peptides (BIOPEP). The predicted number of bioactive peptides released after the SIGIT digestion of GEP ranged from 1 (secretin) to 39 (mucin-5AC), while those for dietary proteins ranged from 1 (gliadin) to 55 (myosin). Angiotensin-I-converting enzyme (ACE-I) inhibitory peptide sequences were found in abundance in both GEP and dietary proteins. The GEP mucin-5AC and the dietary protein myosin were predicted to release the highest number of ACE-I inhibitory peptides (38 and 49 peptides respectively), and were found to be comparable in their potential to release ACE-I inhibitory peptides.

Following SIGIT digestion of eleven representative GEP, nineteen novel GEP-derived peptide sequences were selected by applying quantitative structure-activity relationship rules, and were chemically synthesised. Two novel peptides with the

amino acid sequences RPCF and MIM, showing dipeptidyl peptidase IV (DPP-IV) inhibitory activity and five novel antioxidant (2,2-diphenyl-1-picrylhydrazyl (DPPH)- inhibitory and, or ferric reducing antioxidant power (FRAP) activity) peptides with amino acid sequences CCK, RPCF, CRPK, QQCP and DCR were identified. These results indicate that GEP may contain novel bioactive peptide sequences.

The potential release of bioactive peptides, from four GEP (trypsin, lysozyme, mucin, and serum albumin) and a dietary protein (chicken albumin), in the gastrointestinal tract (GIT) was investigated using an *in vitro* digestion model. The *in vitro* digests were screened for ACE-I-, renin-, platelet-activating factor acetylhydrolase (PAF-AH)-, and DPP-IV-inhibition, and antioxidant activity. All four *in vitro* GEP digests showed ACE-I inhibition comparable to that of the positive control captopril. In comparison to the unfractionated digests, the enriched fractions (<3 and <10 kDa) of lysozyme and serum albumin showed greater renin-, PAF-AH-, and DPP-IV-inhibition, and antioxidant potential. Over 190 peptide sequences were identified from these fractions using mass spectrometry.

Stomach chyme (SC) and jejunal digesta (JD) were collected from growing pigs that were fed a protein-free diet for a period of 3 days. The peptides extracted from SC and JD samples were characterized by SDS-PAGE, and their ACE-I-, DPPH-, and microsomal lipid peroxidation (MLP)- inhibition, FRAP activity determined. Potential bioactive peptides responsible for bioactivity were identified using mass spectrometry. SDS-PAGE analysis showed that all of the samples contained a heterogeneous mixture of peptides. Porcine JD samples inhibited ACE-I and DPPH, while SC samples inhibited MLP. Characterization studies identified over 180

peptide sequences from the enriched fractions of SC and JD samples that showed the highest activity. Further, a porcine serum albumin peptide sequence (FAKTCVADESAENCDKS) was found to be a sub-sequence of a larger sequence identified in the *in vitro* digest of human serum albumin. There was considerable inter-animal variation for the bioactivities. This may be attributed to sampling effects and, or natural variations in the gut contents, thus underlining the complexity involved in *in vivo* release of bioactive peptides.

Together, the results indicate: 1) GEP contain abundant encrypted bioactive peptide sequences; 2) GEP-derived bioactive peptides display a range of bioactivities; 3) GEP-derived bioactive peptides are released during gastrointestinal digestion in pigs; 4) GEP may contain numerous novel bioactive peptide sequences encoded within their primary sequence.

In conclusion, the evidence reported here suggests that, like the dietary proteins, GEP are also a potentially rich source of exogenously-derived bioactive peptides in the gastrointestinal tract. Beyond their primary functions, GEP may act as an important cryptomic source of bioactive peptides, given that the amount of GEP secreted into the gut is equal to or greater than the dietary protein ingested per day, and that up to 80% of GEP are known to be digested.

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Contents

Abstract ..... **I**

Acknowledgements ..... **V**

Abbreviations ..... **XIX**

List of Figures ..... **XXI**

List of Tables ..... **XXVII**

List of Publications ..... **XXXI**

1. Introduction **1**

2. Review of Literature **7**

2.1. Introduction 8

2.1.1. Classification of bioactive peptides *8*

2.2. GIT: A system of peptide-mediated physiological regulation 14

2.2.1. Immunomodulatory system *14*

2.2.2. GIT microbiota *16*

2.2.3. Regulatory peptides of the enteric nervous system (ENS) *17*

2.2.4. The Renin Angiotensin Aldosterone System (RAAS) *18*

2.2.5. Peptide-mediated antioxidant activity *21*

2.2.6. Peptide-mediated inhibition of advanced glycation end products *23*

2.3. Food protein-derived ExBP 25

2.4. GEP: An unexplored source of ExBP 31

2.4.1. Major GEP secretions *31*

2.4.2. Other GEP proteins *36*

2.4.3. Non-secretory components contributing to GEP *37*

2.4.4. Effect of dietary nutrients on GEP secretion *39*

2.5. Current strategies for the investigation of bioactive peptides 43

2.5.1. In silico investigation of bioactive peptides *43*

2.5.2. In vitro investigation of bioactive peptides *45*

2.5.3. In vivo investigation of bioactive peptides *47*

2.6. Objectives 50

2.6.1. Experimental approach *51*

2.6.2. Experimental techniques *52*

3. Gastrointestinal Endogenous Proteins as a Source of Bioactive Peptides - An *In silico* Study 55

3.1. Abstract 56

3.2. Introduction 57

3.3. Materials and Methods 59

3.3.1. Materials *59*

3.3.2. Prediction of the total number of bioactive peptide sequences present in the intact gut endogenous and dietary proteins *62*

3.3.3. Prediction of the total number of bioactive peptides to be released from the digestion of GEP *64*

3.4. Results 65

3.4.1. The total number and frequency (A_O) of bioactive peptide sequences within the amino acid sequence of intact gut endogenous proteins and intact dietary proteins 65

3.4.2. Predicted number and frequency (AD) of bioactive peptides released after gastric digestion of dietary proteins and gut endogenous proteins based on an in silico simulation..... 76

3.4.3. Predicted number and frequency (AD) of bioactive peptides released after gastric and small intestinal digestion of dietary proteins and gut endogenous proteins based on an in silico simulation 80

3.4.4. Predicted number and frequency (AD) of bioactive peptide sequences released after small intestinal digestion of gut endogenous proteins secreted in the small intestine based on an in silico simulation 85

3.5. Discussion..... 88

4. Novel Dipeptidyl Peptidase IV Inhibitory and Antioxidant Peptides Derived from Human Gastrointestinal Endogenous Proteins 101

4.1. Abstract..... 102

4.2. Introduction 102

4.3. Materials and Methods 104

4.3.1. Materials and Reagents 104

4.3.2. Gastrointestinal endogenous proteins (GEP) 105

4.3.3. Preliminary in silico analysis 105

4.3.4. Chemical synthesis of selected peptides..... 108

4.3.5. DPP-IV inhibition 109

4.3.6. DPPH inhibition 110

4.3.7. FRAP bioassay 110

4.3.8. Statistical analysis 111

4.4. Results 111

4.5. Discussion 125

5. Gastrointestinal Endogenous Protein-Derived Bioactive Peptides: An *in vitro* Study of Their Gut Modulatory Potential 141

5.1. Abstract 142

5.2. Introduction 143

5.3. Materials and Methods 145

5.3.1. Materials and Reagents 145

5.3.2. Simulated gastrointestinal digestion of selected gastrointestinal endogenous proteins (GEP) and a food protein comparator 146

5.3.3. Total protein analysis 149

5.3.4. Tris-tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis 149

5.3.5. ACE-I inhibition assay 150

5.3.6. Renin inhibition assay 150

5.3.7. PAF-AH inhibition assay 151

5.3.8. DPP-IV inhibition 151

5.3.9. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)-based total antioxidant capacity (ABTS-TAC) assay 151

5.3.10. DPPH inhibition 152

5.3.11. Electrospray ionization time of flight mass spectrometry (ESI-TOF MS) Characterisation of peptides present in the most active fractions..... 152

5.4. Results 154

5.4.1. Characterization of digests by tris-tricine SDS-PAGE..... 154

5.4.2. Angiotensin-I converting enzyme (ACE-I) inhibition by digests and enriched fractions 156

5.4.3. Renin inhibition by digests and enriched fractions..... 159

5.4.4. Platelet-activating factor-acetylhydrolase (PAF-AH) inhibition by digests and enriched fractions 161

5.4.5. Dipeptidyl peptidase-iv inhibitory (DPP-IV) inhibition by digests and enriched fractions 163

5.4.6. 2,2'-Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid total antioxidant capacity (ABTS-TAC) of digests and enriched fractions 165

5.4.7. DPPH inhibition by digests and enriched fractions 167

5.4.8. Electrospray ionisation time of flight mass spectrometry (ESI-TOF-MS) characterisation of peptides..... 169

5.5. Discussion..... 183

6 Identification of Bioactive Peptides Derived from the Gastrointestinal Endogenous Proteins of Growing Pigs 193

6.1 Abstract..... 194

6.2 Introduction 195

6.3 Materials and methods 197

6.3.1 Materials and Reagents 197

6.3.2 Test diet 197

6.3.3 Experimental design and sample collection 199

6.3.4 Ammonium sulphate precipitation of proteins 200

6.3.5 Total protein analysis 200

6.3.6 Tris-tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis 200

6.3.7 ACE-I inhibition assay 201

6.3.8 FRAP value 201

6.3.9 DPPH inhibition 201

6.3.10 MLP inhibition 202

6.3.11 Electrospray ionization time-of-flight liquid chromatography tandem mass spectrometry (ESI-TOF LC-MS/MS)-based characterisation of peptides 203

6.3.12 Data analysis 205

6.4 Results 207

6.4.1 SDS-PAGE analysis 207

6.4.2 ACE-I inhibition 209

6.4.3 Antioxidant potential 210

6.4.4 ESI-TOF LC-MS/MS characterisation of peptide sequences 213

6.5 Discussion 237

7. Overall Conclusions and Avenues for Future Work 245

7.1. Introduction 246

7.2. Summary of results 246

7.2.1. In silico approach to identify potential bioactive sequences 246

7.2.2. Identification of novel bioactive peptides using in silico digestion, de novo chemical synthesis and in vitro bioactivity assays 248

7.2.3. GEP-derived bioactive peptides: an in vitro digestion study 249

7.2.4. Identifying potential bioactive peptides from porcine GEP: an in vivo study 250

7.3. General discussion of results 252

7.3.1. Classification of bioactive peptides 252

7.3.2. Bioactive potential of GEP is comparable to that of dietary proteins . 253

7.3.3. Digestion of GEP may generate numerous peptides with multiple bioactivities and, or novel sequences 255

7.3.4. Generation of bioactive peptides from GEP in in vivo systems 259

7.3.5. Gut microbiota influences generation of bioactive peptides 260

7.3.6. Epithelial cells as a source of bioactive peptides in the GIT 262

7.3.7. Structural aspects of proteins/peptides governing digestion and subsequent bioactivity 262

7.3.8. Safety and toxicity of GEP-derived bioactive peptides 265

7.3.9. Some important considerations in the study of GEP- and dietary protein-derived bioactive peptides 265

7.4. Conclusions 267

7.5. Avenues for further work 268

8. Bibiology 271

9. Appendices 327

9.1. Statements of contribution to doctoral thesis containing publications 327

9.2. Copyright permissions for adaptations of publications into thesis chapters . 329

9.3. Copies of publications 331

Abbreviations

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

ACE-I: Angiotensin-I-converting enzyme

CaMPDE: Cyclic nucleotide phosphodiesterase 1

DPPH 2,2-Diphenyl-1-picrylhydrazyl

DPP-IV: Dipeptidyl peptidase IV

ENL: Endogenous nitrogen losses

ENS: Enteric nervous system

ESI-TOF LC-MS/MS: Electrospray ionization time-of-flight liquid chromatography tandem mass spectrometry

ESI-TOF MS: Electrospray ionization time-of-flight mass spectrometry

ExBP: Exogenous bioactive peptides

FAO (UN FAO): Food and agriculture organisation of the United Nations

FRAP Ferric reducing antioxidant power

GALT: Gut-associated lymphoid tissue

GEP: Gut endogenous proteins

GIT: Gastrointestinal tract

GLP-2: Glucagon-like peptide-2

MAFP: Methyl arachidonyl fluorophosphonate

MLP inhibition: Microsomal lipid peroxidation inhibition

MWCO: Molecular weight cut-off

NOD: Nucleotide-binding oligomerization domain-like receptors

PAF-AH: Platelet-activating factor acetylhydrolase

QS: Quorum sensing

QSAR: Quantitative structure-activity relationship

RAAS: Renin angiotensin aldosterone system

RFU: Relative fluorescence units

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SIGIT digestion: Simulated *in silico* gastrointestinal digestion

TEnBP: Truly endogenous bioactive peptides

WPI: Whey protein isolate

List of Figures

Figure 1.1 Overview of chapters in the dissertation **5**

Figure 2.1 Schematic representation of the types of bioactive peptides that are known to date. The bioactive peptides derived in the body have been grouped as the truly endogenous bioactive peptides (TEnBP), while those generated in the GIT lumen are grouped as exogenous bioactive peptides (ExBP). **13**

Figure 2.2 The GIT: a complex system relating to numerous regulatory systems ... **15**

Figure 2.3 The potential role of cryptome-derived bioactive peptides in the Renin-Angiotensin-Aldosterone System (RAAS) **19**

Figure 2.4 The major contributors to the potential gut cryptome **33**

Figure 4.1 Schematic representation of the combination of methodologies used for discovery and generation of dipeptidyl peptidase IV (DPP-IV) inhibitory and antioxidant peptides from human gastrointestinal endogenous proteins (GEP). **107**

Figure 4.2 The *in vitro* DPP-IV inhibitory activity (mean ± SD) of select chemically synthesised peptides tested at a sample concentration of 1 mg/mL. Sitagliptin was used as a positive control and was also tested at a concentration of 100 μM. **121**

Figure 4.3 The *in vitro* DPPH inhibition activity (mean ± SD) of selected peptides tested at a concentration of 1 mg/mL. Resveratrol was used as a positive control and was also tested at a concentration of 1 mg/mL. **123**

Figure 4.4 Mean FRAP values (mean ± SD) of selected synthetic peptides. The values are expressed as the Trolox equivalents (μM per mg of peptide). **..... 125**

Figure 5.1 The *in vitro* experimental design and methodologies used to study the angiotensin-I converting enzyme (ACE-I), renin, Platelet-activating factor-acetylhydrolase (PAF-AH) and dipeptidyl peptidase IV (DPP-IV) inhibition, and Total antioxidant capacity by 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl -1-picrylhydrazyl (DPPH) inhibition based antioxidant activity of the gastric and small intestinal hydrolysates and the small intestinal <10 and <3 kDa fractions of gastrointestinal endogenous proteins porcine trypsin, porcine salivary mucin, human recombinant lysozyme and serum albumin, and food protein chicken albumin. SDS-PAGE: Tris-tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis; ESI-TOF MS/MS: Electrospray ionization time of flight mass spectrometry.**.... 148**

Figure 5.2 Tris-tricine SDS-PAGE, of the *in vitro* gastric and small intestinal digests of the gastrointestinal endogenous proteins, porcine trypsin (TRYP), human lysozyme (LYS), porcine salivary mucin (MUC) and human serum albumin (SA), and dietary protein chicken albumin (CA) conducted under reducing conditions. Each protein was analyzed prior to hydrolysis (U) and after gastric (G) and small intestinal digestion (G + SI).**..... 156**

Figure 5.3 Angiotensin-I converting enzyme (ACE-I) inhibition by (A) gastric (G) and small intestinal digests (G + SI) of the gastrointestinal endogenous proteins, porcine trypsin (TRYP), human lysozyme (LYS), porcine

salivary mucin (MUC) and human serum albumin (SA), and dietary protein chicken albumin (CA); and (B) <10 and <3 kDa fractions of the small intestinal digests. All of the samples and the positive control captopril were tested at the concentration of 1 mg/mL. **158**

Figure 5.4 Renin inhibition by (A) gastric (G) and small intestinal digests (G + SI) of the gastrointestinal endogenous proteins, porcine trypsin (TRYP), human lysozyme (LYS), porcine salivary mucin (MUC) and human serum albumin (SA), and dietary protein chicken albumin (CA); and (B) <10 and <3 kDa fractions of the small intestinal digests. All of the samples were tested at the concentration of 1 mg/mL. The positive control Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-Ome was tested at 10 μM. **160**

Figure 5.5 Platelet-activating factor-acetylhydrolase (PAF-AH) inhibition by (A) gastric (G) and small intestinal digests (G + SI) of the gastrointestinal endogenous proteins, porcine trypsin (TRYP), human lysozyme (LYS), porcine salivary mucin (MUC) and human serum albumin (SA), and dietary protein chicken albumin (CA); and (B) <10 and <3 kDa fractions of the small intestinal digests. All of the samples were tested at the concentration of 1 mg/mL. The positive control methyl arachidonyl fluorophosphonate (MAFP) was tested at 260 ng/mL.....**162**

Figure 5.6 Dipeptidyl peptidase IV (DPP-IV) inhibition by (A) gastric (G) and small intestinal digests (G + SI) of the gastrointestinal endogenous proteins, porcine trypsin (TRYP), human lysozyme (LYS), porcine salivary mucin (MUC) and human serum albumin (SA), and dietary protein chicken albumin (CA); and (B) <10 and <3 kDa fractions of the

small intestinal digests. All of the samples were tested at the concentration of 1 mg/mL. The positive control sitagliptin was tested at 100 μM. **164**

Figure 5.7 Total antioxidant capacity by 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) inhibition by (A) gastric (G) and small intestinal digests (G + SI) of the gastrointestinal endogenous proteins, porcine trypsin (TRYP), human lysozyme (LYS), porcine salivary mucin (MUC) and human serum albumin (SA), and dietary protein chicken albumin (CA); and (B) <10 and <3 kDa fractions of the small intestinal digests. All of the samples and the positive control resveratrol were tested at the concentration of 1 mg/mL and the results are expressed in μM trolox equivalents (TE). **166**

Figure 5.8 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition by (A) gastric (G) and small intestinal digests (G + SI) of the gastrointestinal endogenous proteins, porcine trypsin (TRYP), human lysozyme (LYS), porcine salivary mucin (MUC) and human serum albumin (SA), and dietary protein chicken albumin (CA); and (B) <10 and <3 kDa fractions of the small intestinal digests. All of the samples and the positive control resveratrol were tested at the concentration of 1 mg/mL. **168**

Figure 6.1 The *in vivo* and *in vitro* experimental design and methodologies used to study the generation of porcine gastrointestinal endogenous protein (GEP)-derived bioactive peptides in the stomach chyme and jejunal digesta of pigs, following gastrointestinal (GIT) digestion of a protein free diet. **206**

Figure 6.2 Tris-tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the dialysed protein and peptide fractions precipitated from stomach chyme (SC1, SC2, and SC3) and jejunal digesta (JD1, JD2 and JD3) samples obtained from the growing pigs. **207**

Figure 6.3 ACE-I (Angiotensin-I converting enzyme) inhibition for the dialysed protein and peptide fractions precipitated from stomach chyme (SC1, SC2, and SC3) and jejunal digesta (JD1, JD2 and JD3) samples each obtained from three growing pigs and for the positive control captopril. **.....209**

Figure 6.4 FRAP (Ferric reducing antioxidant potential) for the dialysed protein and peptide fractions precipitated from stomach chyme (SC1, SC2, and SC3) and jejunal digesta (JD1, JD2 and JD3) samples each obtained from three growing pigs. **.....211**

Figure 6.5 DPPH (2,2-diphenyl-1-picrylhydrazyl) inhibition for the dialysed protein and peptide fractions precipitated from stomach chyme (SC1, SC2, and SC3) and jejunal digesta (JD1, JD2 and JD3) samples each obtained from three growing pigs and for the positive control (resveratrol). **.....212**

Figure 6.6 MLP (Microsomal lipid peroxidation) inhibition for the dialysed protein and peptide fractions precipitated from stomach chyme (SC1, SC2, and SC3) and jejunal digesta (JD1, JD2 and JD3) samples each obtained from three growing pigs and from the positive control resveratrol. **.....213**

List of Tables

Table 2.1 Examples of recently discovered bioactive peptides from dietary proteins or the food peptidome that are predicted to be released after simulated *in silico* or *in vitro* gastrointestinal tract (GIT) digestion **.....28**

Table 2.2 Overview of experimental techniques used in the study reported here **....52**

Table 3.1 Gut endogenous and dietary proteins examined in the *in silico* study[1] **....59**

Table 3.2 Number (#) of potential bioactive peptides (per protein molecule) identified in the intact endogenous and dietary proteins and the A_O[1] and Y[2] values **.....67**

Table 3.3. Number of potential bioactive peptides (per protein molecule) predicted to be released and A_D[1] value after gastric digestion of both gut endogenous proteins secreted in the mouth and stomach and selected dietary proteins based on an *in silico* digestion model **.....78**

Table 3.4 Number of potential bioactive peptides (per protein molecule) predicted to be released and A_D[1] value after gastric plus small intestinal digestion for gut endogenous proteins secreted in the mouth and stomach and selected dietary proteins based on an *in silico* digestion model **.....81**

Table 3.5 Number of potential bioactive peptides (per protein molecule) predicted to be released and A_D[1] value after small intestinal digestion for gut endogenous proteins secreted in the small intestine based on an *in silico* digestion model **.....86**

Table 3.6 Amino acid[1] sequences of bioactive peptides predicted to be released after mouth to ileum digestion of selected proteins based on an *in silico* digestion model. **.....95**

Table 3.7 Predicted quantity of bioactive peptides (mg/d) released after digestion of either dietary proteins or gut endogenous proteins in the gastrointestinal tract..... **98**

Table 4.1 Details of human gastrointestinal endogenous proteins (GEP) studied in the *in silico* analysis **112**

Table 4.2 The number of known DPP-IV inhibitory peptides and antioxidant peptides predicted to be released after simulated *in silico* gastrointestinal (SIGIT) digestion of selected GEP. **114**

Table 4.3 Molecular weight (MW) and PeptideRanker scores of peptides selected for chemical synthesis and *in vitro* bioassays. **117**

Table 4.4 The IC_{50} values for the two most active synthetic peptides among the total 19 peptides, RPCF and MIM. **121**

Table 4.5 Estimated IC_{50} (± SD) of the antioxidant peptides CCK, RPCF and CRPK. **123**

Table 4.6 Peptide subsequences matching with fragments within the intact novel peptides investigated in the present study (Data collated from BIOPEP (BIOPEP, 2015; Minkiewicz et al., 2008b). **127**

Table 5.1 Gastrointestinal endogenous proteins (GEP) and dietary protein examined in the *in vitro* study**.** **147**

Table 5.2 The protein content of the *in vitro* gastric (G) and small intestinal digests (G + SI) of the gastrointestinal endogenous proteins, porcine trypsin (TRYP), human lysozyme (LYS), porcine salivary mucin (MUC) and human serum albumin (SA), and dietary protein chicken albumin (CA). **155**

Table 5.3 Gastrointestinal endogenous proteins (GEP)-derived peptide sequences found in the <3 and <10 kDa fractions of the *in vitro* small intestinal digests of lysozyme and serum albumin. The samples were analyzed using electrospray ionization time of flight mass spectrometry (ESI-TOF MS). **.....170**

Table 6.1 Ingredient composition of the basal and experimental diet (g/kg dry matter)**.....198**

Table 6.2 The protein content of the dialysed protein and peptide fractions precipitated from stomach chyme (SC1, SC2, and SC3) and jejunal digesta (JD1, JD2 and JD3). **.....208**

Table 6.3 Parent gastrointestinal endogenous proteins (GEP) that released the peptide sequences that were identified from the dialysed protein and peptide fractions precipitated from stomach chyme (SC) and jejunal digesta (JD) of growing pigs fed a protein-free diet. The samples were analysed using electrospray ionization time-of-flight liquid chromatography tandem mass spectrometry (ESI-TOF LC-MS/MS)..**215**

Table 6.4 Gastrointestinal endogenous protein (GEP)-derived peptide sequences that were identified from the dialysed protein and peptide fractions precipitated from stomach chyme (SC3) and jejunal digesta (JD1 and JD3) of growing pigs fed a protein-free diet. The samples were analysed using electrospray ionization time-of-flight liquid chromatography tandem mass spectrometry (ESI-TOF LC-MS/MS). **.....218**

List of Publications

Note: All of my publications have Lakshmi A. Dave as my author name

- **Dave, L. A.,** Hayes, M., Mora, L., Montoya, C. A., Moughan, P. J., & Rutherfurd, S M. (2016). Gastrointestinal endogenous protein-derived bioactive peptides: An *in vitro* study of their gut modulatory potential. International Journal of Molecular Sciences, 17(4), 482. doi:10.3390/ijms17040482.

- **Dave, L. A.,** Hayes, M., Rutherfurd, S. M., & Moughan, P. J. (2016). Novel Dipeptidyl Peptidase IV Inhibitory and Antioxidant Peptides Derived from Human Gastrointestinal Endogenous Proteins. International Journal of Peptide Research and Therapeutics, 22, 355. doi:10.1007/s10989-016-9515-y

- **Dave, L. A.,** Hayes, M., Montoya, C. A., Rutherfurd, S. M., & Moughan, P. J. (2015). Human gut endogenous proteins as a potential source of angiotensin-I-converting enzyme (ACE-I)-, renin inhibitory and antioxidant peptides. Peptides, 76, 30-44. doi: 10.1016/j.peptides.2015.11.003

- **Dave, L. A.,** Montoya, C. A., Rutherfurd, S M., & Moughan, P. J. (2014). Gastrointestinal endogenous proteins as a source of bioactive peptides - An *in silico* study. PLoS ONE, 9(6), e98922. doi: 10.1371/journal.pone.0098922

Chapter 1

1. Introduction

Dietary proteins, apart from being a source of amino acids for body protein synthesis, are now recognised as sources of bioactive peptide sequences (Rutherfurd-Markwick & Moughan, 2005; Shahidi & Zhong, 2008). Physiological effects, such as ACE-I inhibition (Mullally et al., 1997), antioxidant activity (Di Bernardini et al., 2011) and immune-modulation (O'Sullivan et al., 2015), among others, have been attributed to certain dietary protein-derived peptides. The regulatory and therapeutic potential of such biologically active peptides has emerged as an exciting aspect of research in the areas of human nutrition and physiology.

The food protein-derived bioactive peptides are commonly referred to as the exogenous bioactive peptides, while peptides such as enkephalin that are produced by proteolytic processing in the body, are referred to as endogenous peptides (Meisel & Schlimme, 1990). With respect to the former class of bioactive peptides, it is notable that the typical human diet is highly variable in nature. As a result, the supply of dietary protein will also be highly variable as will the amount and type of bioactive peptides produced after the digestion of dietary proteins in the gastrointestinal tract (GIT). This is inconsistent with the idea that the food-derived bioactive peptides form an important regulatory system in the body.

In contrast, a significant and relatively constant amount of protein enters the GIT daily in the form of mucins, serum albumin, digestive enzymes and sloughed epithelial cells. Microorganisms also occupy the GIT and are constantly turned over,

thus contributing significant amount of protein to the gut contents. In comparison to dietary proteins, the gastrointestinal endogenous proteins (GEP) represent a larger and more consistent source of proteins in the GIT. GEP have been reported to undergo simultaneous digestion along with their dietary counterparts (Nasset & Ju, 1961; Souffrant et al., 1993). However, the possibility that GEP may release encrypted bioactive peptide sequences during their digestion has remained unexplored. If GEP prove to be a significant source of bioactive peptides then this would constitute a paradigm shift in our current understanding about the role of GEP. Indeed it may be possible that the role of GEP as bioactive peptide reservoirs may be just as important or more important than their traditionally considered functions.

This dissertation attempts to address the above hypothesis and to understand the relevance of GEP-derived peptides in the context of the gut. The research data are drawn from *in silico*, *in vitro* and *in vivo* digestion studies conducted individually or in an integrated manner.

The discussion presented in Chapter 2 reviews the different themes around bioactive peptides. The different peptide-mediated regulatory mechanisms in the GIT that may be affected by GEP-derived bioactive peptides have been outlined. The major GEP that have the potential to act as sources of bioactive peptides have been discussed in detail. The different strategies to study bioactivity of peptides have also been considered. Further, a new classification of bioactive peptides based on their site of generation has been proposed.

The work described in Chapter 3 is the first exploratory step that seeks to investigate if the protein sequences of key GEP indeed contain any bioactive peptide sequences

that have been previously reported from dietary protein sources. This study also investigates if there exists a possibility for the release of GEP-derived bioactive peptides during simulated *in silico* gastrointestinal (SIGIT) digestion. This approach has primarily considered the primary structure of the GEP and the known cleavage specificity of the GIT enzymes. The results presented compile and summarize the types of bioactivities predicted to be present after simulated *in silico* gastric and small intestinal phases of digestion, and also provides a view of the comparative potential of GEP versus dietary proteins as a source of bioactive peptides.

In the study described in Chapter 4, proteins that represent the major types of GEP secreted into the GIT, including digestive enzymes, hormones, mucins, antimicrobial proteins, and proteins of epithelial and microbial origin were subjected to SIGIT. The online databases used in the *in silico* approach described in Chapter 3 primarily document bioactive peptides from the dietary protein sources. Since GEP as a source of bioactive peptides has not been investigated previously, the possibility that GEP may release novel bioactive peptides that have not yet been studied was considered in the work described in Chapter 4. The peptide sequences identified after SIGIT were analysed based on previously reported structure-activity attributes of dipeptidyl peptidase IV (DPP-IV) inhibitory and antioxidant peptides. Potential peptides with structural attributes that matched known DPP-IV and antioxidant activity were chemically synthesised and tested *in vitro* for bioactivity. In the case of antioxidant activity, two different assays were used to allow an understanding as to whether different peptides might possess different mechanisms of action.

In the study discussed in Chapter 5, which aimed to investigate the release of bioactive peptides, four quantitatively important GEP (trypsin, salivary mucin,

lysozyme and serum albumin) were subjected to a simulated digestion using a static *in vitro* digestion model that closely mimics the physiological conditions in the GIT. The resulting digests were then screened for a range of biological activities specifically relevant to the gut. The digests were enriched further by subjecting them to molecular weight-based filtration (<3 and <10 kDa). This allowed determination of the size-range of peptides that showed the maximum activity. The sequences of peptides in the fractions showing maximum bioactivity were also characterized using mass spectrometry.

Numerous studies on food protein-derived bioactive peptides are *in vitro* investigations, and a very few of these peptides are shown to be released during digestion in animals or humans. Thus, the work outlined in Chapter 6 aimed to study the bioactivity of GEP-derived peptides in growing pigs that were fed a protein-free diet. The use of a protein-free diet in this *in vivo* experiment allowed the sampling of gastric chyme and small intestinal digesta samples that contained digested fractions of GEP alone, without any interference from dietary proteins. Samples were collected at both the gastric and small intestinal stages of digestion and the polypeptides in the samples were assessed for ACE-I inhibition and antioxidant activity. The peptides responsible for the observed activity were characterised using mass spectrometry.

Chapter 7 provides a summary of the major findings of the work and discusses them in the light of the current understanding of bioactive peptides. This chapter also outlines potential aspects for future investigations in the area of GEP-derived bioactive peptides. The layout of the thesis chapters is presented in Figure 1.1. The

details of the approaches used and methodologies have been summarized in Section 2.6 of Chapter 2.

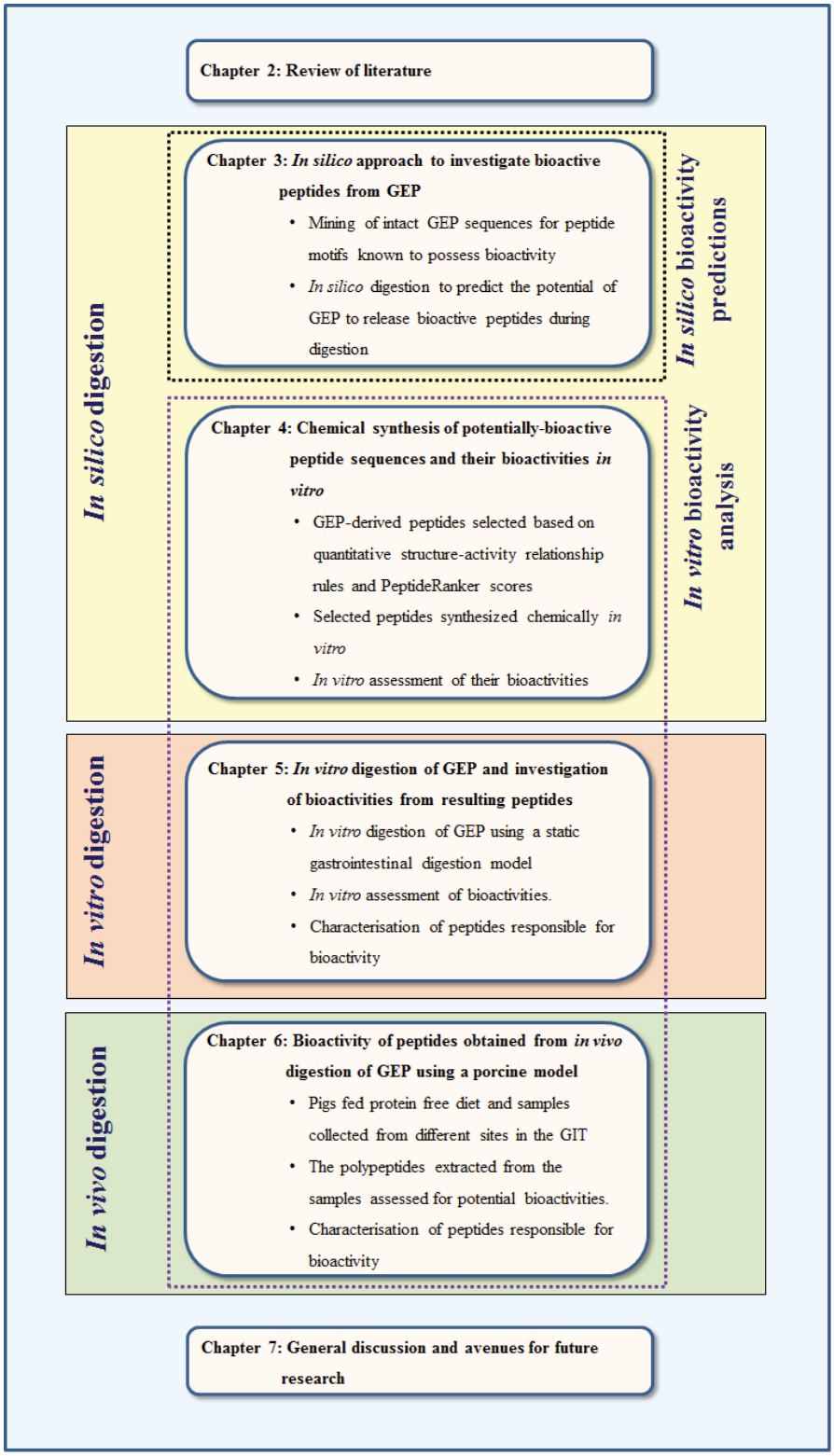

Figure 1.1 *Overview of chapters in the dissertation*

Chapter 2

2. Review of Literature

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Dave, L. A., Hayes, M., Montoya, C. A., Rutherfurd, S. M., & Moughan, P. J. (2015). Human gut endogenous proteins as a potential source of angiotensin-I converting enzyme (ACE-I)-, renin inhibitory and antioxidant peptides. Peptides, 76, 30-44. doi: 10.1016/j.peptides.2015.11.003

2.1. Introduction

Proteins in living organisms carry out a variety of biological functions, these include being vital constituents of cellular materials, acting as regulatory molecules for complex biological processes, and being a source of essential amino acids and energy (FAO Expert Consultation, 2011; Kimmerlin & Seebach, 2005; Voet et al., 2013). In addition, certain amino acid sequences embedded within the primary structure of proteins are also known to influence various physiological functions in the body. These amino acid sequences, after their release from the parent protein, are referred to as biologically active or bioactive peptides (Ferrario, 2002; Karelin et al., 1995; Loh et al., 1984; Meisel et al., 1989; Ner et al., 2015).

The various bioactivities exhibited by bioactive peptides include immune regulation (Politis & Chronopoulou, 2008), antimicrobial activity (Mor & Nicolas, 1994), blood pressure regulation (Atlas, 2007; Sipola et al., 2001), antioxidant activity (Garcia-Nebot et al., 2014), and opioidergic effects (Nyberg et al., 1997).

The following sections discuss the classification and role of bioactive peptides in the regulation of human physiological processes with an emphasis on gut function.

2.1.1. Classification of bioactive peptides

Several bioactive peptides from a diverse range of sources have been reported by a wide number of researchers. The specific role and type of a bioactive peptide will be largely determined by the site of its generation and the proteolytic event that led to its release from the parent protein.

Therefore, in this section, based on the site of their generation, a new classification of bioactive peptides is proposed. Accordingly, bioactive peptides can be broadly classified as being either truly endogenous or exogenous depending on the site in the body where they originate and act. Truly endogenous bioactive peptides (TEnBP) can be defined as peptides produced from the human proteome inside the "body proper", that may either play a role in physiological regulation or exert a health benefit (Karelin et al., 1998a; Kastin & Pan, 2010). Exogenous bioactive peptides (ExBP) are those that are generated outside the "body proper", such as in the lumen of the GIT, which, in anatomical terms, is considered to be external to the body (Franklin, 2015).

2.1.1.1. Truly endogenous bioactive peptides

TEnBP can be further classified into three major types (Fesenko et al., 2015); (1) biosynthetically-derived bioactive peptides (Steiner et al., 1980), (2) directly encoded bioactive peptides (Crappe et al., 2014) and (3) cryptome-protein derived bioactive peptides (Pimenta & Lebrun, 2007).

Biosynthetically-derived bioactive peptides are the oldest known class of bioactive peptides. These peptides are encoded within inactive precursor proteins with a distinct *N*-terminal signal peptide that helps in the release of the protein *via* secretory pathways (Steiner et al., 1980). Biosynthetically-derived peptides become bioactive and perform their primary function following their release and the subsequent proteolytic removal (post-translational modification) of the *N*-terminal signal peptides from the respective precursor proteins (Steiner et al., 1980). Biosynthetically-derived peptides are ubiquitous and play a significant role

in various systems of the body including the respiratory, cardiovascular, renal and gastrointestinal systems and are also known to have an active role in the brain, blood, immune system and also during inflammation and cancerous growth (Gomes et al., 2010; Karelin et al., 1998b; Kastin & Pan, 2010). Common examples of endogenous biosynthetically-derived bioactive peptides include the gastroenteropancreatic and neuroendocrine peptides including gastrin, somatostatin, insulin and the enkephalins (Steiner et al., 1980).

The second group of bioactive peptides are the directly encoded bioactive peptides. These peptides are encoded directly from small open reading frames (sORFs) and lack a *N*-terminal signal peptide and are therefore released directly into the cytoplasm (Crappe et al., 2014). Little is known about their role in human regulatory systems. Examples of directly encoded bioactive peptides include the micropeptides (Andrews & Rothnagel, 2014; Crappe et al., 2014). Micropeptides are also known as polycistronic or short open reading frame encoded polypeptides and are translated from small open reading frames (sORFs) shorter than 100 amino acids in length (Crappe et al., 2014).

Endogenous cryptome-protein derived bioactive peptides are hidden sequences encrypted in body proteins that have their own, well-defined primary functions (Autelitano et al., 2006; Ner et al., 2015; Pimenta & Lebrun, 2007). These bioactive peptides are released following proteolytic degradation of the parent protein in the respective cell or tissue (Karelin et al., 1998a). Cryptome-protein derived bioactive peptides are known as cryptides or crypteins (Pimenta & Lebrun, 2007; Samir & Link, 2011).

Several bioactive peptides from different cryptome proteins of muscle, skin, blood, hair, milk and tears have been identified to date (Gomes et al., 2010; Karelin et al., 1998a). These include the bioactive peptide endostatin from skin protein collagen which can inhibit endothelial cell proliferation (Marneros & Olsen, 2001); hemopressin from the blood protein haemoglobin which plays a role in the regulation of food intake and/or hypotension (Gomes et al., 2010); and the immuno-modulatory peptide fragments from the eukaryotic cell protein ubiquitin (Pasikowski et al., 2011). Only few cryptome-protein derived bioactive peptides have been reported to date, and the study of cryptides is a relatively new field of research (Hattori & Mukai, 2014; Samir & Link, 2011).

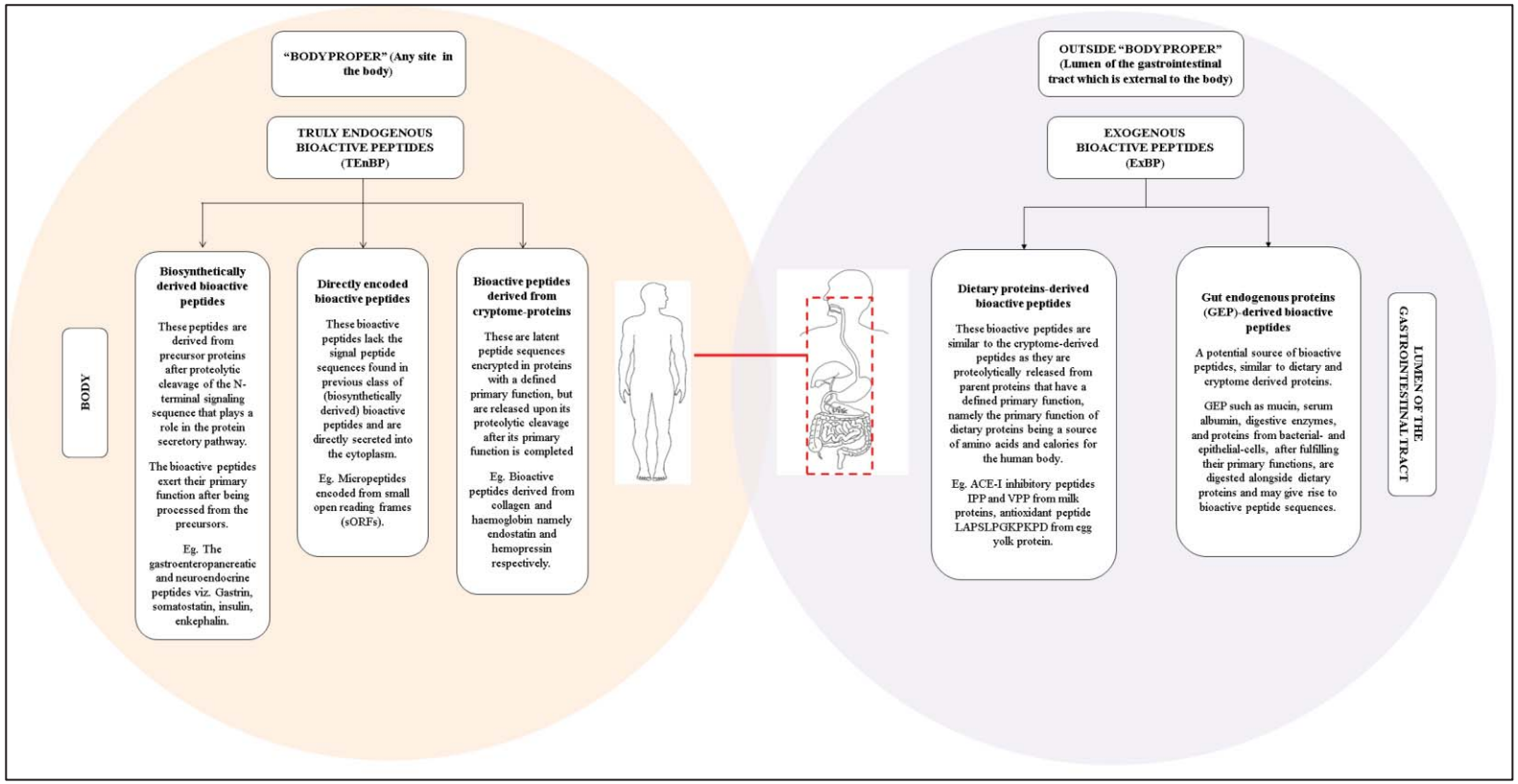
2.1.1.2. Exogenous bioactive peptides

ExBP are bioactive peptides derived from food, such as peptides found in sour milk (Masuda et al., 1996) or generated from the digestion of dietary (Shahidi & Zhong, 2008) or host (endogenous) proteins in the GIT (Dave et al., 2014; Moughan et al., 2014). The GIT is essentially an open-ended tube that is in constant direct contact with the outside environment, and the gut lumen is therefore, external to the body (Franklin, 2015). Food proteins are a well-known source of these ExBP. These bioactive peptides may be generated by the hydrolysis of food proteins during food processing and/or gastrointestinal digestion (Mora & Hayes, 2015). *In vitro* and *in vivo* investigations have demonstrated that bioactive peptides have a myriad of activities and health benefits including antioxidant, antimicrobial, ACE-I and renin inhibitory, immune-modulatory and opioid activities (Barbe et al., 2014; Hayes et al., 2007; Korhonen & Pihlanto, 2006). The bioactive peptides encrypted within dietary proteins, together constitute the food

peptidome (Dallas et al., 2015; Minkiewicz et al., 2008a; Saadi et al., 2015; Sanchez-Rivera et al., 2014).

While dietary exogenous proteins and peptides released in the gut during digestion have been investigated extensively, the possibility that non-dietary proteins that are also present in the digestive tract (GEP) may also give rise to bioactive peptides has only recently received attention (Moughan et al., 2014). Although GEP have been extensively investigated within the context of determining true estimates of amino acid digestibility and dietary amino acid requirements (FAO Expert Consultation, 2011; Moughan & Rutherfurd, 2012; Rutherfurd et al., 2015b), the possibility that GEP may also serve as a source of bioactive peptides has not been extensively considered to date.

It is of note that, although GEP are endogenous by source (i.e. they are synthesised within the body), they are secreted into the lumen of the GIT which is external to the body, and therefore, GEP are an exogenous source of proteins and bioactive peptides. Furthermore, both food proteins and GEP found in the gut have their own, well-defined primary functions. Food proteins are a source of nutrition for the body, while the GEP are secreted into the GIT to facilitate the digestion and absorption of nutrients. Thus, both these protein sources are similar to cryptome proteins since, beyond their primary functions, they may also give rise to bioactive peptides. Figure 2.1 presents the different TEnBP and ExBP found in humans.



(Based on data collated from various sources (Crappe et al., 2014; Kastin & Pan, 2010; Marneros & Olsen, 2001; Minkiewicz et al., 2008a; Moughan et al., 2014; Pimenta & Lebrun, 2007; Steiner et al., 1980; Tanaka et al., 2014))

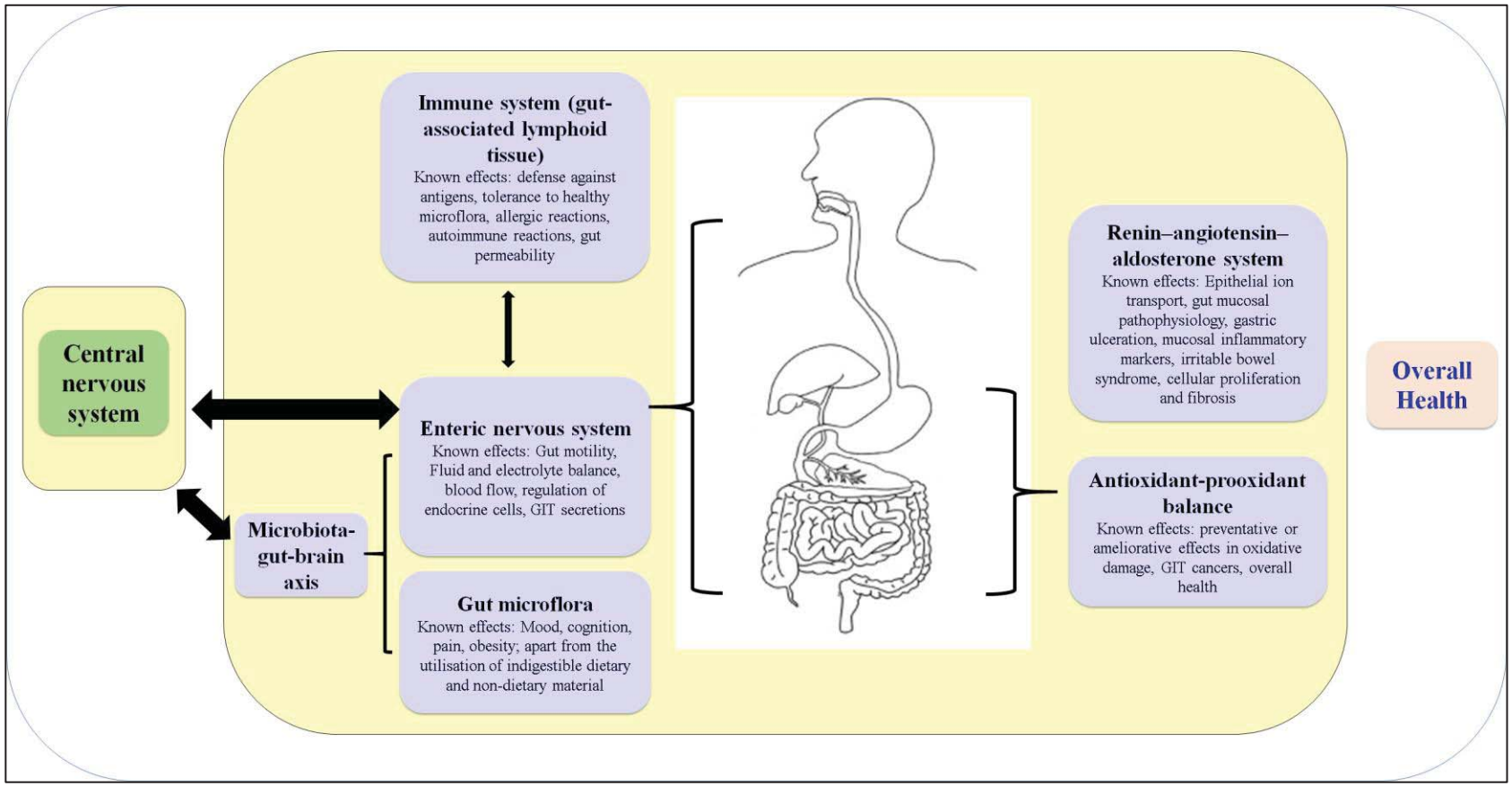
Figure 2.1 *Schematic representation of the types of bioactive peptides that are known to date. The bioactive peptides derived in the body have been grouped as the truly endogenous bioactive peptides (TEnBP), while those generated in the GIT lumen are grouped as exogenous bioactive peptides (ExBP).*

2.2. GIT: A system of peptide-mediated physiological regulation

When considering the role of bioactive peptides with respect to human physiology, it may be questioned if such regulatory peptides have a significant role in the GIT and what is the scope of GEP within the latter context. Besides performing its primary function of nutrient digestion and assimilation, the GIT also houses numerous other regulatory systems, as shown in Figure 2.2.

2.2.1. Immunomodulatory system

The GIT is constantly exposed to external physicochemical stimuli including ingested food, antigens, pathogens and toxins, and internal stimuli such as secretions. Moreover, both the GIT immune system and the gut microbiota are continually responding to these stimuli (Martin & Hine, 2008). The gut-associated lymphoid tissue (GALT) of the GIT is a major immune site and has been reported to contain around 70% of the entire immune system (Vighi et al., 2008). GALT participates extensively in both innate and adaptive immune responses originating from the gut (Vighi et al., 2008). In particular, in response to antigenic stimuli, cytokines and antibodies such as secretory immunoglobulin A are produced by the GALT. The intestinal epithelium and the GALT contain both toll-like receptors and nucleotide-binding oligomerization domain (NOD)-like receptors that are involved in innate immunity and intracellular sensing (Magrone & Jirillo, 2013). It is well known that GALT functioning is dependent on the protein supply to the body (Cunningham-Rundles et al., 2005; McGee & McMurray, 1988; Woodward, 1998), and further that certain amino acids such as glutamine, arginine and cysteine may influence intestinal immunity (Ruth & Field, 2013).



(Based on data collated from various sources (Burokas et al., 2015; Fandriks, 2011; Halliwell et al., 2000; Surai et al., 2004; Vighi et al., 2008))

Figure 2.2 *The GIT: a complex system relating to numerous regulatory systems*

2.2.2. GIT microbiota

The GIT immune system is also affected by the symbiotic bacteria of the gut (O'Hara & Shanahan, 2006). Intestinal microbes can modulate inflammatory responses and impact the production of cytokines (Burokas et al., 2015). Metabolites of the microbiota are also known to interact with NOD-like receptors; and depending on the stimulus, can be either anti- or pro- inflammatory (Magrone & Jirillo, 2013). All the above mentioned stimuli may also affect the population densities of the microbes, a phenomenon that is known to lead to bacterial cell-to-cell communication known as quorum sensing (QS) (Swift et al., 2000). QS is used by the microbiota to regulate their gene expression in response to changing bacterial population densities (Miller & Bassler, 2001).

Interestingly, while numerous microbe-derived QS-peptides are known to serve as signalling molecules (Wynendaele et al., 2013), recent research points to a potential role of analogues of QS-peptides in controlling QS, that may in turn lead to a reduction in the growth of virulent bacteria (LoVetri & Madhyastha, 2010). For example, the synthetic analogue, KBI-3221, of the naturally occurring QS competence-stimulating peptide, was found to prevent biofilm formation by *Streptococcus mutans*, a known caries forming Streptococci (LoVetri & Madhyastha, 2010). Hence, a possible role for food and GEP-derived bioactive peptides may exist in QS of the gut microbiota. The role of gut microbiota as potential sources of bioactive peptides has been explored in detail in Section 2.4.3.2.

2.2.3. Regulatory peptides of the enteric nervous system (ENS)

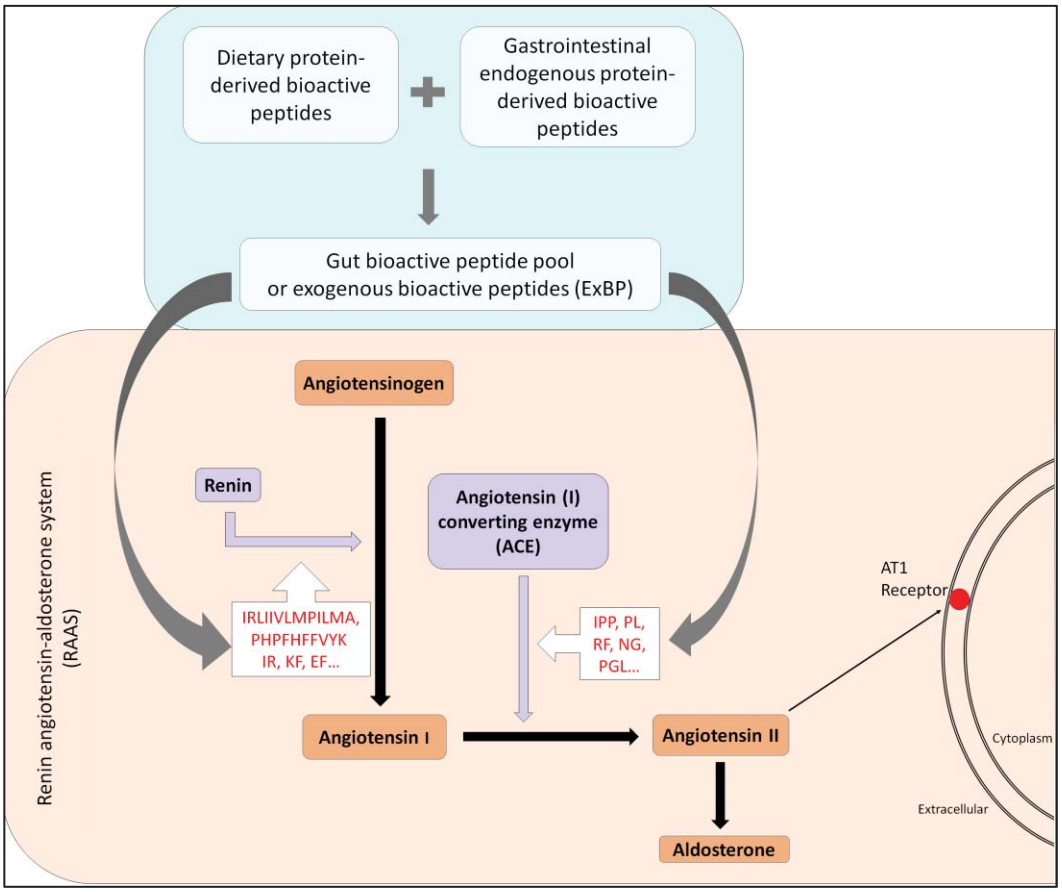
Another major system within the GIT is the autonomous enteric nervous system (ENS). The ENS is responsible for neural regulation of gut motility, fluid and electrolyte balance, blood flow, endocrine hormones, other GIT secretions, gut permeability and absorption (Burokas et al., 2015; Costa et al., 2000; Tack, 2000). ENS regulation is mediated through sensory receptors, primary afferent neurons, inter-neurons, and motor neurons located mostly in the wall of the GIT (Tack, 2000). Peptides can modulate the myenteric GIT musculature and neurons (Tack, 2000). Also, some endogenous bioactive peptides (e.g. the pancreatic polypeptide-fold peptide family), which act as neurotransmitters, can also act as gut hormones (Gehlert, 1998). Certain diet-derived bioactive peptides such as *β*-casomorphin as well as endogenous peptides such as endorphins can both modulate mucus secretion by reacting with the μ-receptors in the jejunum (Trompette et al., 2003). This may suggest that μ-receptors can accept both exogenous and endogenous bioactive peptides. Further, the ENS functioning is related to that of the GALT as neuropeptides are also capable of modulating the mucosal immunity (Genton & Kudsk, 2003). Specific neuropeptide secretions into the GIT lumen may be influenced by the metabolites of the microbiota, and neuropeptides in turn may influence communication between the gut microbiota, gut and the brain (Holzer & Farzi, 2014). This multifaceted interaction has been termed as the microbiota-gut-brain axis (Burokas et al., 2015; Holzer & Farzi, 2014).

2.2.4. The Renin Angiotensin Aldosterone System (RAAS)

Another system that is expressed in the GIT is the renin angiotensin system (RAAS) (Fandriks, 2011; Hallersund et al., 2011). The RAAS is the primary regulator of the fluid and electrolyte balance in the GIT and also blood pressure in the human body.

2.2.4.1. Mechanism of action

Within the RAAS, the enzyme renin acts on angiotensinogen to form angiotensin-I I, which is further hydrolysed to angiotensin-II by the enzyme ACE-I (Johnston, 1990). The enzyme renin is the rate limiting enzyme of the RAAS. The main enzymes involved in RAAS are depicted in Figure 2.3, and the intervention points where bioactive peptides from either food and/or GEP may act are highlighted.



Based on data from (Atlas, 2007; Fitzgerald et al., 2012; Minkiewicz et al., 2008b; Mora & Hayes, 2015; Moughan et al., 2014)).

Figure 2.3 *The potential role of cryptome-derived bioactive peptides in the Renin-Angiotensin-Aldosterone System (RAAS)*

Angiotensin-II, an octapeptide, is a potent vasoconstrictor which causes increased blood pressure. In addition, angiotensin-II stimulates secretion of the hormone aldosterone that can cause extracellular fluid accumulation which can lead to hypertension (Atlas, 2007; Swales, 1994). Thus, inhibition of ACE-I and renin within the RAAS is considered an effective mode of prevention, and treatment for hypertension (Atlas, 2007). Besides regulating fluid and electrolyte balance, the RAAS may also be involved in glucose and amino acid uptake from the GIT, and gut motility (Garg et al., 2012). Evidence suggests that RAAS expression varies in healthy and diseased states and that the modulation of RAAS receptors might, therefore, play a role in alleviating mucosal ulceration, inflammation and carcinogenesis of the GIT (Wegman-Ostrosky et al., 2013).

Several renin inhibitory peptides have been identified to date from dietary sources including pea protein and seaweeds (Fitzgerald et al., 2012). Dietary bioactive peptides can act as competitive, non-competitive or anti-competitive inhibitors of ACE-I, with most inhibitors falling into the category of competitive inhibitors (Kussmann & van Bladeren, 2011). Competitive ACE-I inhibitors bind to the active site of ACE-I or to the enzyme's inhibitor binding site which leads to a reduction in ACE-I activity (Gronhagen-Riska et al., 1987).

2.2.4.2. Characteristics of peptides showing ACE-I inhibition

Numerous studies have attempted to understand the structural aspects of peptides displaying ACE-I inhibition. In general, peptides containing hydrophobic residues at the C-terminal end, and, or branched chain amino acids at the N-terminal have been reported to be effective ACE-I inhibitors (Cheung et al., 1980; FitzGerald et al.,

2004; Pripp et al., 2005). In oligopeptides and tri-peptides showing ACE-I inhibition, the three amino acids at the C-terminal end of the peptide are the determining factor for the bioactivity, with the presence of Y, F, W, P and L in the C-terminal region of the peptide leading to greater ACE-I inhibition (Li & Yu, 2015).

For dipeptides the steric properties and lipophilicity of the C-terminal amino acid determine its bioactive potential (Wu et al., 2006). The presence of positively charged amino acids at the C-terminus of a peptide can also lead to potent ACE-I inhibition (FitzGerald et al., 2004; Gomez-Ruiz et al., 2007).

Quantitative structure activity relationship (QSAR) modelling of predicted ACE-I inhibitory peptides has suggested that the presence of aromatic amino acids at the C-terminal end of the peptide and a positively charged amino acid at the penultimate position and a hydrophobic amino acid at the N-terminal end of a tripeptide are favourable for ACE-I inhibition (Wu et al., 2006).

2.2.5. Peptide-mediated antioxidant activity

In addition to the RAAS and ENS described above, a significant number of antioxidant and pro-oxidant reactions occur in the lumen of the GIT (Halliwell et al., 2000). The GIT is constantly exposed to pro-oxidants including iron, copper, H_2O_2, haem, lipid peroxides, aldehydes and nitrite and xenobiotics present in the diet that can potentially lead to free radical mediated damage in the lumen and also systemically (Halliwell et al., 2000; Pang et al., 2012). Further, the continual renewal of the epithelial layer of the lumen makes it highly susceptible to

genotoxins such as chromium, heterocyclic amines, and mycotoxins (Pool - Zobel et al., 1993; Sugimura, 2000).

2.2.5.1. Mechanism of action

Natural antioxidants such as phytochemicals and peptides released from the diet are believed to counteract the pro-oxidants and may also increase the activity of endogenous antioxidants present in the GIT, thereby playing a protective role against oxidative damage and GIT cancer development (Halliwell et al., 2000; McIntosh et al., 1995). The antioxidant effect of peptides is likely to arise from either single or a combination of free radical scavenging, metal ion chelation or interaction with aldehyde groups that act as oxidants (Chen et al., 1996; Zhou & Decker, 1999). Another mechanism that might be responsible for the antioxidant effect of peptides is the indirect inhibition of the enzymes involved in the generation of free radicals (Halliwell, 1996). Natural antioxidant compounds may also exert their effect by enhancing the expression or functioning of the endogenous antioxidant enzymes catalase, glutathione peroxidase, and superoxide dismutase.

2.2.5.2. Characteristics of peptides with antioxidant bioactivity

The structure of antioxidant peptides is not fully understood, however, the properties of constituent amino acid residues in a peptide play an important role in determining its antioxidant potential. In general, the presence of hydrophobic and aromatic amino acids at the C- and N-terminal ends of the peptide results in antioxidant activity (Chen et al., 1995; Pihlanto, 2006; Udenigwe & Aluko, 2011). Amino acid residues that have ability to act as proton donors, such as Y and W (Hernandez-Ledesma et al., 2005b; Sarmadi & Ismail, 2010), and those containing thiol-group,

such as C (Giles et al., 2003; Taylor & Richardson, 1980) can effectively quench free radicals. Thus, in general, peptides with P, H, Y, W, M and C in their sequences may have a potential to act as antioxidant peptides (Chen et al., 1995; Elias et al., 2008).

Further, Elias et al. (2008) noted that while all of the 20 amino acids may interact with free radicals singly, their combination in peptide sequences could produce greater antioxidant bioactivity than both individual amino acids and also intact proteins (Hernandez-Ledesma et al., 2005a). This greater potential of peptides may be attributed to enhanced stability and unique chemical properties arising from the sequential arrangement of the amino acid residues (Elias et al., 2008). Majumder et al. (2013) further elucidated this mechanism by using a tripeptide IQW from egg protein ovotransferrin. These authors noted that tri-peptide IQW showed significant reduction in tumour necrosis factor induced inflammatory and oxidative markers at a concentration of 50 μmol/L (Majumder et al., 2013). However, at the same concentration, the di-peptides IQ and QW, and the individual amino acids I, Q and W, all of which are present in the tri-peptide, had little or no effect on the same inflammatory markers (Majumder et al., 2013).

2.2.6. Peptide-mediated inhibition of advanced glycation end products

Besides being able to exert an antioxidant effect, bioactive peptides in the GIT might also help prevent damage induced by the pathological modification of proteins. For example, the peptides from Ethawah goat milk, chicken egg lysozyme and lactoferrin are reported to prevent the formation of advanced glycation end products (AGEs) and/or enhance their excretion (Alabdulmohsen et al., 2015; Li et al., 1995; Zheng et al., 2001). AGEs are formed as result of a casacade of reactions between

susceptible proteins and reducing sugars, generally under conditions of prevalent hyperglycaemia or during ageing. AGEs have been implicated in various micro- and macrovascular diseases.

2.2.6.1. Mechanism of action

The proposed mechanism for the prevention of AGE-mediated damage by the above mentioned beneficial proteins/peptides is thought to be a possible sequestration or glycation ligand binding of AGEs (Li, 1998; Zheng et al., 2001), prevention of deposition of AGEs in tissues, and reduction in overexpression of receptors of AGEs (RAGEs) (Cocchietto et al., 2008), or by the inhibition of AGE-RAGE interaction (Fatchiyah et al., 2015). Further, in a D-galactose-induced BALB/c mice model that was used to study glycation related brain and liver tissues and overall oxidative stress levels, it was found that oral administration of the dipeptide NW at a concentration of 40 mg/kg for 4 weeks could reduce the oxidative stress caused by glycation and could also improve spatial memory and learning dysfunctions (Han et al., 2014). Therefore, it could be possible that some of the dietary and GEP-derived bioactive peptides might have the potential to prevent AGE induced damage of the GIT tissue.

Further, in an *in vitro* study, Bohrmann et al. (1999) noted that certain endogenous proteins and peptides might play a role in the prevention of proteopathy. Bohrmann et al. (1999) tested the role of thirteen proteins that form 90% of the proteins found in plasma and cerebrospinal fluid, in the amyloid beta-peptide (A-β) polymerization that occurs during the development of Alzheimer's; and reported that albumin can be a potent inhibitor of amyloid formation. Similarly, certain GEP-derived bioactive

peptides might have a role to play in the prevention of gastrointestinal amyloidosis (Ebert & Nagar, 2008)

From the above discussion, it is evident that the GIT is a complex system comprising numerous regulatory systems, with receptors within these systems being expressed in abundance (Fandriks, 2011; Genton & Kudsk, 2003; Halliwell et al., 2000; Holzer & Farzi, 2014; Vighi et al., 2008). Consequently, it is entirely plausible that TEnBP and ExBP can regulate a variety of functions within the GIT. The current knowledge about food-derived ExBP and the potential of GEP as a source of ExBP are discussed below.

2.3. Food protein-derived ExBP

Following the ingestion of foods, proteins are broken down by enzymes and HCl present in the GIT to form peptides and some of these peptides may have potential physiological modulatory effects and health benefits. However, it remains unproven whether these peptides are present systemically following absorption in quantities high enough to exert a beneficial effect (Miner-Williams et al., 2014b; Segura-Campos et al., 2011). Numerous studies have produced hydrolysates by application of *in vitro* simulated GIT digestion using commercially available enzymes and have shown, using *in vitro* assessment, that at least some of the resultant peptides have biological activity (Cruz-Huerta et al., 2015; Dia et al., 2014; Fujita et al., 1995; Hirota et al., 2011; Marques et al., 2015; Shi et al., 2014; Velarde-Salcedo et al., 2013; Zambrowicz et al., 2015).

Dietary bioactive peptides have been defined as peptides released from food proteins that when ingested or generated in the GIT exert a physiological effect above and

beyond their basic nutritional benefits. In this way, they are similar to the cryptome-protein derived peptides in the TEnBP. Dietary bioactive peptides can produce significant biological activity or regulatory effects locally within the gut or elsewhere in the body (Dalziel et al., 2014; Di Bernardini et al., 2011; Dziuba & Darewicz, 2007; Foltz et al., 2007; Inoue et al., 2011; Majumder et al., 2015; Trompette et al., 2003). Bioactive peptides typically have a chain length of between 2 and 30 amino acids (Pihlanto-Leppala et al., 2000; Shahidi & Zhong, 2008) although a small number of bioactive peptides are known to be longer (Galvez et al., 2001). Lunasin for example, which is a bioactive peptide derived from cereal and soya proteins is 43 amino acids in length and is known to have heart health bioactivities but can also induce allergenic effects (Lule et al., 2015). Apart from their size, other common structural features of dietary bioactive peptides include the presence of hydrophobic amino acid residues in their sequences, a net positive charge and resistance to digestion by GIT proteases and peptidases (Korhonen & Pihlanto, 2003; Muro Urista et al., 2011; Perez Espitia et al., 2012).

Milk proteins are the most extensively studied source of bioactive peptides of food protein origin (FitzGerald et al., 2004; Korhonen & Pihlanto, 2003; Meisel & Schlimme, 1990; Nongonierma & FitzGerald, 2014; Ricci et al., 2010). Casein, in particular β-casein, is known to be a rich source of bioactive peptides and has been widely investigated for this purpose (Boutrou et al., 2013; Han et al., 2008; Iwaniak & Dziuba, 2011; Korhonen & Pihlanto, 2003; Silva & Malcata, 2005), and the major whey proteins including β-lactoglobulin and α-lactalbumin have also been found to contain peptide sequences with different bioactivities (Hernandez-Ledesma et al., 2008; Pihlanto-Leppala, 2000; Yamauchi et al., 2003). Other dietary protein sources

reported to contain bioactive peptides include egg (Miguel & Aleixandre, 2006), meat (Bauchart et al., 2007; Di Bernardini et al., 2011), fish (Urakova et al., 2012), cereals (Cavazos & Gonzalez de Mejia, 2013), soya (Garcia-Nebot et al., 2014) and more recently macro and microalgae (Fitzgerald et al., 2011).

Based on *in silico, in vitro, ex vivo* and *in vivo* models, over 3088 different potential bioactive peptides possessing more than 44 distinct biological activities have been documented to date and these have been compiled into a bioactive peptide database (BIOPEP database; (Minkiewicz et al., 2008b)). The most widely studied bioactivity is ACE-I inhibition (Martinez-Maqueda et al., 2012; Shahidi & Zhong, 2008), followed by antimicrobial and anti-oxidative activities. To date, over 550 ACE-I inhibitory (Iwaniak et al., 2014) and 416 (Dziuba & Dziuba, 2014) antimicrobial peptides have been reported in BIOPEP. Other bioactivities that have been examined include the opioid-agonist/antagonist peptides (Boutrou et al., 2015), hypocholesterolaemic (Wang & de Mejia, 2005), mineral-binding (Guo et al., 2014), dipeptidyl peptidase IV (DPP-IV) inhibitory (Hayes et al., 2015), prolyl endopeptidase inhibitors (Lafarga et al., 2015a) and anticancer peptides (Ortiz-Martinez et al., 2014). As shown in Table 2.1, a variety of food proteins can generate bioactive peptides possessing numerous activities, when subjected to hydrolysis (simulated digestion).

Table 2.1 *Examples of recently discovered bioactive peptides from dietary proteins or the food peptidome that are predicted to be released after simulated in silico or in vitro gastrointestinal tract (GIT) digestion*

Parent Protein/Protein source	**Bioactivity**	***in silico* hydrolysis**	***in vitro* hydrolysis simulating GITdigestion**	**Reference**
Globulin 11S (*Amaranthus hypochondriacus*)	ACE-I inhibition	Precursors of: SG, HPT, NG, GEGR, QG		(Montoya-Rodriguez et al., 2015)
Soymilk hydrolysate and pumpkin bread (containing soybean and pumpkin pulp)	Anti-inflammation		RQRK, VIK, Full hydrolysate	(Dia et al., 2014)
Yellow string beans (*Phaseolus vulgaris*)	Antioxidant		Heat treated bean hydrolysates	(Karas´ et al., 2014)
Amaranth (*Amaranthus hypochondriacus*) proteins	DPP-IV-inhibition		IPI, PPPP, GP, PP, MP, VA (and others)	(Velarde-Salcedo et al., 2013)
Oryzacystatins	Antihypertensive	AKK, LY, VW, others		(Udenigwe, 2015)

Rice bran (*Oryza sativa*) proteins	Antioxidant	LH, HL, LY, others		
	DPP-IV-inhibition	GP, VA, KA, others		
Plant and microalgae ribulose-1,5-bisphosphate carboxylase	Antioxidant	FC and 2 FC precursors		(Je et al., 2015)
Canary seed (*Phalaris canariensis L.*) proteins	DPPIV-inhibition ACE-I inhibition		Hydrolysates	(Estrada-Salas et al., 2014)
Buffalo (*Bubalus bubalis*) casein	Antioxidant		EDVPSER, NAVPITPTL, VLPVPQK, HPHPHLSF	(Shanmugam et al., 2015)
Pork meat	ACE-I inhibition		KAPVA, ER, KLP, RPR, MYPGIA, VIPEL	(Escudero et al., 2010)
Bresaola (Bovine meat)	ACE-I inhibition		PR, VF, VAP, AVFPSIVGRPR (and others)	(Ferranti et al., 2014)
Egg white hydrolysate	ACE-I inhibition		RADHP, YPI	(Miguel et al., 2007)

Although digestion of food begins in the mouth, protein digestion in the GIT begins in the stomach through the action of HCl and pepsin. Protein and peptides that exit the stomach continue to be digested in the small intestine. The small intestinal proteases can be broadly divided into endo-peptidases and exo-peptidases (Goodman, 2010). The major pancreatic endo-peptidases, trypsin, chymotrypsin, and elastase cleave peptide bonds in proteins and oligopeptides (Desnuelle, 1986). Once the digestion of proteins is completed, the resulting amino acids and small peptides are absorbed by carrier-mediated transport mechanisms (Matthews, 1972; Moughan & Stevens, 2012; Silk, 1977; Silk & Clark, 1974; Silk, 1974). The di- and tri-peptides subsequently undergo digestion at the brush border membrane and in the cytosol of the enterocytes within the small intestine (Caspary, 1992).

At any given time, the proteins present in the GIT will be a mixture of both the dietary proteins and GEP, including microbial protein (Moughan & Rutherfurd, 2012). Consequently, like the dietary proteins, the GEP are also exposed to the proteases in the GIT and are digested (Souffrant et al., 1993) resulting in the generation of GEP-derived peptides, some of which may possess bioactivity (Moughan et al., 2014). Consequently, the GEP may also constitute a potential gut cryptome, but GEP derived bioactive peptides would have to be present in sufficient quantities to exert physiological effects.

2.4. GEP: An unexplored source of ExBP

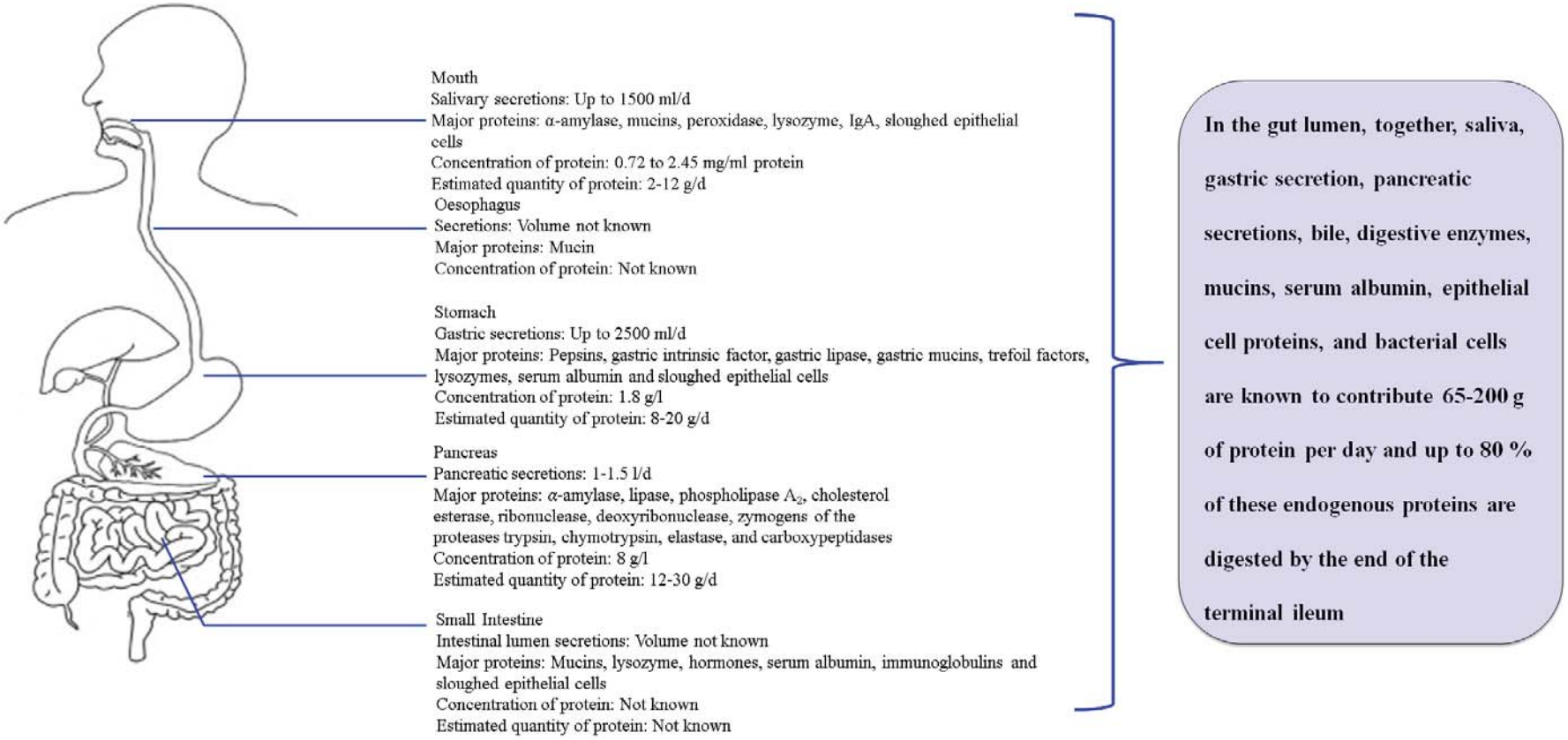
GEP represent a quantitatively significant and much more constant supply of protein in the GIT than dietary proteins (Lobley, 2003). Indeed, it has been reported that in a healthy individual, the nitrogen present in the GEP is equivalent to or greater than that present from food proteins when an individual consumes a balanced diet (Nasset & Ju, 1961). Furthermore, up to 80% of the GEP are digested and re-absorbed into the body (Souffrant et al., 1993) and it is possible that during digestion bioactive peptides may be released from the parent proteins. It is of note that the abovementioned value of 80% is an average across different types of GEP that may be reabsorbed, while individually, different GEP may have different rates of digestion and reabsorption. Henceforth, in this dissertation, the 80% value mentioned should be considered as that referring to average reabsorption rate across GEP. The total gut lumen bioactive peptide pool or ExBP should therefore be considered as a summation of the food- and GEP-derived bioactive peptides.

2.4.1. Major GEP secretions

The gastrointestinal system of an adult human is approximately 9 m in length (DeSesso & Jacobson, 2001) and includes the GIT and the accessory organs of the salivary glands, liver, gall bladder and pancreas (Reed & Wickham, 2009). The GIT secretes a wide range of proteins and other compounds to fulfil its major functions of food digestion and assimilation.

Figure 2.4 details the various GEP secreted in the main sections of the GIT, and the estimated quantities of GEP derived from the different parts of the GIT. Overall, the total amount of GEP secreted per day can be between 65-200 g (Brunser et al., 1992;

Nasset, 1964; Nasset & Ju, 1961). The major proteins secreted along the GIT have been documented in various comprehensive reviews (Miner-Williams et al., 2012, 2014a; Miner-Williams et al., 2009; Moughan et al., 2007). A brief overview, of the major GEPs secreted along the major sections of the GIT that may contribute to the pool of GEP-derived bioactive peptides, is presented below (Figure 2.4).



*(Data collated from various source**s** (Brunser et al., 1992; FAO Expert Consultation, 2011; Moughan & Rutherfurd, 2012; Nasset, 1964; Nasset & Ju, 1961; The UniProt Consortium, 2012))*

***Figure 2.4** The major contributors to the potential gut cryptome*

Saliva is a major contributor to the GEP pool in the mouth. Around 800-1500 mL of saliva is secreted daily (van Lennep et al., 1986) and has a protein concentration range of between 0.72 to 2.45 mg/mL (Lin & Chang, 1989). Saliva contains the enzymes amylase and lipase, as well as mucins and other proline-rich proteins (Carpenter, 2013). To date 1166 proteins have been reported in the proteomes of the parotid and submandibular salivary glands (Denny et al., 2008) and 738 of these are also found in the oral epithelial cells (Ghosh et al., 2012). The mandibular salivary glands also secrete a wide range of bioactive proteins including nerve growth factor, epidermal growth factor, renin, tonin and certain gastrointestinal peptide hormones (Denny et al., 2008). After the buccal cavity, the oesophagus primarily secretes mucus proteins, or mucins (Namiot et al., 1994) for which there are 21 known genes sequenced to date. A number of these genes are reportedly specifically expressed in various segments of the GIT (Gremel et al., 2015).

In the stomach, apart from HCl, the gastric fluid mainly consists of a mixture of mucins, other secreted soluble proteins, and proteins from sloughed epithelia from the gastric mucosa (Kon et al., 2008). The gastric fluid proteome comprises 265 different proteins (Kam et al., 2011) with the main proteins present in gastric secretions being pepsins, gastric intrinsic factor, gastric lipase, gastric mucins and trefoil factors.

Another significant source of GEP entering the gut lumen includes proteins from the liver, e.g. bilary secretions. Besides biliary pigments, bile also contains plasma proteins and peptides such as glutathione (Barbhuiya et al., 2011; Farina et al., 2014). Small amounts of thyroid and steroid hormones are also known to be present

in bile. Indeed, bile is known to contain over 2,500 proteins including plasma proteins and immunoglobulins (Barbhuiya et al., 2011).

Within the small intestine, the pancreatic secretions enter the GIT lumen through the duodenum. The pancreatic juice contains over 170 different proteins (Gronborg et al., 2004). The main exocrine secretions of the pancreas are bicarbonate ions, α-amylase, lipase, phospholipase A2, cholesterol esterase, ribonuclease and deoxyribonuclease, and zymogens of the proteases trypsin, chymotrypsin, elastase, and carboxypeptidases (DeSesso & Jacobson, 2001). Apart from the pancreatic and biliary secretions entering the small intestine, the small intestine itself secretes electrolytes, mucins, lysozyme, hormones and immunoglobulins; and also receives protein from large quantities of cells that are continually sloughed off from the active small intestine mucosa.

While it is well established that the digestion and absorption of most food proteins is virtually complete anterior to the terminal ileum, a small quantity (5-10%) of undigested dietary and endogenous proteins, peptides and free amino acids will enter the colon, in addition to a significant amount of protein that is secreted into the colon itself (Jackson, 2000). The large resident population of microbes in the colon can also digest protein and may yield bioactive peptide sequences that are different to those generated by the action of gastrointestinal proteases in the upper GIT. Further, a small amount of protein and peptide assimilation is reported to occur in the colon (Smith & Macfarlane, 1998), therefore it is possible that a small amount of peptide absorption may also take place in the colon.

2.4.2. Other GEP proteins

Apart from the proteins present in the gastrointestinal secretions described above, there are other proteins that contribute to the GEP content. These include most notably, serum albumin (Brassinne, 1974; Franks et al., 1963), immunoglobulins, and lysozyme (Atuma et al., 2001; Schrager & Oates, 1978; Tabak, 1995; Tabak et al., 1982; The UniProt Consortium, 2012). All the gut hormones, including cholecystokinin, somatostatin, ghrelin, gastrin and secretin that control the GIT functioning and are secreted in minute quantities along the GIT, may also contribute to GEP (Holdcroft, 2000; Kojima et al., 1999).

2.4.3. Non-secretory components contributing to GEP

2.4.3.1. Epithelial cells

The surface area of the permeable epithelial lining or mucosa of the GIT is very large in an adult human (approximately 32 m^2) (Helander & Fandriks, 2014). The mucosa maintains a dynamic equilibrium and is rapidly turned over, being completely replaced every two to three days (Creamer et al., 1961) with up to 250 g of mucosal epithelia shed every day (Creamer et al., 1961). In a study that examined the digesta of pigs fed a casein-based diet, it was reported that mucosal cellular protein, contributed as much as 5% of the total nitrogen at the terminal ileum (Miner-Williams et al., 2009), it is of note that up to 80% of proteins from these desquamated cells would be digested and absorbed prior to the terminal ileum. Excluding proteins that are of microbial origin, mucosal cell proteins comprise the largest, non-secretory contributors to the GEP derived from the body. Consequently, regular shedding of the mucosal epithelium is reported to contribute as much as 50 to 78 g/d of protein (Leblond & Walker, 1956).

2.4.3.2. Microbiome

Although microbial proteins are not actually host endogenous proteins, they are generally included in estimations of the GEP. The gut microflora of humans has a synergistic relationship with the host. The gut microflora are involved in the breakdown of nutrients such as fibre, vitamin synthesis and energy metabolism (Krajmalnik-Brown et al., 2012). The microbiota also play a role in mucosal homeostasis and immune defence (Lozupone et al., 2012). The diverse range of microbes, predominantly made up of bacteria, and minor colonies of archaea and

eukaryota (Savage, 1977), form a symbiotic community that is dynamic and responds to stimuli such as the diet and health status (e.g. the intake of fibre or antibiotics for humans in disease states) of the individual (Turroni et al., 2008). Although, the vast majority of microbes in the GIT inhabit the large intestine (10^{11}-10^{12} bacteria/g of colonic contents), a significant number of microbes also inhabit the upper GIT of the adult human, particularly the distal small intestine (10^{8} bacteria/g dry weight of ileal contents) (Canny & McCormick, 2008). The total bacterial cells of the gut are estimated to be 10^{18} (Turroni et al., 2008) and are ten-fold greater than the number of human cells (O'Hara & Shanahan, 2006). As a result, gut microbes may be a significant source of protein in the gut lumen.

It has been reported that in pigs fed a casein-based semi-synthetic diet, microbial protein was the greatest contributor (45%) to the endogenous nitrogen at the terminal ileum. Similarly in humans fed a casein-based diet, bacterial protein contributed up to 70% of the total nitrogen concentration of the gastrointestinal contents (Miner-Williams et al., 2014a). Although only a fraction (5-30%) of the gut flora population inhabit the upper GIT (Finegold, 1969), microbial proteases may generate bioactive peptide fragments while utilising proteins as substrates for their metabolic activities. This is an important consideration since most *in vitro* models used to study the generation of bioactive peptides from dietary proteins only include gastrointestinal proteases and do not consider proteases of microbial origin (Minekus et al., 2014).

2.4.4. Effect of dietary nutrients on GEP secretion

It is known that dry matter and various dietary nutrients can impact GEP secretions (Boisen & Moughan, 1996; Deglaire et al., 2008; Deglaire et al., 2006; Hodgkinson & Moughan, 2007; Miner-Williams et al., 2014a; Montagne et al., 2001; Ravindran et al., 2009). Several studies have examined the effects of various dietary components such as dietary protein, fibre and anti-nutritional factors such as lectins, tannins and enzyme inhibitors, on the flow of GEP (Butts et al., 1993b; Claustre et al., 2002; Cowieson et al., 2004; Deglaire et al., 2008; Deglaire et al., 2006; Gilani et al., 2005; Gilani & Sepehr, 2003; Ouellet et al., 2002). The effects of fibre, amino acids, peptides and proteins are discussed here.

The effect of dietary fibre intake on GEP secretion has been the subject of several reviews (Boisen & Moughan, 1996; Vickery, 1950). Morel et al. (2003) that have examined the effects of non-starch polysaccharides on growth and GEP secretions in pigs. The study involved feeding pigs with either β-glucan, a highly degradable polysaccharide or arabinoxylan, a polysaccharide with significant water-holding properties. It was found that β-glucan increased mucin secretion and ileal endogenous amino acid flows (Morel et al., 2003). Similar results have been noted in broiler chickens, where dietary cellulose levels in the diet substantially impacted the flow of crude protein and amino acids at the terminal ileum (Kluth & Rodehutscord, 2009). While the exact mechanism by which dietary fibre affects endogenous nitrogen losses (ENL) is not clear, several possible mechanisms have been suggested. These include 1) alteration of endogenous secretions due to the higher viscosity created by the fibre and 2) physical abrasion of the gastrointestinal lumen by dietary fibre (Leterme et al., 1992).

Different proteins and peptides from different protein sources can influence GEP secretions and composition differently. In growing rats, increasing the amount of zein (a protein devoid of lysine) in the diet increased the mean flow of endogenous lysine at the terminal ileum (Hodgkinson & Moughan, 2007). In a separate study using the same model, Deglaire et al. (2008) found that alimentation with diets containing intact casein led to a greater ileal endogenous protein flow in comparison to diets based on hydrolysed casein. However, when compared to free amino acids as the sole nitrogen source in diets, hydrolysed casein also increased the endogenous protein flows at the terminal ileum (Deglaire et al., 2007). Similar trends were reported by Han et al. (2008) for rats, by (Butts et al., 1993a) for pigs and by Moughan et al. (2005) for human subjects, wherein the ENL at the terminal ileum increased when hydrolysed casein was included in the diet.

From the above studies, it is clear that the form of bovine casein (unhydrolysed or hydrolysed) can impact the ENL. In general, the ingestion of bovine casein hydrolysates led to an increase in the ENL when compared with the flows obtained with a protein-free diet. This increase was more than that observed for diets containing amino acids but less than the increase brought about by the intact casein. Furthermore, two independent studies, Ravindran et al. (2009) and Hodgkinson et al. (2000) have reported that feeding increasing concentrations of enzyme hydrolysed casein to male broiler chickens and growing pigs respectively, increased the GEP flows in a dose-dependent manner.

A recent comprehensive study (Rutherfurd et al., 2015a) sheds light on how different protein sources may impact the GEP flows. The authors investigated the effect of feeding protein hydrolysates from five different protein sources including

gelatin, beef, casein, soy protein isolate and lactalbumin to male rats. It was found that basal endogenous ileal amino acid flows were different for the peptide hydrolysates derived from the different protein sources. The authors suggested that the differences may arise from the effects of potentially different bioactive peptides arising from digestion of the different protein sources used in the study (Rutherfurd et al., 2015a).

The full extent to which the dietary bioactive peptides can influence GEP secretion and therefore elicit GEP-derived bioactive peptides is presently not well understood. However, it is well-known that peptides released from dietary proteins in the GIT can stimulate secretion of GEP (Moughan et al., 2007). Zoghbi et al. (2006) reported that based on a mucin-producing rat colon adenocarcinoma cell line study, *β*-casomorphin-7 (a bovine milk *β*-casein derived bioactive peptide), increased the secretion of mucin by directly affecting the secretory activity of the goblet cells, inducing the expression of Mucin-5AC gene and by activating the μ-opioid receptors (Zoghbi et al., 2006). These findings are in agreement with the previously reported findings of Claustre et al. (2002) who reported that enzymatic hydrolysates of casein and lactalbumin and purified *β*-casomorphin-7 caused mucin release in isolated vascularly perfused rat jejunum. Han et al. (2008) investigated the effect of hydrolysed casein on the mRNA expression of mucin genes in the small intestine of rats and reported that the gene expression of mucin-3 was significantly increased by the hydrolysed casein diet in comparison to protein-free or synthetic amino acid diets. Several other studies have also reported that bioactive peptides can bind to specific receptors in the gut and thereby modulate gut related biological processes, for example, gut motility and satiety (Moller et al., 2008; Moughan et al., 2007;

Shahidi & Zhong, 2008). It is evident that bioactive peptides present within food proteins and released during digestion in the GIT can have an effect on gut physiology. The possibility that GEP may act as a source of bioactive peptides, and that dietary bioactive peptides can in turn modulate the secretion of the GEP offers a unique opportunity to manipulate gut modulatory processes by fine-tuning either of the two elements. Therefore, it is clearly important that the GEP are studied within the context of being a source of bioactive peptides. This apparent interdependency between food and GEP-derived peptides can then be exploited to obtain an optimal supply of endogenous and exogenously derived bioactive peptides in the gut.

In conclusion, the above discussion indicates that GEP may act as a potential source of bioactive peptides when subjected to digestion in the GIT, and hence the GEP could be considered as a gut cryptome. The GEP and food peptidomes may together constitute the population of ExBP in the GIT. The study of the gut cryptome as a source of bioactive peptides, therefore, emerges as an important research area that merits further investigation.

2.5. Current strategies for the investigation of bioactive peptides

This section provides an overview of the key strategies employed to investigate bioactive peptides.

2.5.1. *In silico* investigation of bioactive peptides

The *In silico* approach is one of the most widely used methods to mine bioactive sequences from a given polypeptide sequence. This approach is often the first step in the investigation of bioactive peptides, primarily because it is simple to carry out, does not require specialized equipment and can be completed within a short time (Iwaniak & Dziuba, 2011). An array of proteolytic enzymes with different specificities could be used for generating the peptides *in silico* and then compared with previously documented sequences responsible for multiple bioactivities. This approach could also be used to carefully fine-tune the cleavage of the polypeptide so as to release desired bioactive sequences under controlled conditions.

In this approach, online tools (SIB & ExPASy, 2014) are used to simulate proteolytic events for a given protein. When studying potentially bioactive peptides released in the GIT, three major gut proteases (pepsin, trypsin and chymotrypsin) and a few of the other minor proteases found in the GIT are used in the bioinformatics based simulation of gastrointestinal digestion. The resulting peptide sequences are then compared with those of known bioactive peptides compiled in various databases (Minkiewicz et al., 2009; Minkiewicz et al., 2008b; Wang & Wang, 2004). Thus, this allows prediction of the bioactive peptide motifs that may be released after digestion and the stage of digestion where release occurs. This

approach has been used in the work described in Chapters 3 and 4 to mine bioactive sequences from GEP.

Minkiewicz et al. (2008b) have compiled a list of different databases that are available for processing information, available from previously reported bioactive peptide sequences. These authors note that peptide sequence information could be processed using four major databases: BIOPEP (Olsztyn, Poland), EROP (Moscow, Russia), SwePep (Uppsala, Sweden) and PepBank (Cambridge, MA) (Minkiewicz et al., 2008b). The latter three databases contain TEnBP sequences generated in the body from endogenous proteins (Minkiewicz et al., 2008b). On the other hand, the BIOPEP database focusses on sequences from proteins of food origin (Minkiewicz et al., 2008b), implying that the sequences present in this database may be ExBP. Thus, for the *in silico* studies presented in this dissertation, the database BIOPEP has been used for identifying bioactive sequences encrypted in GEP proteins.

While *in silico* techniques and online tools are important in the investigations of potential bioactive peptides, the results of such studies must be interpreted with some caution. The *in silico* approach using the BIOPEP database only takes into account the primary structure of the proteins and the known specificity of the selected protease, and hence may not be a true representation of the gastrointestinal conditions *in vivo* (Minkiewicz et al., 2008b; SIB & ExPASy, 2014). The limitations of this approach have been discussed in detail later in Chapters 3 and 7.

It is likely that under *in vitro* and *in vivo* digestion conditions peptides may retain a part of their secondary structure which in turn may affect their molecular properties and hence bioactivity. Some databases providing information about the secondary

structure of the peptides include UniProt (The UniProt Consortium, 2007) and EVA (Costantini et al., 2007) , but such types of software also have their own limitations (Minkiewicz et al., 2008b) as they may not have been integrated with databases that rely on the primary structure to predict bioactivity.

Recently, more sophisticated molecular docking and structure-activity prediction methods for the prediction of bioactive peptides and the study of their mechanisms of action, have also been used for studying food-derived peptides (Nongonierma & FitzGerald, 2016; Pina & Roque, 2009; Zhang et al., 2015a). These methods were primarily developed for drug design but are now being adapted for the purpose of investigating dietary bioactive peptides.

2.5.2. *In vitro* investigation of bioactive peptides

In this approach, the protein of interest is subjected to *in vitro* proteolytic events to obtain a mixture of peptides with varying chain-lengths, free amino acids, and a small residual amount of the intact protein. The proteolysis of a given protein can be brought about by events such as the action of gut proteases, fermentation by microbes, processing or the action of any other specific protease (Minkiewicz et al., 2008b; Moller et al., 2008). For the purpose of this review, the discussion is restricted to gastrointestinal digestion.

A vast majority of the studies on food-derived bioactive peptides have been carried out using *in vitro* methods (Shahidi & Zhong, 2008). Several static *in vitro* digestion models have been used to date for investigating the release of the bioactive peptides from various food proteins and these have been the subject of various reviews (Guerra et al., 2012; Hur et al., 2011; McClements & Li, 2010). Broadly, these

models range from simple static digestion models using enzymes at optimum pH to generate proteolytic products (Kaur et al., 2010), to more advanced often dynamic digestion models that attempt to stimulate the *in vivo* conditions as closely as possible (Guerra et al., 2012; Hur et al., 2011; Oomen et al., 2002). The latter advanced models incorporate additional relevant biological constituents such as bile salts, and mucins by using gastrointestinal "juices" extracted from humans or animals (Ulleberg et al., 2011), or by using commercially available constituents.

Recently, a standardised INFOGEST model of simulated gastrointestinal digestion has been developed by the EU-COST Infogest network to study the *in vitro* digestion of food/macromolecules (Minekus et al., 2014). This model outlines the parameters for digestion that are relevant in physiological conditions. Using such a method, developed as an outcome of international consensus, would allow a better comparison of results between studies, and hence has been used for the *in vitro* digestion of GEP in Chapter 5.

The bioactivity of the hydrolysates or peptides produced after *in vitro* digestion can be tested using chemical, enzymatic or cell culture-based *in vitro* assays (Nongonierma & FitzGerald, 2016) which have been the subject of extensive reviews as described in detail by several authors elsewhere (Apak et al., 2013; Ben Henda et al., 2013; Blois, 1958; Cervato et al., 1999; Cushman & Cheung, 1971; Elias et al., 2008; Gronhagen-Riska et al., 1987; Hayakari et al., 1978; Hettiarachchy et al., 2012; Huang et al., 2005; Lea, 2015; Lu et al., 2010).

Apart from *in vitro* bioactivity assays, a few studies have also utilised *ex vivo* techniques to assess the bioactive potential of peptides. *Ex vivo* techniques involve

the use of cells extracted from animal tissues or tissues, or the use of biological fluids such as plasma and serum (Hirota et al., 2011). However, *in vitro* digestion and analytical techniques for the determination of bioactivity are unable to simulate the exact physiological conditions that occur *in vivo*, and therefore, the actual bioavailability, metabolism and physiological effect of the peptides is likely to vary *in vivo* (Foltz et al., 2010).

2.5.3. In vivo investigation of bioactive peptides

The final frontier in the investigation of bioactive peptides is to test their occurrence and physiological efficacy *in vivo*. In comparison to *in vitro* analysis, the investigation of bioactive peptides using *in vivo* methods has received less interest likely due to the challenges involved in such studies. In general, *in vivo* studies are time-consuming and involve higher costs. They also require more planning with regards to the use of appropriate experimental models, ethics of animal use, and optimal methods of sample collection and analysis. *In vivo* studies may be undertaken using animals as models for humans or human subjects directly.

The two main aspects that require consideration while planning *in vivo* studies on bioactive peptides are: a) whether bioactive peptides are generated from a given protein source during gastrointestinal digestion, and b) the ability of such peptides to be absorbed and reach the target tissue unaffected.

Among various food proteins, the liberation of bioactive peptides from milk proteins in the GIT has been widely studied. Different models have been used to investigate the release of bioactive peptides *in vivo* including calves, rats, pigs and healthy humans. The most preferred animal model for such studies is the pig model due to

the similarity in gut enzymes and the digestive physiology of pigs to that of humans (Deglaire et al., 2009). Meisel and Frister (1989) and more recently Barbe et al. (2014) using a pig model reported the release of a number of bioactive peptides *in vivo* with a range of bioactivities including ACE-I inhibition, opioid agonist, immunomodulatory, and antioxidant activity upon the digestion of milk casein fractions: α_{s1}, α_{s2}, β and κ- caseins. The results of this study correlate well with those of Boutrou et al. (2013) who reported similar bioactivities from casein-derived peptides released in the human jejunum. Interestingly, a comparison of results from these three studies reveals that the peptides with the same sequences exhibited similar bioactivities in both pig and human models. These peptides (from α_{s1}-casein) ranged from 4-30 residues in chain-length.

The structural attributes of the peptides that may be absorbed through the gut are not fully known, however, it is generally recognised that peptides of shorter chain length (2-5 amino acid residues) (Roberts et al., 1999) and peptides containing proline residues at the C-terminal may resist digestion (FitzGerald & Meisel, 2000), indicating that these peptides may be available for absorption. Notwithstanding this, peptides with chain lengths of up to 14 (Grimble et al., 1986) and 51 (Roberts et al., 1999) amino acid residues have also been reported to be absorbed. Shimizu et al. (1997) note a positive correlation between the hydrophobicity of peptides and their bioavailability.

The behaviour of a majority of the bioactivities associated with food-derived bioactive peptides are purportedly systemic in nature. Hence, the potential effect of these peptides will be highly dependent on their bioavailability *in vivo*. While there are very few studies that map the transit of specific dietary peptides to the assumed

target tissue (Masuda et al., 1996) or measure their absolute concentrations (Jeong et al., 2010) achieved *in vivo*, there are some studies that have explored the release of bioactive peptides in the gastrointestinal tract. For example, the ingestion of proteins and peptides from various food sources including milk, meat and barley have been found to exert effects such as opioid (Boutrou et al., 2013), antihypertensive (Bauchart et al., 2007; Boutrou et al., 2013), inhibition of platelet aggregation (Chabance et al., 1998), and suppression of tumours (Jeong et al., 2010) *in vivo*.

In an animal model or human subject, the detection of "active" peptides and a study of their systemic transit are complicated by multiple factors such as: a) the presumably low concentration of active peptides, b) the dynamic activity of luminal-brush border and plasma peptidases, c) the short plasma- and elimination half-life of peptides, and d) the presence of numerous peptides of body origin (Dallas et al., 2015; Foltz et al., 2010; Sato et al., 2008). This implies that, very few peptides might in fact enter systemic circulation unchanged, and further only a minimal concentration of bioactive peptides may be achieved in a target tissue. However, there are several peptide-mediated regulatory systems that are functioning within the GIT itself wherein various bioactive peptides may play a significant role.

With respect to the bioactive peptide classification that has been promulgated in this review, it is of note that in the case of TEnBP, the concept of bioavailability is not highly relevant, while in case of the ExBP, bioavailability is a key determining factor for the efficacy and any potential health effect. As the TEnBP are largely generated and/or present in the same location as where they exert their effect, and are also cell or tissue-specific in nature (eg. haemorphin-7 that is generated and found in plasma (Karelin et al., 1999) and in the brain (Murillo et al., 2006)), the

issue of bioavailability is irrelevant. However, it is clear from the discussion in this section, that for ExBPs to have a positive health effect they must either bind to cells or receptors present on the luminal side of the GIT, and/or they must survive GIT digestion, be absorbed into the bloodstream and reach the target tissue at an appropriate concentration to exert an effect.

Thus, in the context of this dissertation, the scope of the work presented in the subsequent chapters is restricted to investigating whether GEP are a source of bioactive peptides with measurable bioactivity that is relevant to the GIT itself.

In conclusion, in this dissertation, all of the three key approaches of *in silico*, *in vitro* and *in vivo* investigations discussed above have been used to investigate whether GEP are a source of bioactive peptides, and to examine the relevance of these bioactive peptides with respect to gut physiology.

2.6. Objectives

The main objective of the current study was to determine if GEP can be a source of bioactive peptides with gut modulatory potential. The experiments in this work were designed to study a wide range of gut relevant bioactivities, while also aiming to characterise both previously known and any potentially novel peptides responsible for the observed bioactivities.

2.6.1. Experimental approach

The experimental studies in this work have been divided into three main parts.

1. Bioinformatics-based determination of the bioactive potential of GEP
    - Investigation of the bioactive potential of a large range of GEP as whole intact proteins
    - Documentation of the resultant peptides obtained after simulated *in silico* gastric and small intestinal digestion of GEP
    - Analysing the bioactive potential of GEP based on the number of bioactive peptide sequences (previously documented sequences) predicted to be released after simulated *in silico* gastric and small intestinal digestion
    - Chemical synthesis of novel, potential bioactive peptide sequences and *in vitro* testing of bioactivity
2. Investigation of the *in vitro* gut modulatory potential of GEP
    - Analysing the GEP trypsin, mucin, lysozyme and serum albumin
    - Investigation of a range of gut relevant enzyme inhibitory (including ACE-I-, renin, PAF-AH, DPP-IV inhibition) peptides and the antioxidant potential of *in vitro* gastrointestinal digests of the GEP
    - Characterisation of the peptides responsible for the observed bioactivities
3. Investigation of the *in vitro* bioactivity of peptides obtained from an *in vivo* digestion of porcine GEP

- Collection of gastric (chyme) and small intestinal (digesta) endogenous contents from growing pigs that were fed a protein-free diet
- Purification of GEP and GEP-derived peptides from the chyme and digesta and testing for *in vitro* bioactivities.
- Characterisation of the peptides responsible for the observed bioactivities

2.6.2. Experimental techniques

Table 2.2 below provides a brief overview of the different experimental techniques used in the study reported here.

Table 2.2 *Overview of experimental techniques used in the study reported here*

Study	**Technique**
Preliminary mining of GEP	*In silico* databases: for extraction of GEP sequences
	BIOPEP database: for examining the presence of bioactive peptide sequences (previously documented/known)
	Peptide cutter tool: for simulating gastrointestinal digestion
	PeptideRanker tool: for ranking novel peptide sequences based on their structure and potential bioactivity
Chemical synthesis of peptides	Microwave assisted solid phase peptide synthesis
In vitro digestion	EU-COST INFOGEST *in vitro* digestion model

In vitro bioactivity assay	Enzyme inhibition bioassays: ACE-I inhibition, renin inhibition, PAF-AH inhibition, DPP-IV inhibition Antioxidant: FRAP, DPPH inhibition, ABTS, total antioxidant capacity based on Fe^{2+} reduction, MLP inhibition
Characterisation of protein and peptides	Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) Electrospray Ionization Time of Flight Mass Spectrometry (ESI-TOF MS) or Electrospray ionization time-of-flight liquid chromatography tandem mass spectrometry (ESI-TOF LC-MS/MS)
In vivo	Protein-free diet based animal (growing pig) model Purification of protein using ammonium sulphate-based precipitation

Chapter 3

3. Gastrointestinal Endogenous Proteins as a Source of Bioactive Peptides - An *In silico* Study

The contents of this chapter were published in a peer-reviewed journal article and have been adapted with permission from:

Dave, L. A., Montoya, C. A., Rutherfurd, S M., & Moughan, P. J. (2014). Gastrointestinal endogenous proteins as a source of bioactive peptides - An *in silico* study. *PLoS ONE, 9*(6), e98922. doi: 10.1371/journal.pone.0098922

3.1. Abstract

Dietary proteins are known to contain bioactive peptides that are released during digestion. Endogenous proteins secreted into the gastrointestinal tract represent a quantitatively greater supply of protein to the gut lumen than those of dietary origin. Many of these endogenous proteins are digested in the gastrointestinal tract but the possibility that these are also a source of bioactive peptides has not been considered. An *in silico* prediction method was used to test if bioactive peptides could be derived from the gastrointestinal digestion of gut endogenous proteins. Twenty six gut endogenous proteins and seven dietary proteins were evaluated. The peptides present after gastric and intestinal digestion were predicted based on the amino acid sequence of the proteins and the known specificities of the major gastrointestinal proteases. The predicted resultant peptides possessing amino acid sequences identical to those of known bioactive peptides were identified. After gastrointestinal digestion (based on the *in silico* simulation), the total number of bioactive peptides predicted to be released ranged from 1 (gliadin) to 55 (myosin) for the selected dietary proteins and from 1 (secretin) to 39 (mucin-5AC) for the selected gut endogenous proteins. Within the intact proteins and after simulated gastrointestinal digestion, ACE-I inhibitory peptide sequences were the most frequently observed in both the dietary and endogenous proteins. Among the dietary proteins, after *in silico* simulated gastrointestinal digestion, myosin was found to have the highest number of ACE-I inhibitory peptide sequences (49 peptides), while for the gut endogenous proteins, mucin-5AC had the greatest number of ACE-I inhibitory peptide sequences (38 peptides). Gut endogenous proteins may be an important source of bioactive

peptides in the gut particularly since gut endogenous proteins represent a quantitatively large and consistent source of protein.

3.2. Introduction

The main role of dietary proteins is to provide amino acids for body protein synthesis (FAO WHO and UNU, 2007). However, investigations over the last two decades have shown that dietary protein can also be a source of latent bioactive peptides (from 2 to greater than 40 amino acids long) that when released during digestion in the gastrointestinal tract can act as modulators of various physiological functions (Meisel, 1997; Rutherfurd-Markwick & Moughan, 2005; Shahidi & Zhong, 2008). These peptides are reported to possess a range of effects including antihypertensive, cholesterol-lowering, antioxidant, anticancer, immunomodulatory, antimicrobial, opioid, antiobesity and mineral binding effects (Meisel & Schlimme, 1990; Moller et al., 2008; Shahidi & Zhong, 2008). The most extensively studied dietary sources of these bioactive peptides include milk, egg, meat, soya and cereal proteins (Muro Urista et al., 2011; Rutherfurd-Markwick & Moughan, 2005; Ryan et al., 2011; Shahidi & Zhong, 2008). The bioactive peptides released during the digestion of dietary proteins are believed to act either within the gastrointestinal tract or are possibly absorbed into the bloodstream where they may act systemically (Boutrou et al., 2013; Moughan et al., 2007; Rutherfurd-Markwick & Moughan, 2005; Segura-Campos et al., 2011).

The supply of dietary proteins, and therefore the supply of gastrointestinal bioactive peptides derived from those proteins, will likely be highly variable as humans do not consume the same foods or amounts of food on a day to day basis. However, a

considerable amount of endogenous (non-dietary) protein is also present in the lumen of the gastrointestinal tract during digestion, consisting of proteins such as mucins, serum albumin, digestive enzymes, protein within sloughed epithelial cells and microbial protein, and this material may be a source of bioactive peptides (Moughan et al., 2014) . When compared to dietary protein, gut endogenous proteins represent a larger and more constant supply of protein in the gastrointestinal tract (Fuller & Reeds, 1998; Moughan & Rutherfurd, 2012; Souffrant et al., 1993), with endogenous nitrogen entering the digestive tract of humans being quantitatively equal or greater than the dietary nitrogen intake (Erickson & Kim, 1990; Miner-Williams et al., 2012; Miner-Williams et al., 2009; Moughan & Rutherfurd, 2012; Nyachoti et al., 1997). In a study conducted using pigs fed a casein-based diet, it has been reported that up to 80% of endogenous proteins are digested and reabsorbed by the end of the small intestine (Souffrant et al., 1993). During digestion a wide range of endogenous protein-derived peptides are likely to be generated, but the biological activity of such endogenously sourced gut peptides has not yet been considered. Potentially, gut endogenous proteins could be an important source of gut bioactive peptides given the amount of endogenous proteins present in the gastrointestinal tract. This study aimed to use an *in silico* approach to investigate whether known bioactive peptide sequences are present within the amino acid sequences of endogenous proteins secreted along the gastrointestinal tract and whether these bioactive peptides may potentially be released during enzymatic digestion in the human gastrointestinal tract. To our knowledge, the present study is the first to show that the amino acid sequences of gut endogenous proteins hold within them

abundant bioactive peptide sequences and that the possibility exists that these peptides are released during gastrointestinal digestion.

3.3. Materials and Methods

3.3.1. Materials

Twenty six known human gut endogenous proteins with well characterised amino acid sequences were examined. Additionally, 7 dietary proteins, which have been reported to contain bioactive peptides, were also examined (Erdmann et al., 2008; Minkiewicz et al., 2008b; Shahidi & Zhong, 2008; Terashima et al., 2010; Vercruysse et al., 2009). The proteins analysed are shown in Table 3.1.

Table 3.1 *Gut endogenous and dietary proteins examined in the in silico study*[1]

Protein/peptide classified based on the function in the body, with accession number[1]	Site of secretion within gastrointestinal tract	Chain length of mature protein[2] (No. of amino acid residues)
Lubrication, maintenance of integrity of tissue lining, cell signaling, immunity		
Mucin-2 (Q02817)	Small intestine and colon	5159
Mucin-3A (Q02505)	Small intestine	2520
Mucin-3B (Q9H195)	Small intestine and colon	901
Mucin-5AC (P98088)	Stomach, oesophagus and proximal duodenum	5003

Mucin-6 (Q6W4X9)	Stomach	2417
Mucin-7 (Q8TAX7)	Salivary gland -mouth	355
Mucin-13 (Q9H3R2)	Stomach, small intestine and colon	493
Mucin-15 (Q8N387)	Small intestine and colon	311
Mucin-20 (Q8N307)	Throughout the gut	684
Maintenance of colloid osmotic pressure and acid-base balance and transport		
Serum albumin (P02768)	From plasma into stomach and intestine	591
Enzymes in digestion		
Chymotrypsinogen B (P17538)	Pancreas	245
Chymotrypsinogen B_2 (Q6GPI1)	Pancreas	245
Gastric triacylglycerol lipase (P07098)	Stomach	379
Pancreatic amylase (P04746)	Pancreas	496
Pancreatic triacylglycerol lipase (P16233)	Pancreas	449
Pepsin (P00790)	Stomach	373
Salivary amylase (P04745)	From salivary gland into mouth	496
Trypsin (P07477)	Pancreas	232

Hormones		
Cholecystokinin (P06307)	Small intestine	95
Gastrin (P01350)	Stomach, duodenum, pancreas	80
Promotilin (P12872)	Small intestine (also affects gastric activity)	90
Secretin (P09683)	Duodenum (also affects gastric pH)	103
Somatostatin (P61278)	Stomach, intestine, pancreas	92
Other proteins/peptides involved in the regulation of specific processes in the digestive tract/immunity		
Gastric inhibitory peptide (P09681)	Stomach	132
Gastric intrinsic factor (P27352)	Stomach	399
Lysozyme C (P61626)	Throughout the gut	130
Dietary proteins		
β-casein, Bovine milk (P02666)	-	209
Gliadin, Wheat (P02863)	-	266
Glutenin, Wheat (P10385)	-	337
Glycinin, Soya (P04347)	-	492

Ovalbumin, Chicken egg (P01012)	-	386
Actin[3], chicken meat (P60706)	-	375
Myosin[3], chicken meat (P13538)	-	1939

[1]Compiled from the UniProtKB Protein Database (The UniProt Consortium, 2015).

[2]The given chain length excludes signal peptide.

[3]Initiator methionine not removed from the intact protein sequence (chain length inclusive of the initiator methionine).

3.3.2. Prediction of the total number of bioactive peptide sequences present in the intact gut endogenous and dietary proteins

To predict the number of bioactive peptide sequences encoded within the gut endogenous and dietary proteins, the amino acid sequence of each protein was obtained from an online protein database (The UniProt Consortium, 2015). The amino acid sequence of each protein was examined for the presence of known bioactive peptide sequences using an online bioactive peptide database (Minkiewicz et al., 2008b). The latter database contained the amino acid sequences of 2609 known bioactive peptides with 48 different bioactivities known to be bioactive based on either *in vitro* or *in vivo* studies (Iwaniak & Dziuba, 2009; Minkiewicz et al., 2008b).

The bioactive peptide database was used according to the instructions given and peptides possessing one or more of the following bioactivities were identified

antiamnestic, ACE-I inhibitor, antithrombotic, stimulating (glucose uptake-, -vasoactive substance release), regulating (ion flow-, stomach mucosal membrane activity-, phosphoinositol mechanism peptide-), antioxidative, bacterial permease ligand, inhibitor (dipeptidyl peptidase IV-, dipeptidyl-aminopeptidase IV-, dipeptidyl carboxypeptidase-, cyclic nucleotide phosphodiesterase (CaMPDE)-, neuropeptide-), hypotensive, activating ubiquitin mediated proteolysis. The total number of bioactive peptide sequences identified in the intact proteins was recorded for each gut endogenous and dietary protein.

The bioactive peptide frequency (A) and the relative bioactive peptide frequency (Y) are often used to describe the potency of proteins as sources of bioactive peptides (Minkiewicz et al., 2008b). In the present study the frequency of bioactive peptide sequences within the intact protein (A_O) was calculated as follows:

$$A_0 = \left(\frac{a_0}{N}\right) x\ 100$$

where, a_O is the total number of identified bioactive peptides present in the protein or the number of bioactive peptides with a specific activity based on the BIOPEP database (Minkiewicz et al., 2008b), N is the total number of amino acid residues within the protein.

The relative frequency of occurrence of bioactive peptides with a specific activity (Yj)[%]:

$$Yj = (Aj / \sum_{j=1}^{l} Aoj) \times 100$$

where, Aoj is the number of peptides with a specific activity, l is the total number of peptide sequences across all activity categories present within the protein, j is the specified activity.

Prediction of the frequency of potential bioactive peptide sequences present in the gastrointestinal tract after simulated digestion in the stomach, stomach and small intestine and small intestine alone (AD)

$$A_D = a_D/N \ x\ 1000$$

where, a_D is the number of identified bioactive peptides present after the simulated (*in silico*) digestion and N is the total number of amino acid residues within the protein.

3.3.3. Prediction of the total number of bioactive peptides to be released from the digestion of GEP

A prediction of the number of bioactive peptides that would be released from gut endogenous proteins and dietary proteins after upper gastrointestinal tract digestion was made using an *in silico* simulation based on the amino acid sequence of the proteins and the reported specificity of the major proteases present in the gastrointestinal tract. The site of secretion of the gut endogenous proteins was also taken into account. For the gut endogenous proteins secreted in the mouth and stomach and for the dietary proteins, gastric digestion was simulated *in silico* based on the amino acid sequence of the dietary or gut endogenous protein and the specificity of pepsin (Gasteiger et al., 2005; Keil, 1992). Gastric and small intestinal digestion was predicted based on the specificity of pepsin, trypsin and chymotrypsin (Gasteiger et al., 2005; Keil, 1992). For endogenous proteins secreted in the small

intestine, only small intestinal digestion was simulated taking into account the reported specificity of trypsin and chymotrypsin only. The amino acid sequences of the endogenous and dietary proteins were obtained from a protein sequence database as described above (The UniProt Consortium, 2015). The *in silico* simulated digestion was conducted using an online Peptide Cutter tool application (Gasteiger et al., 2005). The amino acid sequence of each of the predicted resultant peptides for each of the gut endogenous and dietary proteins was then compared to the amino acid sequence of known bioactive peptides using an online bioactive peptide sequence database (Minkiewicz et al., 2008b).

3.4. Results

3.4.1. The total number and frequency (A_O) of bioactive peptide sequences within the amino acid sequence of intact gut endogenous proteins and intact dietary proteins

Among the dietary proteins studied, the amino acid chain length of the proteins varied from 209 (*β*-casein) to 1939 (myosin) amino acids, while for the gut endogenous proteins the range was from 80 (human gastrin) to 5159 (human mucin-2) amino acids (Table 3.1). The total number of bioactive peptide sequences identified and their corresponding potential bioactivities, within the amino acid sequences of the intact gut endogenous and dietary proteins are shown in Table 3.2. In addition, the A_O values for each activity and for all the activities considered along with Y values for each of the proteins are also shown.

The total number of bioactive peptides, present within the amino acid sequences of the intact protein molecules for the gut endogenous proteins, ranged from 46 peptides for somatostatin to 2507 peptides for Mucin-5AC (Table 3.2).

Table 3.2 *Number (#) of potential bioactive peptides (per protein molecule) identified in the intact endogenous and dietary proteins and the A_O[1] and Y[2] values*

	Variable	Activity category[3]										Overall A_O[4]	Total No. of bioactive peptides [5]
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**		
Endogenous protein													
Mucin-2	#	34	1680	31	80	80	117	7	211	11	19		
	A_O	7	326	6	16	16	23	1	41	2	4	436	2250
	Y	2	75	1	4	4	5	0	9	0	1		
Mucin-3A	#	12	534	12	87	12	40	0	103	5	8		
	A_O	5	212	5	35	5	16	0	41	2	3	326	821
	Y	1	65	1	11	1	5	0	13	1	1		
Mucin-3B	#	5	221	7	44	6	9	0	47	3	6		
	A_O	6	245	8	49	7	10	0	52	3	7	391	352
	Y	1	63	2	13	2	3	0	13	1	2		
Mucin-5AC	#	104	1490	100	111	100	141	5	374	15	31	501	2507

	A_O	21	298	20	22	20	28	1	75	3	6		
	Y	4	59	4	4	4	6	0	15	1	1		
Mucin-6	#	30	726	33	53	32	96	0	184	8	14		
	A_O	12	300	14	25	13	40	0	76	3	6	494	1193
	Y	3	61	3	5	3	8	0	15	1	1		
Mucin-7	#	2	124	4	11	2	15	0	68	2	3		
	A_O	6	349	11	31	6	42	0	192	6	8	654	232
	Y	1	53	2	5	1	6	0	29	1	1		
Mucin-13	#	5	129	5	31	7	20	2	31	3	4		
	A_O	10	262	10	63	14	41	4	63	6	8	487	240
	Y	2	54	2	13	3	8	1	13	1	2		
Mucin-15	#	0	92	0	18	0	13	0	16	1	0		
	A_O	0	296	0	58	0	42	0	51	3	0	457	142
	Y	0	65	0	13	0	9	0	11	1	0		
Mucin-20	#	17	207	17	28	18	18	0	46	0	17		
	A_O	25	303	25	41	26	26	0	67	0	25	541	370
	Y	5	56	5	8	5	5	0	12	0	5		
Serum albumin	#	0	199	0	31	2	37	4	50	6	6	574	339
	A_O	0	337	0	52	3	63	7	85	10	10		

	Y	0	59	0	9	1	11	1	15	2	2		
Chymotrypsinogen B	#	3	83	4	14	3	9	1	21	2	2		
	A_O	12	339	16	57	12	37	4	86	8	8	584	143
	Y	2	58	3	10	2	6	1	15	1	1		
Chymotrypsinogen B2	#	2	81	3	14	2	9	1	22	2	2		
	A_O	8	331	12	57	8	37	4	90	8	8	567	139
	Y	1	58	2	10	1	6	1	16	1	1		
Gastric triacylglycerol lipase	#	1	138	1	15	1	14	3	32	3	10		
	A_O	3	364	3	40	3	42	8	84	8	26	586	222
	Y	0	62	0	7	0	7	1	14	1	5		
Pancreatic alpha amylase	#	3	183	3	7	5	26	0	29	4	4		
	A_O	6	369	6	14	10	52	0	58	8	8	540	268
	Y	1	68	1	3	2	10	0	11	1	1		
Pancreatic triacylglycerol lipase	#	2	167	2	18	2	25	2	26	6	1		
	A_O	4	372	4	40	4	56	4	58	13	2	563	253
	Y	1	66	1	7	1	10	1	10	2	0		
Pepsin A	#	3	140	2	18	3	20	2	25	2	4	598	223
	A_O	8	375	5	48	8	54	5	67	5	11		

	Y	1	63	1	8	1	9	1	11	1	2		
Salivary amylase	#	3	184	3	7	5	26	0	28	4	4		
	A_O	6	371	6	14	10	52	0	56	8	8	540	268
	Y	1	69	1	3	2	10	0	10	1	1		
Trypsin	#	3	81	3	8	5	8	0	14	1	1		
	A_O	13	349	13	34	22	34	0	60	4	4	539	125
	Y	2	65	2	6	4	6	0	11	1	1		
Cholecystokinin	#	0	38	0	4	1	3	0	9	0	3		
	A_O	0	400	0	42	11	32	0	95	0	32	611	58
	Y	0	66	0	7	2	5	0	16	0	5		
Gastrin	#	4	33	4	8	4	11	1	9	0	0		
	A_O	50	413	50	100	50	138	13	113	0	0	950	76
	Y	5	43	5	11	5	14	1	12	0	0		
Promotilin	#	1	32	1	6	1	4	1	10	1	0		
	A_O	11	356	11	67	11	44	11	111	11	0	644	58
	Y	2	55	2	10	2	7	2	17	2	0		
Secretin	#	3	50	3	7	3	4	0	13	0	1		
	A_O	29	485	29	68	29	39	0	126	0	10	835	86
	Y	3	58	3	8	3	5	0	15	0	1		

Somatostatin	#	0	27	0	2	0	3	0	9	0	3		
	A_O	0	293	0	22	0	33	0	98	0	33	500	46
	Y	0	59	0	4	0	7	0	20	0	7		
Gastric inhibitory peptide	#	1	33	2	9	2	2	2	13	0	4		
	A_O	8	250	15	68	15	15	15	98	0	30	515	68
	Y	1	49	3	13	3	3	3	19	0	6		
Gastric intrinsic factor	#	8	113	8	23	9	15	2	30	2	7		
	A_O	20	283	20	58	23	38	5	75	5	18	559	223
	Y	4	51	4	10	4	7	1	13	1	3		
Lysozyme C	#	1	45	1	1	2	4	0	8	1	5		
	A_O	8	346	8	8	15	31	0	62	8	38	538	70
	Y	1	64	1	1	3	6	0	11	1	7		

Dietary protein													
β-casein, Bovine milk	#	10	127	6	15	6	17	1	31	1	0		
	A_O	48	608	29	72	29	81	5	148	5	0	1105	231
	Y	4	55	3	6	3	7	0	13	0	0		
Gliadin, Wheat	#	1	94	0	11	0	11	0	22	1	1		
	A_O	4	353	0	41	0	41	0	83	4	4	797	212
	Y	0	44	0	5	0	5	0	10	0	0		
Glutenin, Wheat	#	2	82	0	7	0	7	0	28	1	3		
	A_O	6	243	0	21	0	21	0	83	3	9	439	148
	Y	1	55	0	5	0	5	0	19	1	2		
Glycinin, Soya	#	2	163	4	22	2	20	0	29	2	3		
	A_O	4	331	8	45	4	41	0	59	4	6	510	251
	Y	1	65	2	9	1	8	0	12	1	1		
Ovalbumin, Chicken egg	#	1	133	1	22	2	23	0	22	3	3		
	A_O	3	345	3	57	5	60	0	57	8	0	554	214
	Y	0	62	0	10	1	11	0	10	1	0		

Actin, chicken meat	#	2	149	2	18	3	15	0	28	1	4		
	A_O	5	397	5	48	8	40	0	75	3	11	597	224
	Y	1	67	1	8	1	7	0	13	0	2		
Myosin, chicken meat	#	1	641	4	112	6	127	37	93	11	23		
	A_O	1	331	2	58	3	65	19	48	6	12	553	1072
	Y	0	60	0	10	1	12	3	9	1	2		

[1] A_O is the frequency of occurrence of bioactive fragments in a protein sequence, calculated as $A_O = a_O/N \times 1000$

where, a_O is the total number of identified bioactive peptides present in the protein or the number of bioactive peptides with a specific activity based on the BIOPEP database (Minkiewicz et al., 2008b), N is the total number of amino acid residues within the protein.

[2]Y is the relative frequency of occurrence of bioactive fragments with a specific activity in a protein sequence, calculated as $Yj = (Aj / \sum_{j=1}^{l} Aoj) \times 100$

where, Aoj is the number of peptides with a specific activity, l is the total number of peptide sequences across all activity categories present within the protein, j is the specified activity.

[3]1 antiamnestic, 2 ACE-I -inhibitor, 3 antithrombotic, 4 stimulating (glucose uptake-, -vasoactive substance release), 5 regulating (ion flow-, stomach mucosal membrane activity-, phosphoinositol mechanism peptide-), 6 antioxidative, 7 bacterial permease ligand, 8 inhibitor (dipeptidyl peptidase IV inhibitor-, dipeptidyl-aminopeptidase IV inhibitor-, dipeptidyl carboxypeptidase-, CaMPDE-, neuropeptide-), 9 hypotensive, 10 activating ubiquitin mediated proteolysis.

[4]Overall A_O represents the total number of amino acid sequences corresponding to known bioactive peptides identified per protein molecule across all bioactivity categories normalised for amino acid chain length.

[5]The total number of bioactive peptides represents the total number of amino acid sequences corresponding to known bioactive peptides identified per protein molecule across all bioactivity categories (not just the 10 bioactivity categories shown above).

When based on the sub-classes of proteins presented in Table 3.1, the total number of identified bioactive peptide sequences present within the amino acid sequences of the intact protein molecules ranged from 142 - 2507 for the mucins, 339 for serum albumin, 125 - 268 for the digestive enzymes, 46 - 86 for the hormones and 68 - 223 for the remaining "other" proteins. For the dietary proteins, the total number of identified bioactive peptide sequences present within the amino acid sequence of the intact proteins ranged from 148 for glutenin to 1072 for myosin.

Among the observed bioactivity categories, angiotensin-I converting enzyme (ACE-I) inhibitory peptide sequences were present in the largest numbers for all of the examined dietary and gut endogenous proteins with Y ranging from 43% for gastrin to 75% for mucin-2 for the gut endogenous proteins and from 44% for gliadin to 67% for actin. For the gut endogenous proteins, the A_O for the ACE-I inhibitory peptide sequences ranged from 212 for mucin-3A to 485 for secretin while for the dietary proteins A_O for the ACE-I inhibitory peptide sequences ranged from 243 for glutenin to 608 for β-casein.

In addition to the 10 most abundantly observed bioactive peptide categories presented in Table 3.2, peptide sequences reportedly possessing other bioactivities were also observed in a few select proteins. For example, opioid peptide sequences were present within the amino acid sequences of all of the dietary proteins but only a few of the endogenous proteins. Similarly, coeliac toxic peptide sequences were present within the amino acid sequences of the wheat proteins gliadin and glutenin only.

3.4.2. Predicted number and frequency (AD) of bioactive peptides released after gastric digestion of dietary proteins and gut endogenous proteins based on an in silico simulation

The number of bioactive peptides (and their corresponding predicted bioactivities) predicted to be released after gastric digestion of gut endogenous proteins secreted in the mouth and stomach and of dietary proteins based on an *in silico* simulation of gastric digestion are presented in Table 3.3. The total number of bioactive peptides predicted to be released after gastric digestion of the gut endogenous proteins ranged from 0 to 12 bioactive peptides per protein molecule for lysozyme C and serum albumin respectively. When grouped into the protein subclasses shown in Table 3.1, the total number of predicted bioactive peptides after digestion was 1 - 11 peptides per molecule for the mucins, 12 for serum albumin, 2 - 8 for the digestive enzymes, 0 - 2 for the hormones and 0 - 4 for the "other" proteins. For the dietary proteins, between 1 (glutenin and gliadin) and 11 (myosin) bioactive peptides were predicted to be released per protein molecule after gastric digestion. When the number of predicted bioactive peptides was presented in relation to the number of amino acids in each protein, the A_D value for the mucins, serum albumin, digestive enzymes, hormones and "other" proteins was 1 - 6, 20, 4 - 21, 0 - 22 and 0 - 10 respectively. For the dietary proteins, the A_D value ranged from 3 for (glutenin and actin) to 14 for (β-casein).

Bioactive peptides with ACE-I inhibitory activity were predicted to be present after gastric digestion in higher numbers compared to peptides in the other activity categories with a total of 51 ACE-I inhibitory peptides predicted to be present post-digestion across all of the examined proteins as compared to 0 - 22 predicted

peptides for all of the other activity categories. Serum albumin and myosin were predicted to yield the largest number of ACE-I inhibitory peptides after peptic digestion with 8 ACE-I inhibitory peptides per protein molecule. This was closely followed by 7 ACE-I inhibitory peptides for mucin-5AC. Considerably fewer ACE-I inhibitory peptides were predicted (0 - 4 peptides per molecule) for the remaining gut endogenous and dietary proteins. The other predicted bioactivities with identified peptides were stimulating (glucose uptake-), inhibitor (dipeptidyl peptidase IV-, dipeptidyl-aminopeptidase IV-), and antioxidative activities and activation of ubiquitin mediated proteolysis.

Table 3.3. *Number of potential bioactive peptides (per protein molecule) predicted to be released and A_D[1] value after gastric digestion of both gut endogenous proteins secreted in the mouth and stomach and selected dietary proteins based on an in silico digestion model*

Protein	**Activity[2]**					**Overall A_D**	**Total No. of predicted bioactive peptides released[3,4]**
	2	**4**	**6**	**8**	**10**		
Endogenous protein							
Mucin-5AC	7	1				2	8
Mucin-6	4	5	2	3		5	11
Mucin-7		1	1	1		6	2
Mucin-13	1					2	1
Mucin-20	1					1	1
Serum albumin	8	3	1	3		20	12
Gastric triacylglycerol lipase	4	3		3	1	21	8
Pepsin A		1	1	1		5	2
Salivary amylase	1	1				4	2
Gastrin			1			13	1

Promotilin	2					22	2
Secretin						0	0
Somatostatin			1			11	1
Gastric inhibitory peptide		1		1		8	1
Gastric intrinsic factor	2	1		2		10	4
Lysozyme C						0	0
Dietary protein							
β-casein, Bovine milk	2	1	1	1		14	3
Gliadin, Wheat	1			1	1	4	1
Glutenin, Wheat	1					3	1
Glycinin, Soya	4					8	4
Ovalbumin, Chicken egg	4	1		1		13	5
Actin, chicken meat	1					3	1
Myosin,	8	3		4	1	6	11

chicken meat

[1]A_D is the frequency of occurrence of bioactive peptides after digestion of the protein, calculated as $A_D = a_D/N$ x 1000, where, a_D is the number of identified bioactive peptides present after the simulated (*in silico*) digestion and N is the total number of amino acid residues within the protein.

[2]2 ACE-I inhibitor, 4 stimulating (glucose uptake-, -vasoactive substance release), 6 antioxidative, 8 inhibitor (dipeptidyl peptidase IV inhibitor-, dipeptidyl-aminopeptidase IV inhibitor-, dipeptidyl carboxypeptidase-, CaMPDE-, neuropeptide-), 10 activating ubiquitin mediated proteolysis.

[3]The total number of peptides released is a summation of all the bioactive peptides predicted to be released after digestion of the intact proteins.

[4]Some of the predicted bioactive peptides have more than one activity. Hence, the total number of bioactive peptides released may be less than the summation of the number of bioactive peptides from the individual activity categories.

3.4.3. Predicted number and frequency (AD) of bioactive peptides released after gastric and small intestinal digestion of dietary proteins and gut endogenous proteins based on an in silico simulation

The total number of bioactive peptides predicted to be released after gastric and small intestinal digestion *in silico* for the gut endogenous proteins secreted into the mouth and stomach, and that therefore underwent digestion in the stomach and small intestine, ranged from 1 peptide per protein molecule for secretin to 39 peptides per protein molecule for mucin-5AC (Table 3.4). When the proteins were divided into

subclasses based on their functions as shown in Table 3.1, the predicted bioactive peptides released per protein molecule were 2 – 39 for the mucins, 22 for serum albumin, 4 – 15 for the digestive enzymes, 1 – 5 for the hormones and 3 – 10 for the "other" proteins.

Table 3.4 *Number of potential bioactive peptides (per protein molecule) predicted to be released and A_D[1] value after gastric plus small intestinal digestion for gut endogenous proteins secreted in the mouth and stomach and selected dietary proteins based on an in silico digestion model*

Protein	**Activity**[2]							**AD**	**Total No. of predicted bioactive peptides released**[3,4]
	2	**4**	**5**	**6**	**8**	**9**	**10**		
Endogenous protein									
Mucin-5AC	38			3				8	39
Mucin-6	17	4		5	3			9	22
Mucin-7	2	2		3	3	1		17	6
Mucin-13	6							12	6
Mucin-20	2							3	2

Serum albumin	17	3		4	2		37	22
Gastric triacylglycerol lipase	10	3		2	3		40	15
Pepsin A	3	1		1	3	1	11	4
Salivary amylase	8	2	1	3			22	11
Gastrin	2			1			38	3
Promotilin	4			1			56	5
Secretin	1						10	1
Somatostatin	1			1			22	2
Gastric inhibitory peptide	1	1			1		23	3
Gastric intrinsic factor	6	1		2	2		25	10
Lysozyme C	3			1			31	4
Dietary proteins								
β-casein, Bovine milk	8	1		1	1		38	8

Gliadin, Wheat	1							4	1
Glutenin, Wheat	2			1				9	3
Glycinin, Soya	10			4	3	1		30	15
Ovalbumin, Chicken egg	7	1		3	1			23	9
Actin, chicken meat	7			1	1			24	9
Myosin, chicken meat	49	4	1	4	5	1	1	28	55

[1]A_D value is the frequency of occurrence of bioactive peptides after digestion of the protein, calculated as $A_D = a_D/N \times 1000$ where, a_D is the number of identified bioactive peptides present after the simulated (*in silico*) digestion and N is the total number of amino acid residues within the protein.

[2]2 ACE-I inhibitor, 4 stimulating (glucose uptake-, -vasoactive substance release), 5 regulating (ion flow-, stomach mucosal membrane activity-), 6 antioxidative, 8 inhibitor (dipeptidyl peptidase IV inhibitor-, dipeptidyl-aminopeptidase IV inhibitor-, dipeptidyl carboxypeptidase-, CaMPDE-, neuropeptide-), 9 hypotensive, 10 activating ubiquitin mediated proteolysis.

[3]The total number of peptides released is a summation of all the bioactive peptides predicted to be released after digestion of the intact proteins.

[4]Some of the predicted bioactive peptides have more than one activity. Hence the total number of bioactive peptides released may be less than the summation of the number of bioactive peptides from the individual activity categories.

For the dietary proteins, the predicted number of bioactive peptides released after digestion (*in silico*) ranged from 1 for gliadin to 55 for myosin. When the size of the proteins were taken into account, the predicted A_D value for the mucins, serum albumin, digestive enzymes, hormones and "other" proteins was 3 – 17, 37, 11 – 40, 10 – 56 and 23 – 31 respectively. For the dietary proteins, the predicted A_D value ranged from 4 for gliadin to 38 for *β*-casein.

After *in silico* simulated gastric and small intestinal digestion, the most abundant bioactive peptides predicted to be present were the ACE-I inhibitory peptides ranging from 1 peptide per protein molecule for secretin, somatostatin, gastric inhibitory peptide and gliadin to 38 for mucin-5AC. Other gut endogenous proteins from which notable amounts of ACE-I inhibitory peptides were predicted to be released were mucin-6 (17 peptides per molecule), serum albumin (17 peptides per molecule) and gastric triacylglycerol lipase (10 peptides per molecule). Among the food proteins evaluated, myosin was predicted to yield the greatest number of ACE-I inhibitory peptides (49 peptides per molecule). Other bioactive peptides predicted to be present after gastric and small intestinal digestion (based on an *in silico* simulation) across all proteins were glucose uptake-or vasoactive substance release-stimulating (0 – 4 per molecule), dipeptidyl peptidase IV- or dipeptidyl-

aminopeptidase IV-inhibitor (0 – 5 peptides per molecule), antioxidative (0 – 5 peptides per molecule), ion flow- or stomach mucosal membrane activity- regulating (0 -1 peptides per molecule), and hypotensive (0 -1 peptides per molecule) peptides and peptides activating ubiquitin mediated proteolysis (0 – 1 peptides per molecule).

3.4.4. Predicted number and frequency (AD) of bioactive peptide sequences released after small intestinal digestion of gut endogenous proteins secreted in the small intestine based on an in silico simulation

For endogenous gut proteins that are secreted into the small intestine (for example, the pancreatic enzymes and small intestinal mucins) and therefore would not be subject to digestion in the stomach, an *in silico* analysis of the bioactive peptides that would be predicted to be released after intestinal digestion alone was performed (Table 3.5).

Table 3.5 *Number of potential bioactive peptides (per protein molecule) predicted to be released and A_D[1] value after small intestinal digestion for gut endogenous proteins secreted in the small intestine based on an in silico digestion model*

Protein	Activity[2]					A_D	Total No. of predicted bioactive peptides released[3,4]
	2	5	6	8	9		
Endogenous protein							
Mucin-2	18	2	4	4	4	5	24
Mucin-3A	12					5	12
Mucin-3B	9					10	9
Mucin-13	3					6	3
Mucin-15	1		2			6	2
Mucin-20	4					6	4
Serum albumin	9		6	1	1	24	14
Chymotrypsinogen B	5					20	5
Chymotrypsinogen B2	5					20	5
Pancreatic amylase	12	1	5			28	14

Pancreatic triacylglycerol lipase[5]	5			13	6
Trypsin	3		1	17	4
Cholecystokinin	1	1		21	2
Gastrin	2			25	2
Promotilin	1		1	22	2
Secretin	1			10	1
Somatostatin	2			22	2
Lysozyme C	2			15	2

[1]A_D value is the frequency of occurrence of bioactive peptides after digestion of the protein, calculated as $A_D = a_D/N \times 1000$ where, a_D is the number of identified bioactive peptides present after the simulated (*in silico*) digestion and N is the total number of amino acid residues within the protein.

[2]2 ACE-I inhibitor, 5 regulating (ion flow-, stomach mucosal membrane activity-, phosphoinositol mechanism peptide-), 6 antioxidative, 8 inhibitor (dipeptidyl peptidase IV inhibitor-, dipeptidyl-aminopeptidase IV inhibitor-, dipeptidyl carboxypeptidase-, CaMPDE-, neuropeptide-), 9 hypotensive.

[3]The total number of peptides released is a summation of all the bioactive peptides predicted to be released after digestion of the intact proteins.

[4]Some of the predicted bioactive peptides have more than one activity. Hence the total number of bioactive peptides released may be less than the summation of the number of bioactive peptides from the individual activity categories.

[5]Pancreatic triacylglycerol is predicted to release 1 immunostimulating peptide. Peptide not shown in the table, but is reflected in the corresponding total number of predicted bioactive peptides released.

For the proteins listed in Table 3.5 mucin-2, serum albumin and pancreatic amylase had the greatest predicted numbers of bioactive peptides released, with 24, 14 and 14 bioactive peptides respectively per molecule; while secretin had the least (1 peptide per molecule). Within the subclasses of proteins based on protein function and presented in Table 3.1, the predicted number of bioactive peptides released after digestion was 2 – 24 peptides per molecule for the mucins, 14 peptides per molecule for serum albumin, 4 – 14 peptides per molecule for the digestive enzymes and 1 - 2 peptides per molecule for the hormones and 2 peptides per molecule for lysozyme C. The corresponding A_D values were 5 – 10 for the mucins, 24 for serum albumin, 13 – 28 for the digestive enzymes and 10 - 25 for the hormones and 15 for lysozyme

3.5. Discussion

All of the protein amino acid sequences were sourced from the UniProt Protein Knowledgebase, a standard repository of protein sequences related information (The UniProt Consortium, 2015). BIOPEP, the database of bioactive peptides used in this chapter, is a widely recognised and utilised tool for the bioinformatics based prediction of bioactive peptides in a given amino acid sequence (Dziuba et al., 1999; Iwaniak & Dziuba, 2011; Minkiewicz et al., 2008b). The associated bioactivity of

the peptide sequences listed in the BIOPEP database is documented and continually updated based on previous and on-going *in vitro* and *in vivo* studies (Cheung et al., 2009; Iwaniak & Dziuba, 2009; Minkiewicz et al., 2008b; Minkiewicz et al., 2011). The resultant peptides generated after simulated gastrointestinal digestion were predicted using Peptide Cutter, an enzymatic cleavage prediction software (Gasteiger et al., 2005), that is hosted by the ExPASY server, a standard tool used in bioinformatics and mass spectrometry-based studies (Wilkins et al., 1999).

The findings of the present study are based on an *in silico* gastrointestinal digestion prediction-model. The model is based on the amino acid sequence (primary structure) of the intact proteins and knowledge about the specificity of proteases in the gastrointestinal tract. Being an *in silico* model, it cannot be concluded with certainty that the purported bioactive peptides will be generated after the actual *in vivo* gastrointestinal tract digestion of gut endogenous proteins. However, there are similarities between data generated in the presently reported study and data generated in other *in silico, in vitro* and *in vivo* studies. For example, in the present study *β*-casein was found to be the greatest potential source of bioactive peptides, including ACE-I inhibitory peptides. This finding is consistent with another *in silico* study that examined a range of food proteins and predicted that bovine caseins were the greatest source of ACE-I inhibitory peptides (Iwaniak & Dziuba, 2009). In addition, Boutrou et al. (2013) investigated the kinetics of the release of peptides from either casein or whey proteins in the jejunum of humans, and reported that *β*-casein released both larger numbers of bioactive peptide fragments and generated peptides with a diverse range of bioactivities. Moreover, and in line with our own findings, *in vitro* studies have shown that the antihypertensive peptides VPP and IPP

present in the amino acid sequence of bovine *β*-casein, which are known to be released during lactobacilli-based fermentation of milk (Nakamura et al., 1995), are not released during enzymatic digestion using an *in vitro* digestion model that simulated digestion in the gastrointestinal tract (Ohsawa et al., 2008).

Overall, the *in silico* technique used in the presently reported study does demonstrate that large numbers of bioactive peptide sequences do exist within the amino acid sequences of endogenous proteins that may be cleavable by the digestive enzymes and it is likely that in the process of digestion within the gut, bioactive peptides would be liberated from the gut endogenous proteins, particularly given that it is known from *in vivo* studies that as much as 80% of the endogenous protein secreted into the gastrointestinal tract is digested and reabsorbed (Souffrant et al., 1993). The presently reported study does not include analysis of two major contributors to the non-dietary nitrogenous losses in the gut, namely, bacterial proteins and the sloughed epithelial cells. Also factors that may influence *in vivo* protein digestion in the gastrointestinal tract, such as, the tertiary structure of the proteins, the effects of food processing on protein digestion, and the influence of bacterial enzymatic digestion have not been taken into account. An attempt has been made, however, to analyse a range of gut endogenous proteins secreted at different sites within the gut and with known amino acid sequences.

All of the dietary and gut endogenous proteins evaluated in the present study contained large numbers of peptide sequences within the greater amino acid sequence of the intact protein that corresponded to the sequences of known bioactive peptides, at least based on the BIOPEP bioactive peptide database (Minkiewicz et al., 2008b). Furthermore, the total number of bioactive peptide sequences present in

the overall amino acid sequence of the intact proteins varied across both dietary and gut endogenous proteins, although the range was much greater for the endogenous proteins. The mucin proteins generally contained the greatest number of bioactive peptide sequences while the hormone molecules contained the least. In comparison with the dietary proteins, 16 of the 26 gut endogenous proteins contained a similar or greater number of bioactive peptide sequences per molecule. This suggests that based on amino acid sequence, the gut endogenous proteins may contain quantitatively significant amounts of bioactive peptides. In general, for both the food and gut endogenous proteins, smaller proteins contained comparatively fewer bioactive peptide sequences when compared to the larger proteins. The latter observation indicated, not unexpectedly, that the longer the amino acid chain of a protein, the higher the probability of finding peptide sequences that correspond to previously studied and reported bioactive peptides documented in the BIOPEP database (Minkiewicz et al., 2008b).

If the gut endogenous proteins and food proteins are considered in terms of the potential bioactive profile (the relative number of bioactive peptide sequences within each bioactivity category), both gut endogenous and dietary proteins were similar with ACE-I inhibitory peptide sequences being present in the greatest numbers. This may be attributed to the fact that ACE-I inhibitory peptides have been researched more extensively in comparison to all of the other bioactivities and hence the bioactive peptide database used in the present study contains a much higher proportion of known ACE-I inhibitory peptides as compared to bioactive peptides with other activities (Martinez-Maqueda et al., 2012; Shahidi & Zhong, 2008). Both the gut endogenous proteins and the dietary proteins seem to contain remarkably

similar relative numbers of bioactive peptides within each activity category particularly given the very different amino acid sequences across the different proteins. For example, ACE-I inhibitory peptides comprised 43 – 75% of the total number of bioactive peptides found across the proteins while inhibitor peptides comprised 10 – 29%, antioxidative peptides comprised 3 – 14%, stimulating peptides comprised 3 – 13% and hypotensive peptides comprised 0 - 2%. Overall, large numbers of bioactive peptide sequences were observed in the intact gut endogenous protein amino acid sequences. In comparison to the dietary proteins examined in the present study, gut endogenous proteins were similar in terms of being a potential source of bioactive peptides.

Significant numbers of bioactive peptides were predicted to be released after gastric digestion (based on an *in silico* digestion model) of both food and gut endogenous proteins; however the numbers predicted were only 0 - 3.5% (average across all examined proteins = 1.0%) of the total number of bioactive peptide amino acid sequences identified in the intact amino acid sequences of each protein. It would appear, based on the *in silico* prediction used in the present study, that for both the dietary and gut endogenous proteins most of the predicted bioactive peptide sequences present in the intact proteins would not be released during gastric enzymic digestion. In terms of the bioactive peptides that were predicted to be released after gastric digestion, the gut endogenous proteins appeared to be similar to the dietary proteins both in terms of the total number of predicted bioactive peptides and the number of predicted bioactive peptides normalised for the amino acid chain length of the protein (A_D values).

The number of bioactive peptides predicted to be released after gastric and small intestinal digestion combined were considerably higher compared to gastric digestion alone but were still much fewer in comparison to the number of bioactive peptide amino acid sequences identified within the intact protein (3.3% of the total number of the identified bioactive peptides were predicted to be released across protein sources). It was predicted that after combined gastric and small intestinal digestion, many endogenous proteins were an equal source of bioactive peptides compared to the selected dietary proteins with a mean A_D across all of the endogenous proteins of 23 compared to 22 for the dietary proteins. Moreover at least two of the endogenous proteins had a greater A_D value in comparison with β-casein, a known rich source of bioactive peptides.

Not all gut endogenous proteins are secreted ubiquitously throughout the gastrointestinal tract (The UniProt Consortium, 2015). For example while serum albumin is known to be secreted into both the stomach and the small intestine (Brassinne, 1974; Herczeg, 1975), trypsin is only secreted into the duodenum and therefore is only subject to digestion in the small intestine. For proteins that are secreted in the small intestine, digestion in the gastrointestinal tract was predicted based on an *in silico* model for small intestinal digestion alone with the two major intestinal enzymes trypsin and chymotrypsin. The number of bioactive peptides predicted to be present after small intestinal digestion alone were much fewer in comparison to those predicted after both gastric and intestinal digestion. For example, the total number of bioactive peptides predicted to be released after small intestinal digestion of serum albumin (14 bioactive peptides per protein molecule) was much lower than that predicted for gastric and intestinal digestion (22 bioactive

peptides per protein molecule). Despite this, the results of the present study would predict that gut endogenous proteins secreted into the small intestine also appear to be significant sources of bioactive peptides.

After small intestinal digestion alone, the predicted released bioactive peptides possessed fewer bioactivities. For example, across all of the examined proteins (gut endogenous and dietary) the bioactive peptides predicted to be released after gastric and intestinal digestion had collectively up to 7 different bioactivities, while after small intestinal digestion alone, the predicted bioactive peptides collectively had only up to 3 different bioactivities, with an exception of serum albumin and mucin-2 which were predicted to release bioactive peptides in two additional bioactivity categories. Furthermore, for proteins that are secreted in both the stomach and small intestine, the same protein was predicted to release different bioactive peptide sequences in terms of total number and amino acid sequence depending on the site of digestion (gastric + small intestinal vs. small intestinal alone; Table 3.6).

For the most abundantly predicted bioactivity, ACE-I inhibition, based on the present *in silico* digestion model, it would appear that on a per molecule basis, gut endogenous proteins may be similar to dietary proteins in terms of the potential to release ACE-I inhibitory peptides in the upper gastrointestinal tract as a result of digestion.

The majority of the bioactive peptide sequences present in the amino acid sequence of the intact gut endogenous protein and after "*in silico"* digestion were di- or tri-peptides, while for the dietary proteins, bioactive peptides of 6 to 9 amino acids in length were also observed (Table 3.6).

Table 3.6 *Amino acid[1] sequences of bioactive peptides predicted to be released after mouth to ileum digestion of selected proteins based on an in silico digestion model.*

Protein	Bioactivity[2]	Gastric	Gastric + Small intestinal	Small intestinal
Gut endogenous				
Serum albumin				
	2	KA, IA, LF, QK, GM, RL, VE, AA	IA, LF, PR, LY, QK, AW, EK, GM, EY, AR, EK, KP, VR, VPK, VE, VK, AA	LF, LY, AW, NY, EY, AR, VF, VPK, TF
	4	LV, LL	LV	
	6	LHT	LHT, KP	LK, LY, TY
	8	KA, LL		EF
	9			EF
Somatostatin				
	2	AA	AA, QK	NF, TF
	6	EL	EL	
Dietary				
β-casein, bovine milk				

2	HL, PLP	AR, VK, HK, EMPFPK, HL, PLP, GPFPIIV
4	LI, LL	LI, LL
6	HL	HL
8	VA, LL	VA, LL
Ovalbumin, Chicken egg		
2	KE, YAEERYPIL, KG, EK	LY, LW, EK, VY, PR, GR
4	LL	LL
6		LK, LY, VY
8	LL	LL

[1]**A**ll amino acids are denoted using 'the one-letter notation for amino acid sequences' from the International Union of Pure and Applied Biochemistry and International Union of Biochemistry, 1971**:** Alanine = A; Arginine = R; Asparagine = N; Aspartic acid = D; Cysteine = C; Glutamic acid = E; Glutamine = Q; Glutamine or Glutamic acid = Z; Glycine = G; Histidine = H; Isoleucine = I; Leucine = L; Lysine = K; Methionine = M; Phenylalanine = F; Proline = P; Serine = S; Threonine = T; Tryptophan = W; Tyrosine = Y; Valine = V.

[2]2 ACE-I inhibitor, 4 stimulating (glucose uptake-, -vasoactive substance release), 6-antioxidative, 8 inhibitor (dipeptidyl peptidase IV inhibitor-, dipeptidyl-aminopeptidase IV inhibitor-, dipeptidyl carboxypeptidase-, CaMPDE-, neuropeptide-), 9 hypotensive.

The 3 known opioid agonist peptides in *β*-casein (5 to 11 amino acid long, data not shown) were also longer in chain length than the average bioactive peptide chain length observed in the gut endogenous proteins. In terms of the amino acid composition of the gut endogenous proteins evaluated, it is of note that, many of them contain significant amounts of glycine or proline or both, and it has been reported that a high content of glycine and proline is related to a higher probability of finding bioactive peptide fragments (Minkiewicz et al., 2011).

The results presented in in this study make no attempt to investigate the efficacy of bioactive peptides but rather provides an *in silico* prediction of the number and types of bioactive peptides that potentially can be generated in the gastrointestinal tract during digestion.

To put the current findings into context an attempt was made to predict the amounts of bioactive peptides that may be released into the gastrointestinal tract per day from either dietary protein or gut endogenous protein sources (Table 3.7). For the daily dietary protein intake, the food proteins examined in the presently reported study were used as ingredients for a theoretical diet. This model diet was formulated to contain approximately 40 g of protein which represents the Food and Agricultural Organisation of the United Nations' (FAO) recommended daily protein intake for a healthy adult weighing 60 kg (FAO WHO and UNU Expert Consultation, 2007). The proportion of each individual dietary protein was derived based on a model diet assumed to contain 127 g of dairy products, 128 g of wheat-based products, 25 g of soya products, 44 g (1, medium sized) egg and 46 g of roasted chicken (the vegetables, fruits, fats and sugars in the diet were omitted from the present estimations as they contain negligible amounts of proteins).

Table 3.7 *Predicted quantity of bioactive peptides (mg/d) released after digestion of either dietary proteins or gut endogenous proteins in the gastrointestinal tract*

	Predicted mean quantity of bioactive peptides released[1] (mg/g protein)	**Estimated quantity of bioactive peptides released after digestion in the gastrointestinal tract (mg/d)**		**Predicted (total) amount of bioactive peptides (mg/d)**
		Protein source[2]		
Dietary protein				
β-casein, bovine milk	87	Dairy	348	1842
Gliadin, wheat Glutenin, wheat	14	Wheat products	196	
Glycinin, soya	69	Soya products	207	
Ovalbumin, chicken egg	54	Chicken egg products	324	
Actin, chicken meat Myosin, chicken meat	59	Chicken meat	767	
Gut endogenous protein[3]				

Mucin			
Serum albumin			
Pepsin A	56	2689	2689
Gastrin			
Lysozyme C			

[1]Estimated based on the predicted total number of bioactive peptides released after gastric and small intestinal digestion (from Table 3.4), and the moles and molar masses of the respective proteins; and considering that the majority of the predicted bioactive peptides are 'dipeptides'. All of the evaluated food proteins are used as a model for the remaining proteins in the respective food product.

[2]The model diet is based on a recommended diet for a healthy adult weighing 60 kg, supplying 0.66 g/kg body weight protein per day, amounting to a protein intake of 40 g per day, designed to comply with the FAO recommendations (FAO WHO and UNU Expert Consultation, 2007); whereby dairy, wheat, soya products, chicken egg products, chicken meat contribute 4, 14, 3, 6 and 13 g of protein respectively; Protein content of food products estimated based on the USDA Nutrient Data Laboratory database (Nutrient Data Library, 2012)(Nutrient Data Library, 2012)(Nutrient Data Library, 2012)(Nutrient Data Library, 2012).

[3]Calculated based on Moughan, 2011 (Moughan, 2011), using the amount of gut endogenous protein nitrogen secreted into the gastrointestinal tract, but, excludes protein nitrogen derived from epithelial and bacterial cells.

The amount of endogenous protein secreted into the gastrointestinal tract was estimated based on the reported amounts of gut endogenous protein nitrogen secreted into the gastrointestinal tract, but excludes protein nitrogen derived from epithelial and bacterial cells (Moughan, 2011). Based on the model diet, it is predicted that in a healthy adult, dietary proteins may contribute 1842 mg, while the gut endogenous proteins (excluding microbial protein and sloughed cells) may yield up to 2689 mg of bioactive peptides per day. Given that microbial protein and sloughed cells, which make up approximately two thirds of the total gut non-dietary protein, were not included in the latter prediction it is likely that the amount of bioactive peptides derived from gut endogenous proteins would be much higher.

In conclusion, based on an *in silico* prediction it would appear that gut endogenous proteins may be an important and diverse source of bioactive peptides, in comparison with food proteins, particularly given that gut endogenous proteins are likely to be present in the gastrointestinal tract at a more constant concentration and composition than proteins derived from the diet. However, further *in vitro* and *in vivo* work is needed to corroborate the *in silico* predictions of the present study.

Chapter 4

4. Novel Dipeptidyl Peptidase IV Inhibitory and Antioxidant Peptides Derived from Human Gastrointestinal Endogenous Proteins

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Dave, L. A., Hayes, M, Rutherfurd, S M., & Moughan, P. J. (2016). Novel Dipeptidyl Peptidase IV Inhibitory and Antioxidant Peptides Derived from Human Gastrointestinal Endogenous Proteins. International journal of peptide research and therapeutics, 22, 355. doi:10.1007/s10989-016-9515-y

4.1. Abstract

Human gastrointestinal endogenous proteins (GEP) include the proteins mucins, serum albumin, digestive enzymes, and proteins from sloughed epithelial and microbial-cells. GEP play a vital role in the digestion of food, but are also simultaneously digested by proteases and peptidases of the gastrointestinal tract (GIT). Recent studies suggest that during gastrointestinal digestion, similar to dietary proteins, GEP may also give rise to bioactive peptides. In the present study, the protein sequences of 11 representative GEP were subjected to simulated *in silico* GIT (SIGIT) digestion. Following SIGIT digestion, 19 novel GEP-derived peptide sequences were selected using quantitative structure activity relationship rules for chemical synthesis. The peptides were then tested for their *in vitro* dipeptidyl peptidase IV (DPP-IV) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition, and for their ferric reducing antioxidant power (FRAP). Two novel DPP-IV inhibitory peptides with the amino acid sequences RPCF (IC_{50}=800.51± 49.00 μM) and MIM (IC_{50}=1056.78 ± 61.11 μM), and five novel antioxidant peptides CCK, RPCF, CRPK, QQCP and DCR were identified. The results of this study indicate that GEP are a significant source of bioactive peptides with potential novel bioactive peptide fragments within their sequences.

4.2. Introduction

Human gastrointestinal endogenous proteins (GEP) are a major source of non-dietary protein in the gastrointestinal tract (GIT) (Moughan & Rutherfurd, 2012). GEP include the digestive enzymes, hormones, mucins, serum albumin and other soluble proteins as well as proteins from desquamated epithelial cells (Moughan et al., 2014). In addition, proteins released from the GIT microbiome although not

strictly endogenous are often included in the GEP estimates. Between 65-200 g GEP is secreted into the GIT lumen on a daily basis (Chapter 2). During the digestion of dietary proteins, GEP are also exposed to gut proteases such as pepsin in the stomach, and trypsin, chymotrypsin and other di- and tripeptidases in the small intestine; and as a result significant amounts of GEP are digested and reabsorbed (Souffrant et al., 1993).

Dietary proteins are a well-known source of bioactive peptides and it has recently been suggested that like dietary proteins, GEP may also constitute a resource for bioactive peptide generation (Moughan et al., 2014). Bioactivities associated with food-derived peptides include among others, inhibition of enzymes key to the regulation of blood pressure including angiotensin-I converting enzyme (ACE-I), renin and dipeptidyl peptidase (IV) (DPP-IV), the latter of which also plays a role in the control of insulin secretion (Salles et al., 2015). Other bioactivities include antimicrobial, immunomodulatory, and antioxidant effects (Shahidi & Zhong, 2008). Food-derived bioactive peptides are believed to exert their effects either locally within the gut (Claustre et al., 2002) or systemically following absorption from the GIT (Masuda et al., 1996). DPP-IV is a widely expressed multifunctional serine peptidase belonging to the peptidase family S9 (prolyl-oligopeptidase family) (Rawlings et al., 2012), that plays an important role in the cardiovascular system, glucose uptake, insulin secretion and prevention of hyperglycemia, the latter being a hallmark for the development of diabetes mellitus (Holst, 2004; Salles et al., 2015). DPP-IV is also involved in the processing of gastrointestinal hormones that regulate satiety (Reinehr et al., 2010).

Apart from the various digestive and enzymatic processes occurring in the GIT, the gut lumen is also exposed to a large number of pro-oxidants and toxins (Halliwell et al., 2000). Antioxidant peptides play an important role in the prevention of oxidative and inflammatory damage to the lining of the GIT, and in the regulation of various immune-modulatory functions mediated through the GIT (Blau et al., 1999). Further, exogenously-derived antioxidant peptides also help in the activity of host endogenous antioxidant enzymes (Blau et al., 1999). To date, numerous peptides from dietary proteins with DPP-IV inhibitory and antioxidative effects have been reported using *in vitro* and *in vivo* models (Li-Chan et al., 2012; Power et al., 2013). However to date, there exists no similar experimental evidence for peptides derived from the GIT digestion of GEP.

The aim of this work was to apply *in silico* methodologies to identify GEP-derived peptide sequences with DPP-IV inhibitory and antioxidant activities. This chapter examines the role of GEP-derived novel bioactive peptides. The bioactivities of identified peptide sequences were confirmed by chemical synthesis of the peptides, *in vitro* bioassays and identification of the DPP-IV IC_{50}, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) inhibition and ferric reducing antioxidant power (FRAP) values for selected peptides.

4.3. Materials and Methods

4.3.1. Materials and Reagents

Sitagliptin (positive control for DPP-IV inhibition assay), DPPH radical, methanol antioxidant resveratrol 2,4,6-Tripyridyl-s-Triazine (TPTZ), Iron(III) chloride hexahydrate, ascorbic acid and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic

acid (TROLOX) were supplied by Sigma Aldrich (St. Louis, USA). The DPP-IV inhibition assay kit was supplied by Cambridge BioSciences (Cambridge, England, UK). All other chemicals used were of analytical grade (≥99% purity). Unless otherwise stated, all reagents were made using Milli-Q® de-ionised water.

4.3.2. Gastrointestinal endogenous proteins (GEP)

The primary structures (amino acid sequences) of eleven GEP protein (Table 4.1) including mucin 7 (MUC7- accession number Q8TAX7), serum albumin (SALB- accession number P02768), pepsin (PSN- accession number P0DJD7), salivary amylase (SAMY- accession number P04745), trypsin (TRYP- accession number P07477), cholecystokinin (CCK- accession number P06307), somatostatin-28 (SOM28- accession number P61278), Ghrelin-28 (GHR28- accession number Q9UBU3), lysozyme C (LYSC- accession number P61626), keratin, type II cytoskeletal 6A from oral mucosa (KERT- accession number P02538) and clustered regularly interspaced short palindromic repeat (CRISPR)-associated endoribonuclease Cas2 from *Lactobacillus rhamnosus*, strain ATCC 53103 / GG (GGCAE- accession number C7TEQ4) were obtained from the UniprotKB database (http://www.uniprot.org/help/uniprotkb).

4.3.3. Preliminary *in silico* analysis

Selected proteins were subjected to simulated digestion using an *In silico* model as previously described in Chapter 3 using Peptide Cutter tool located at http://web.expasy.org/peptide_cutter/ (Gasteiger et al., 2005). The GEP (Table 4.1) were subjected to a simulated *in silico* GIT (SIGIT) digestion using a combination of 3 major gut proteases, pepsin (pH >2.0; EC 3.4.23.1), trypsin (EC 3.4.21.4) and chymotrypsin high-specificity (EC 3.4.21.1) to simulate gastric and small intestinal

digestion as reported in Chapter 3. Since TRYP is secreted in the small intestine, it was subjected to SIGIT using trypsin and chymotrypsin. Novel peptide sequences, i.e short peptides with 2-5 amino acids chain length and with no previously attributed bioactivities, were obtained following a detailed search of the BIOPEP database tool located at http://www.uwm.edu.pl/biochemia/index.php/en/biopep (Minkiewicz et al., 2008b). The amino acid sequences of these novel peptides were then analysed using the known quantitative structure activity relationship (QSAR) properties for DPP-IV and antioxidant peptides (Al-Masri et al., 2008; Lacroix & Li-Chan, 2012; Li et al., 2011; Patil et al., 2015b). In addition, the potential of the selected novel peptide sequences to exert any biological effects associated with peptides was evaluated using the online tool PeptideRanker located at http://bioware.ucd.ie/~compass/biowareweb/Server_pages/peptideranker.php (Mooney et al., 2012b). Nineteen peptide sequences were selected for chemical synthesis and further *in vitro* bioactivity assessment based on their peptide ranker scores. Eight peptides were selected as potential DPP-IV inhibitory peptides. Nine peptides were selected for their potential to inhibit DPPH and all nineteen peptides were assessed for their ability to inhibit FRAP. The GEP sequences were mined for potential, novel DPP-IV and antioxidant peptides using the detailed methodology shown in Figure 4.1.

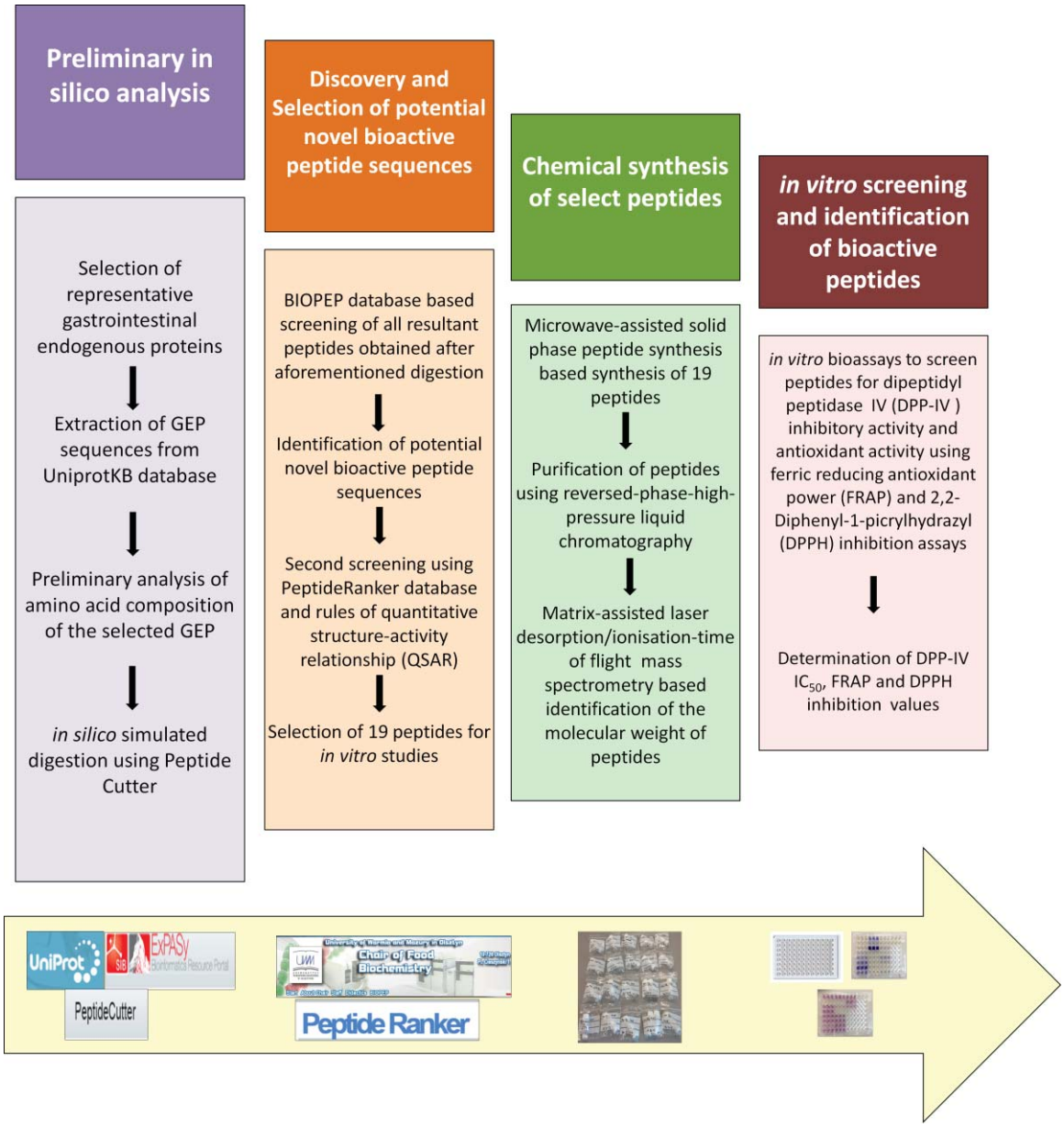

Figure 4.1 *Schematic representation of the combination of methodologies used for discovery and generation of dipeptidyl peptidase IV (DPP-IV) inhibitory and antioxidant peptides from human gastrointestinal endogenous proteins (GEP).*

The amino acid sequences of the selected eleven GEP proteins that are secreted and/released into the gastrointestinal tract (GIT) on a daily basis were obtained from the protein database UniprotKB (http://www.uniprot.org/help/uniprotkb). The protein sequences were

then subjected to simulated in silico gastrointestinal (SIGIT) digestion using the online tool Peptide Cutter (Gasteiger et al., 2005). The resultant peptides predicted to be present after SIGIT digestion were then screened against the online bioactive peptide database BIOPEP (Minkiewicz et al., 2008b) to identify novel peptide sequences. The rules of quantitative structure-activity relationship (QSAR) and the online PeptideRanker tool (Mooney et al., 2012a) were used to further evaluate the bioactive potential of the novel peptide sequences. 19 novel sequences were selected and chemically synthesised for in vitro confirmation of their DPP-IV inhibitory and/or antioxidant potential.

4.3.4. Chemical synthesis of selected peptides

The selected peptides were synthesised using a microwave-assisted solid phase peptide synthesis (MW-SPPS) process employing a Liberty CEM microwave peptide synthesiser (Mathews, NC, USA. H-Ala-HMPB-ChemMatrix) and H-Ile-HMPB-ChemMatrix resins (PCAS Biomatrix Inc., Quebec, Canada) were used for peptide synthesis. Each synthesised peptide was purified using reversed-phase-high-pressure liquid chromatography (RP-HPLC) on a Semi Preparative Jupiter Proteo (4 μm, 90Å) column (Phenomenex, Cheshire, UK). For each synthesised peptide, the HPLC fraction containing the peptide was identified based on the molecular weight of the compounds in each fraction which was in turn determined using matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry. The fractions were then pooled and freeze-dried on a Genevac HT 4X lysophiliser (Genevac Ltd., Ipswich, UK).

4.3.5. DPP-IV inhibition

DPP-IV inhibition activity was determined using a DPP-IV inhibitor screening assay kit (Cayman Chemical Company) in accordance with the manufacturer's instructions. Briefly, triplicate samples were each combined with methanol to obtain a final concentration of 1 mg/mL. A solution of human recombinant DPP-IV enzyme was prepared in assay buffer (20 mM Tris-HCL containing 100 mM NaCl and 1 mM EDTA, pH 8.0) and kept on ice prior to use. The substrate solution contained 5 mM Gly-Pro-Aminomethylcoumarin (AMC) in assay buffer. Sitagliptin, a known DPP-IV inhibitor, was used as a positive control. The test samples and the positive control solution were incubated with the combined enzyme and substrate solutions in a covered microwell plate at 37 °C for 30 min. The fluorescence intensity of the liberated AMC was measured using a FLUOstar Omega microplate reader (BMG LABTECH Gmbh, Offenburg, Germany) with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The percentage inhibition of DPP-IV was calculated as follows:

$$\%\ Inhibition\ DPP\text{-}IV = \frac{[RFU\ DPP\text{-}IV\ activity - RFU\ Inhibitor]}{RFU\ DPP\text{-}IV\ activity} \times 100$$

Where, RFU DPP-IV activity is the fluorescence (measured in relative fluorescence units (RFU)) of the 100% activity of DPP-IV enzyme without addition of any inhibitor, and RFU Inhibitor is the RFU in the presence of the Sitagliptin or the test peptides.

The DPP-IV IC_{50} values were determined for the most active peptides by plotting the percentage inhibition as a function of a range of concentrations of the active peptides.

4.3.6. DPPH inhibition

The DPPH inhibition assay was performed in accordance with previously described methods (Goupy et al., 2003; Nicklisch & Waite, 2014) with minor modifications. Briefly, triplicate solutions (1 mg/mL) of the peptide samples and the positive control (resveratrol) were prepared in methanol. The peptide and positive control solutions (285 µL) were each combined with a freshly prepared DPPH/methanol solution in a Nunc™ 96-well microWell™ plate to give a final DPPH concentration of 100 µM. The plate was incubated in the dark at room temperature (RT) for 30 min, and the absorbance was read at 515 nm. The control reaction mixture containing DPPH but no peptide was also incubated as described above. The percentage DPPH inhibition was calculated using the following equation:

$$\% \, Inhibition \, DPPH = \frac{[Abs \, Control - Abs \, Inhibitor]}{Abs \, Control} \times 100$$

Where, Abs Control is the absorbance of the DPPH solution without any resveratrol or test peptides and Abs Inhibitor is the absorbance of the tested samples or positive control.

4.3.7. FRAP bioassay

The ferric reducing ability related antioxidant potential of peptides was studied using the FRAP method previously described by Benzie and Strain (1996) with modifications as suggested by Bolanos de la Torre et al. (2015). The assay was performed in a Nunc™ 96-well microWell™ plate. Briefly, solutions containing the peptide samples (1 mg/mL) were prepared in Milli Q® water. Trolox at a concentration range of 15-420 µM was used to generate a standard curve. The FRAP working solution was freshly prepared by mixing 10:1:1:1.4 of acetate buffer (300

mM, pH 3.6), ferric chloride (20 mM in Milli Q® water), 2,4,6-Tripyridyl-s-Triazine (TPTZ) (10 mM in 40 mM HCl) and Milli Q® water respectively. The reaction was initiated by adding 280 µL of FRAP working solution to 20 µL of the test compound (chemically synthesised peptides) or standard. The samples were incubated at 37 °C in the dark for 30 min, and the absorbance was read at 593 nm. The FRAP values are expressed as µM Trolox equivalents per mg of peptide.

4.3.8. Statistical analysis

For each activity (DPPH-IV inhibition, DPPH inhibition and FRAP activity), each of the 19 synthetic peptides were analysed using three independent replicates (n=3). Results are presented as mean values ± standard deviation

4.4. Results

The amino acid chain length, molecular weight and UniprotKB accession numbers of the 11 GEP selected for use in the present study are given in Table 4.1. The chain length of the selected GEP ranged from 27 amino acids for Ghrelin-28 to 591 amino acids for serum albumin.

Table 4.1 *Details of human gastrointestinal endogenous proteins (GEP) studied in the in silico analysis*

	Protein		Uniprot Accession ID	Abbreviation	Chain length (Amino acids)	Mass (Da)
Host (Human) proteins	Secreted	Mucin 7	Q8TAX7	MUC7	355	39159
		Serum albumin	P02768	SALB	591	69367
		Pepsin	P0DJD7	PSN	373	41977
		Salivary amylase	P04745	SAMY	496	57768
		Trypsin	P07477	TRYP	232	26558
		Cholecystokinin	P06307	CCK	95	12669
		Somatostatin-28	P61278	SOM28	28	3151
		Ghrelin-28	Q9UBU3	GHR28	27	3245
		Lysozyme C	P61626	LYSC	130	16537
	Epithelial origin	Keratin, type II cytoskeletal 6A	P02538	KERT	564	60045

		(oral mucosa)				
Non-host proteins	Microbial origin	CRISPR-associated endoribonuclease Cas2 (*Lb. rhamnosus* (strain ATCC 53103 / GG))	C7TEQ4	GGCAE	101	11874

Following SIGIT digestion of the 11 selected GEP, over 500 resultant peptides were predicted to be released, and 73 of these peptide sequences corresponded to previously reported bioactive peptides documented in the BIOPEP database. Of these 73 bioactive peptides, 21 known DPP-IV inhibitory peptides and 16 known antioxidant peptides were identified (Table 4.2). MUC7, SALB, PSN, SAMY, TRYP, CCK, SOM28, GHR28, LYSC, KERT, GGCAE were each predicted to release 2, 2, 4, 0, 0, 0, 0, 0, 0, 7, and 6 DPP-IV inhibitory peptides, and 3, 4, 1, 3, 1, 0, 1, 0, 1, 2 and 0 antioxidant peptides respectively, after SIGIT. Following BIOPEP screening, from the total of over 427 unique peptides, 134 novel peptides between 2-5 amino acids in chain length were ranked using the PeptideRanker tool. Of the selected 134 peptides, 19 peptides with PeptideRanker scores closer to 1 were chosen for chemical synthesis and further analysis.

Table 4.2 *The number of known DPP-IV inhibitory peptides and antioxidant peptides predicted to be released after simulated in silico gastrointestinal (SIGIT) digestion of selected GEP.*

Protein[a]	**Number of known bioactive peptides generated after simulated *in silico* gastrointestinal digestion**[b]	
	DPP-IV inhibitory	**Antioxidant**
MUC7	2	3
SALB	2	4
PSN	4	1
SAMY	0	3
TRYP[c]	0	1
CCK	0	0
SOM28	0	1
GHR28	0	0
LYSC	0	1
KERT	7	2
GGCAE	6	0

[a] Mucin 7 (MUC7), serum albumin (SALB), pepsin (PSN), salivary amylase (SAMY), trypsin (TRYP), cholecystokinin (CCK), somatostatin-28 (SOM28), Ghrelin-28 (GHR28), lysozyme C (LYSC- accession number P61626), keratin, type

II cytoskeletal 6A from oral mucosa (KERT) and CRISPR-associated endoribonuclease Cas2 from *Lactobacillus rhamnosus*, strain ATCC 53103 / GG (GGCAE)

[b] SIGIT digestion for all proteins excepting TRYP was performed usingpepsin (pH >2.0; EC 3.4.23.1), trypsin (EC 3.4.21.4) and chymotrypsin high-specificity (EC 3.4.21.1) to simulate gastric and small intestinal digestion as previously reported. (Dave et al., 2014)

[c] TRYP which is secreted in the small intestine was subjected to SIGIT using trypsin and chymotrypsin alone

The bioactivity of peptides is known to be a direct result of 1) the independent properties of the constitutive amino acids and 2) the position of these amino acids within the peptide sequence (Li et al., 2011; Mooney et al., 2012b). Consequently, it is useful to examine the primary structure of the peptide as a means of pre-screening peptides for potential bioactivity prior to conducting more detailed investigations. Novel peptide sequences were analysed using QSAR to identify peptides with potential antioxidant and DPP-IV inhibitory activities. The online programme PeptideRanker (Mooney et al., 2012b) was also used to rank peptides in terms of their potential bioactivities. PeptideRanker ranks peptides on a scale from 0-1.0, and peptides closer to a score of 1.0 are predicted to have the greatest bioactive potential based on the amino acid composition and their location in the peptide (Mooney et al., 2012b).

Further, any peptide with a score ≥ 0.5 is considered to be bioactive (Mooney et al., 2012b). Nineteen peptides (Table 4.3) were selected for chemical synthesis and their

bioactivities assessed *in vitro*. The ranking performed by the PeptideRanker tool is a cumulative analysis based on all of previously studied, bioactivities associated with peptides, and not for individual bioactivities.

The PeptideRanker scores of the 19 selected peptides ranged from 0.46 (peptides AHP, CK and GSR) to 0.99 (peptide FPQW). The remaining top four peptides other than FPQW had amino acid peptide sequences corresponding to RPCF, MIM, GVF and AAF, with Peptide Ranker scores of 0.98, 0.87, 0.85 and 0.83, respectively. The majority of the selected peptides were tri-peptides (12 in total). Five tetra-peptides, 1 penta-peptide and 1 dipeptide were also chemically synthesised.

Following chemical synthesis, eight synthetic peptides (CK, MIM, FPQW, MPSDR, QQCP, RHPY, RPCF and SHF) were tested for DPP-IV inhibition activity. These peptides were selected for DPP-IV inhibition testing as previous studies indicated that dipeptides with a P residue at the C-terminal end or peptides with P at position 2 flanked by A and G residues on either side can potentially inhibit DPP-IV (Al-Masri et al., 2008; Hatanaka et al., 2012; Lafarga et al., 2014). Further, peptides with the sequence X-P where X is a hydrophobic amino acid and P is at the penultimate N-terminal position can also be DPP-IV inhibitors, as can tri-peptides with a GP-X sequence, wherein X can be a hydrophobic or non-hydrophobic residue (Hsu et al., 2013).

Table 4.3 *Molecular weight (MW) and PeptideRanker scores of peptides selected for chemical synthesis and in vitro bioassays.*

Peptide	**MW (kDa)**	**PeptideRanker[a] Score**	**Precursor protein[b]**
FPQW	0.58	0.99	PSN
RPCF	0.52	0.98	SALB
MIM	0.39	0.87	GGCAE
GVF	0.32	0.85	SALB
AAF	0.31	0.83	SALB
AHF	0.37	0.82	SAMY
SHF	0.39	0.80	MUC7
CCK	0.35	0.68	SALB
DCR	0.39	0.65	SAMY
CRPK	0.50	0.65	MUC7
QQCP	0.47	0.61	SALB
VAW	0.37	0.59	LYSC
RHPY	0.57	0.54	SALB
MSY	0.40	0.54	SAMY
MPSDR	0.60	0.54	SAMY
AAC	0.26	0.54	SALB

GSR	0.32	0.46	KERT
CK	0.25	0.46	SAMY
AHP	0.32	0.46	SAMY

All amino acids in peptide sequences above are denoted using 'the one-letter notation for amino acid sequences' from the International Union of Pure and Applied Biochemistry and International Union of Biochemistry, 1971.

[a] http://bioware.ucd.ie/~compass/biowareweb/Server_pages/peptideranker.php (Mooney et al., 2012b)

[b] Protein abbreviations: mucin 7 (MUC7), serum albumin (SALB), pepsin (PSN), salivary amylase (SAMY), trypsin (TRYP), cholecystokinin (CCK), somatostatin-28 (SOM28), Ghrelin-28 (GHR28), lysozyme C (LYSC- accession number P61626), keratin, type II cytoskeletal 6A from oral mucosa (KERT) and CRISPR-associated endoribonuclease Cas2 from *Lactobacillus rhamnosus*, strain ATCC 53103 / GG (GGCAE).

The peptide QQCP was also examined since an extensive search of the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep; Minkiewicz et al. (2008b)) showed that peptides containing glutamine were likely to be DPP-IV inhibitors. FPQW was tested since it had the greatest PeptideRanker score, while CK was tested as it was the sole dipeptide in the present study, The peptides MIM and MPSDR were selected as out of the 396 DPP-IV inhibitory peptides documented in BIOPEP, over 30 contain an M residue at the N-terminal. In addition, FPQW, MPSDR and RPCF were also selected because of the presence of P residue at

position 2, a sequence characteristic known to be present in DPP-IV inhibitors (Nongonierma & FitzGerald, 2014).

Nine peptides with the amino acid sequences (SHF, RHPY, RPCF, MSY, CRPK, CCK, DCR, VAW, QQCP) were tested for their ability to inhibit DPPH. These peptides were selected since, based on free radical-based studies, it has been suggested that peptides with hydrophobic and aromatic amino acids or bulky amino acid residues in the C-terminal position have antioxidant activity (Li & Li, 2013; Udenigwe & Aluko, 2011). Additionally, peptides containing cysteine and methionine residues were also selected due to the potential of the thiol group to participate in redox reactions (Giles et al., 2003; Udenigwe & Aluko, 2011). A majority of the synthesised peptides had amino acid sequences that indicated potential reducing ability, as the presence of aromatic hydrophobic amino acids such as Y, W and F and other hydrophobic residues such as M, A, V, L and I is known to favour the antioxidant capacity of peptides (Hernandez-Ledesma et al., 2005b; Pihlanto, 2006; Sarmadi & Ismail, 2010), in addition to the sulphur containing amino acids C and M are also known to contribute to the ferric reducing ability of peptides (Udenigwe & Aluko, 2011). Thus the antioxidant capacity of all of the 19 peptides were investigated using the FRAP assay method. However, only 14 peptides (VAW, CK, FPQW, CCK, MPSDR, CRPK, GVF, MIM, AHP, RHPY, MSY, RPCF, AAF AND QQCP) showed meaningful FRAP.

The DPP-IV inhibition values obtained for the selected peptides are presented in Figure 4.2. Among the selected synthetic peptides, the peptide RPCF was found to inhibit human DPP-IV by 77.8 (± 2.38)%. The peptide MIM inhibited DPP-IV by 65.8 (± 3.97)% while MPSDR and FPQW inhibited DPP-IV by 58.8 (± 2.31)% and

54.1 (± 2.21)%, respectively. Although QQCP and SHF were predicted to have DPP-IV inhibitory potential, they did not inhibit human DPP-IV enzyme *in vitro*. Further, the IC_{50} values of the two peptides that inhibited DPP-IV to the greatest extent were determined. The IC_{50} values were calculated by plotting the percentage inhibition as a function of the concentration of the peptide. RPCF and MIM had DPP-IV inhibition IC_{50} values of 801 μM and 1057 μM respectively (shown in Table 4.4). Sitagliptin (IC_{50} = 18-19 nM) (Thomas et al., 2008), an oral, hypoglycaemic drug (PubChem CID: 4369359) that is known to inhibit DPP-IV by competitive inhibition was used as a positive control in the assays at a final concentration of 100 μM, in accordance with the assay protocol, and was found to inhibit DPP-IV by 99.8 (± 0.14)%.

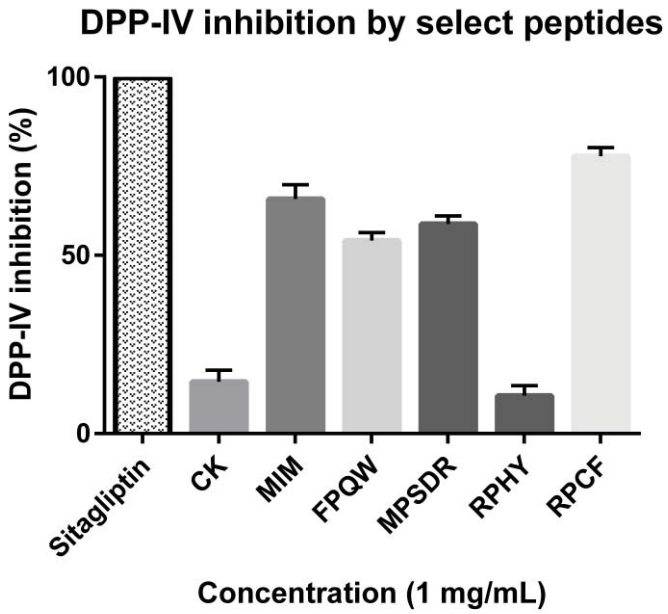

Figure 4.2 *The in vitro DPP-IV inhibitory activity (mean ± SD) of select chemically synthesised peptides tested at a sample concentration of 1 mg/mL. Sitagliptin was used as a positive control and was also tested at a concentration of 100 µM.*

Table 4.4 *The IC_{50} values for the two most active synthetic peptides among the total 19 peptides, RPCF and MIM.*

Peptide	DPP-IV inhibition IC_{50}[a] (µM)
RPCF	800.51 ± 49.00
MIM	1056.78 ± 61.11

[a] *The IC_{50} is defined as the concentration of a test compound required to achieve half maximal inhibition of the agonist/substance (McNaught & Wilkinson, 1997).*

Of the peptides assessed for DPPH inhibition (Figure 4.3), CCK, RPCF, QQCP, DCR and CRPK were found to be strong DPPH inhibitors with similar extents of inhibition with DPPH % inhibition values of 92.0 (± 0.65), 91.0 (± 2.00), 91.2 (± 0.80), 91.0 (± 0.34) and 90.4 (± 0.58)% respectively. Moreover, the latter peptides inhibited DPPH to a similar extent to that of the positive control, Resveratrol (89.9 ± 0.35). The peptides SHF and VAW inhibited DPPH by approximately 50%. The remaining two peptides RHPY and MSY were found to inhibit DPPH by less than 20%. The IC_{50} values for the most potent DPPH scavenging peptides CCK, RPCF and CRPK (Table 4.5) were estimated to be 28.95 ± 0.34, 497.88 ± 113.45 and 91.81 ± 17.64 μM, respectively. The IC_{50} of resveratrol was found to be 72 ± 11.1 μM.

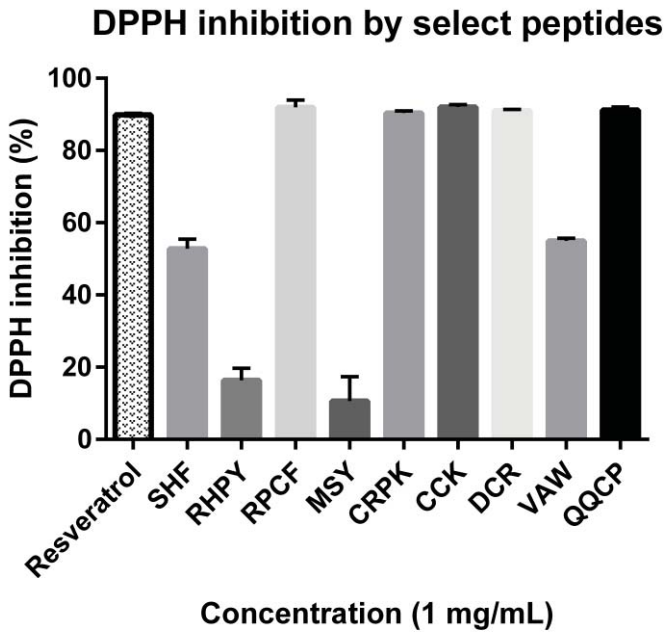

Figure 4.3 *The in vitro DPPH inhibition activity (mean ± SD) of selected peptides tested at a concentration of 1 mg/mL. Resveratrol was used as a positive control and was also tested at a concentration of 1 mg/mL.*

Table 4.5 *Estimated IC_{50} (± SD) of the antioxidant peptides CCK, RPCF and CRPK.*

Peptide	DPPH inhibition IC_{50} (μM)
CCK	28.95 ± 0.34
RPCF	497.88 ± 113.45
CRPK	91.81 ± 17.64

The antioxidant activity of the 14 selected synthetic peptides was also investigated in terms of their ferric reducing ability or electron transfer capacity using the FRAP bioassay. The FRAP values for the 14 peptides was expressed as μM Trolox equivalents per mg of peptide and the results are shown in Figure 4.4. The peptide CCK was found to have the greatest FRAP with a value of 1035.0 (± 92.53) μM Trolox equivalent per mg of peptide. CCK had a 4.5-fold higher FRAP than VAW, which had the second highest FRAP value of all the peptides tested. VAW had a FRAP of 229.4 (± 2.60) μM Trolox equivalent per mg of peptide. This was followed by the dipeptide CK which had a FRAP of 214.2 (± 19.81) μM Trolox equivalent per mg of peptide. The peptides MSY, QQCP AHP, AAF, RPCF, FPQW, MIM, GVF, RHPY and CRPK showed meaningful FRAP ranging from 109.9 (± 6.4) to 24.7 (± 3.85) μM Trolox equivalent per mg of peptide, in comparison to the pentapeptide MPSDR which showed negligible FRAP of 4.7 (± 2.07) μM Trolox equivalent per mg of peptide.

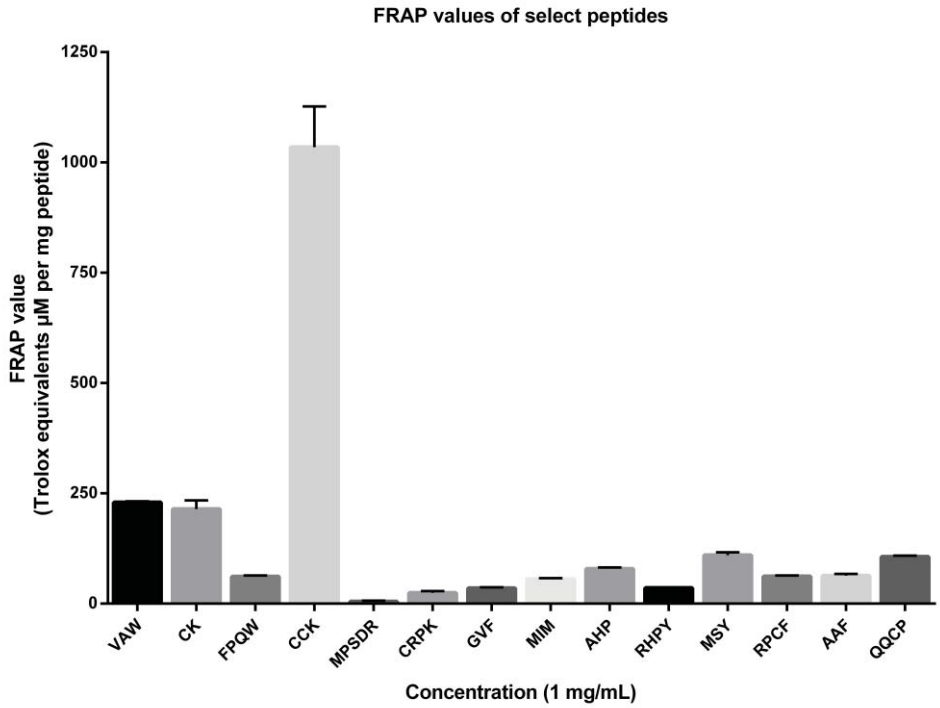

Figure 4.4 *Mean FRAP values (mean ± SD) of selected synthetic peptides. The values are expressed as the Trolox equivalents (µM per mg of peptide).*

4.5. Discussion

Bioactive peptides sequences have been reported from a wide range of proteins and include endogenous bioactive peptides (TEnBP), such as those present in haemoglobin (Lafarga et al., 2014) or exogenously derived bioactive peptides (ExBP) such as those present in dietary as well as endogenous proteins such as the GEP-derived bioactive peptides (Chapter 2, Shahidi and Zhong (2008)). GEP, as a potential source of bioactive peptides, are among the least unexplored. To date no reports exist concerning the release of DPP-IV inhibitory and antioxidant peptides from the GEP. For the GEP examined in the present study (SAMY, TRYP, CCK, SOM28, GHR28 and LYSC, CCK, GHR28 and GGCAE), it was predicted that following SIGIT digestion, 134 short bioactive peptides (2-5 amino acids in chain

length) would be released from the above mentioned proteins. Moreover, following general observations based on previously reported QSAR findings and reports about the constituting amino acids in DPP-IV and antioxidant peptides by other workers (Al-Masri et al., 2008; Lacroix & Li-Chan, 2012; Li & Yu, 2015; Patil et al., 2015b), it was predicted that a number of the peptides that were predicted to be released after SIGIT digestion may possess DPP-IV inhibitory or antioxidant activity. In addition, 14 of the selected 19 peptides chosen for chemical synthesis were predicted to be potential antioxidant peptides. Based on the rules of QSAR, of the 19 selected peptides, the peptides SHF, RHPY, RPCF, MSY, CRPK, CCK, DCR, VAW, and QQCP were predicted to have DPPH inhibitory activity, while all of the 19 peptides were predicted to have ferric ion reducing potential (FRAP). Peptide sequences from all of the selected proteins except for the proteins CCK, GHR28 and GGCAE were predicted to have FRAP or DPPH inhibitory potential. The human protein SALB is a known antioxidant (Taverna et al., 2013), while bovine and porcine serum albumins are known sources of opioid, ACE-I inhibitory, antioxidant and a few DPP-IV inhibitory and gut-motility modulatory peptides (Bah et al., 2013; Takahashi et al., 1998). Of the seven synthesised peptides that are present in SALB, the peptide RPCF was found to be a potent DPP-IV inhibitor with an IC_{50} of 801 μM. The IC_{50} of the peptide RPCF is significantly lower than that of several other known dietary peptidic DPP-IV inhibitors listed in Table 4.6, and is within the generally comparable values reported for peptides from various food sources ranging from cereals to meat proteins (BIOPEP, 2015; Minkiewicz et al., 2008b).

Table 4.6 *Peptide subsequences matching with fragments within the intact novel peptides investigated in the present study (Data collated from BIOPEP (BIOPEP, 2015; Minkiewicz et al., 2008b).*

Protein	Peptide	Peptide Subsequence match found in BIOPEP	Source protein	Reported bioactivity[a]	Reported EC_{50} (µM)	Activity discovered in the present study[a,b]	Estimate of IC_{50} in the present study (µM)
PSN	FPQW	FP	cheese whey protein	ACE-I inhibitor	315	DPP-IV inhibitory; Antioxidant (FRAP)	-
			Rice bran	DPP-IV inhibitor	363		
			Milk hydrolysates	ACE-I inhibitor	0		
		PQ	Dipeptide library/Soy protein hydrolysate	DPP-IV inhibitor	0		
		QW	Dipeptide library/Soy protein	DPP-IV inhibitor	0		

			hydrolysate				
SALB	RPCF	RP	-	ACE-I inhibitor	180	DPP-IV inhibitory; Antioxidant (DPPH inhibition and FRAP)	DPP-IV inhibition IC_{50} = 801
			Rice bran	DPP-IV inhibitor	2240		
		CF	Shark meat hydrolysate	ACE-I inhibitor	1.96		
GGCAE	MIM	IM	Dipeptide library/Soy protein hydrolysate	DPP-IV inhibitor	0	DPP-IV inhibitory; Antioxidant (FRAP)	DPP-IV inhibition IC_{50} = 1057
		MI	Dipeptide library/Soy protein hydrolysate	DPP-IV inhibitor	0		
SALB	GVF	VF	Sardine muscle	ACE-I inhibitor	9.2	Antioxidant (FRAP)	-
			Dipeptide library/Soy protein	DPP-IV inhibitor	0		

			hydrolysate			
			-	ACE-I inhibitor	4600	
		GV	Dipeptide library/Soy protein hydrolysate	DPP-IV inhibitor	0	
			-	ACE-I inhibitor	190	
		AF	Dipeptide library/Soy protein hydrolysate	DPP-IV inhibitor	0	
SALB	AAF					Antioxidant (FRAP) -
			-	ACE-I inhibitor	620	
		AA	Spanish dry-cured ham	DPP-IV inhibitor	9400	
			Milk hydrolysates	ACE-I inhibitor	0	
SAMY	AHF	AH				- -
			egg white albumin	Antioxidant	0	

			Dipeptide library/Soy protein hydrolysate	DPP-IV inhibitor	0		
		HF	Dipeptide library/Soy protein hydrolysate	DPP-IV inhibitor	0		
MUC7	SHF	HF	Dipeptide library/Soy protein hydrolysate	DPP-IV inhibitor	0	Antioxidant (DPPH inhibition)	-
		SH	Dipeptide library/Soy protein hydrolysate	DPP-IV inhibitor	0		
SALB	CCK	No peptide susbsequence match found	-	-	-	Antioxidant (DPPH inhibition and FRAP)	-

SAMY	DCR	No peptide susbsequence match found	-	-	-	Antioxidant (DPPH inhibition)	-
MUC7	CRPK	RP	-	ACE inhibitor	180	Antioxidant (DPPH inhibition and FRAP)	-
			Rice bran	DPP-IV inhibitor	2240		
		PK	Dipeptide library/Soy protein hydrolysate	DPP-IV inhibitor	0		
SALB	QQCP	QQ	Dipeptide library/Soy protein hydrolysate	DPP-IV inhibitor	0	Antioxidant (DPPH inhibition and FRAP)	-
LYSC	VAW	AW	Finnish cereals	ACE inhibitor	10	Antioxidant (DPPH inhibition and FRAP)	-
			marine bivalve (Mactra	Antioxidant	0		

			veneriformis)				
			milk protein	DPP-IV inhibitor	0		
		VA	-	DPP-IV inhibitor	168.24		
SALB	RHPY	HP	Milk hydrolysates	ACE-I inhibitor	0	DPP-IV inhibitory; Antioxidant (DPPH inhibition and FRAP)	-
			Rice bran	DPP-IV inhibitor	2820		
		PY	Dipeptide library/Soy protein hydrolysate	DPP-IV inhibitor	0		
		RH	Dipeptide library/Soy protein hydrolysate	DPP-IV inhibitor	0		
SAMY	MSY	SY	Garlic	ACE-I inhibitor	66.3	Antioxidant (DPPH inhibition and FRAP)	-
				DPP-IV inhibitor	0		

SAMY	MPSDR	MP	rice bran	DPP-IV inhibitor	870	DPP-IV inhibition and antioxdant (FRAP)	-
		DR	Dipeptide library/Soy protein hydrolysate	DPP-IV inhibitor	0		
		PS	Dipeptide library/Soy protein hydrolysate	DPP-IV inhibitor	0		
SALB	AAC	AA	-	ACE-I inhibitor	620	-	-
			Spanish dry-cured ham	DPP-IV inhibitor	9400		
KERT	GSR	GS	-	ACE-I inhibitor	3800	-	-
SAMY	CK	No peptide susbsequence match found	-	-	-	DPP-IV inhibition and antioxdant	

						(FRAP)	
SAMY	AHP	AH	Milk hydrolysates	ACE-I inhibitor	0	Antioxidant (FRAP)	-
			egg white albumin	Antioxidant	0		
			Dipeptide library/Soy protein hydrolysate	DPP-IV inhibitor	0		
		HP	Milk hydrolysates	ACE-I inhibitor	0		
			Rice bran	DPP-IV inhibitor	2820		

All amino acids in peptide sequences are denoted using 'the one-letter notation for amino acid sequences' from the International Union of Pure and Applied Biochemistry and International Union of Biochemistry, 1971.

[a] ACE-I, angiotensin-I converting enzyme; DPP-IV, Dipeptidyl peptidase

[b] DPPH, 2,2-Diphenyl-1-picrylhydrazyl; FRAP, Ferric reducing antioxidant power

While to date, the peptide RPCF has not been identified as a bioactive peptide, the peptides RP and CF, both within the RPCF peptide, have previously been reported to have ACE-I inhibitory activity, and RP, from rice bran has also been reported to have DPP-IV inhibitory activity (IC_{50} of 2240 μM). Interestingly, the IC_{50} of the larger peptide RPCF was 2.8-fold lower than the IC_{50} of the peptide RP. Diprotinin A, an antihyperglycaemic drug with the peptide sequence IPI is reported to have an EC_{50} of 7.4 μM (Maruyama et al., 1996), while DPP-IV inhibitory peptides derived from food source are reported to have a wide range of IC_{50} values ranging from 5860.0 μM for peptide PP derived from rice bran hydrolysate (Hatanaka et al., 2012), to 37.8 μM for peptide WR found in the milk protein lactoferrin (Nongonierma & FitzGerald, 2013).

The protein and consequent bioactive peptide contribution of microbial protein is often overlooked since the overall total quantities in the gut are assumed to be low. A large number of the gut microflora inhabit the lower GIT, however, and the total microbial protein content may contribute up to 45% of the GEP in mammals (Miner-Williams et al., 2009). In this work, the microbial protein GGCAE was predicted as a source of the peptide MIM after SIGIT digestion, and the tripeptide was found to be a potent inhibitor of DPP-IV, with an IC_{50} of 1057 μM. Besides SALB, the proteins PSN and SAMY (major gut digestive enzymes) were also found to be a source of the DPP-IV inhibitory peptides FPQW and MPSDR. Given that DPP-IV is abundantly expressed in the brush border membranes of the small intestine, the role of DPP-IV inhibitors in the GIT is important. A recent study carried out by Waget et al. (2011) reported that upon oral administration of the well-known DPP-IV inhibitor sitagliptin to GLP-1 receptor of wild type mice and GLP-1 receptor lacking

mice, the former showed improved glucose tolerance while the latter group showed no change. The latter results suggest that DPP-IV inhibitors within the GIT may prevent GLP-1 degradation related hyperglycaemia.

The antioxidant potential of the synthetic peptides was assessed using the DPPH inhibition assay and the FRAP bioassay. The protein SALB was a source of DPPH scavenging peptides including CCK, RPCF and QQCP. The protein MUC7 also contained a potent DPPH inhibitory peptide with the sequence CRPK and a moderate DPPH inhibitory peptide with the sequence SHF. The GEP SAMY and LYSC also contained DPPH inhibitory peptides DCR and VAW respectively. The peptides CCK, RPCF, QQCP, DCR and CRPK inhibited DPPH to a significant extent (over 90% inhibition). The antioxidant properties of a peptide are determined by its amino acid composition and the electronic property, hydrophobicity and molecular size of the amino acid residues (Udenigwe & Aluko, 2011). All of the peptides that inhibited DPPH to the greatest extent contained cysteine which in turn contains a sulfhydryl group that is highly susceptible to oxidation and is likely to be largely responsible for the reducing abilities of the latter peptide (Udenigwe & Aluko, 2011). Apart from cysteine residues, DPPH can also be quenched by hydrophobic amino acids, amino acids with higher electronic density (such as proline, histidine, tryptophan), and amino acids with less bulky structures or low molecular weights (such as glycine, valine and threonine) (Udenigwe & Aluko, 2011). The IC_{50} of the peptide CCK (28.95 ± 0.34 μM) was also the lowest among the three best DPPH inhibitors in the present study (CCK, RPCF, and CRPK) and is also comparable to two potent milk protein-derived peptides (WC and VW), which

are known to have an EC_{50} (IC_{50}) in the range of 0.26 mM for WC and 654.2 mM for VW (Nongonierma & FitzGerald, 2015a).

The SALB-derived peptide CCK was also found to have an exceptionally high FRAP activity (1035.00 ± 92.53 μM Trolox equivalent/mg of peptide). In comparison, methanolic extracts of different varieties of Gauva, a fruit that contains exceptionally high amounts of ascorbic acid (50-300 mg/100 g fresh weight basis), were reported to have FRAP activity in the range of 15.5 (± 1.4) to 33.3 (± 1.4) μM Trolox equivalent/g of fresh weight of the fruits (Thaipong et al., 2006). The average FRAP values of serum from healthy subjects has been reported to be around 418 μM Trolox equivalent/L (Cao & Prior, 1998). As in the case of DPPH inhibition, the high FRAP value of CCK is also likely due to the presence of cysteine residues in the peptide, since it is known that the presence of cysteine, methionine and glutamine residues is known to greatly enhance the ferric reducing ability of protein hydrolysates (Aluko, 2012b; Karel et al., 1966). The present findings are in agreement with the findings of Iskandar et al. (2015) who studied the effect of high hydrostatic pressure pretreatment and a modified digestion protocol on the antioxidant properties of whey protein isolate (WPI). Iskandar et al. (2015) reported that the high pressure treatment enhanced both the *in vitro* digestibility and FRAP values of the WPI, and the higher FRAP values were attributed to the high content of cysteine and methionine in WPI. Our findings are also consistent with those of Udenigwe and Aluko (2011), who have reported, using a partial least squares regression model, that cysteine residues contribute positively towards the ferric ion reducing potential of a peptide.

For many of the 19 synthesised peptides investigated in the present work, there were smaller previously reported bioactive peptide sequences (Minkiewicz et al., 2008b) present within the peptides sequence (Table 4.6). For example, the LYSC-derived peptide VAW contains the previously reported bioactive peptides VA and AW (BIOPEP, 2015). The dipeptide VA was reported to be present in cereals protein, bivalve mollusks (*Mactra veneriformis*) and milk protein and was reported to have antioxidative, ACE-I and DPP-IV inhibitory activity, while VA is documented to be a DPP-IV inhibitor in the BIOPEP database. Similarly, the SAMY-derived novel peptide MSY contains the peptide SY within its structure. SY, present in garlic protein, has been reported to have ACE-I and DPP-IV inhibitory activity. Conversely, several of the synthesised peptides investigated in the present study have been reported to be present within amino acid sequence of larger bioactive peptides. For example, the peptide VAW is present in the peptide Soystatin (VAWWMY), a known hypocholesterolemic peptide that is present in soyabean glycinin (Nagaoka et al., 2010). Similarly, MSY is encrypted in the antioxidative peptide LMSYMWSTSM that was detected in the hydrolysate of lecithin-free egg yolk proteins (Park et al., 2001). It is interesting to note that that there are some of the same bioactive peptide sequences present in both food proteins and GEP (Lafarga et al., 2014; Minkiewicz et al., 2015). Further, the ubiquitous presence of common subsequences also highlights the need to investigate each protein and peptide fragment in its own right, in order to determine (i) the most potent sources of bioactive peptides, and (ii) which proteins will yield the bioactive peptide sequences *in vivo* after GIT digestion, at a physiologically relevant concentration.

Given that this study utilised *in silico* and *in vitro* approaches, it cannot be concluded that the novel peptides discovered here will be generated in sufficient quantities in the GIT to exert a physiological effect either locally within the GIT or systemically. Notwithstanding these limitations, this study has demonstrated that it is possible that 11 novel peptides, four with potential DPP-IV inhibitory activities (RPCF, MIM, MPSDR and FPQW) and eight with antioxidant properties (CCK, RPCF, QQCP, DCR, CRPK, SHF, VAW, CK) may be derived from the GIT digestion of GEP.

In conclusion, in the present study, using a combination of an *in silico* approach, chemical synthesis of peptides and *in vitro* bioassay techniques, the potential of GEP as a source of novel bioactive peptides was examined. The key findings of this work include two potent DPP-IV inhibitory peptides with the amino acid sequences RPCF and MIM and five antioxidative peptides with the amino acid sequences CCK, RPCF, CRPK, DCR and QQCP. This work is the first to demonstrate the potential of GEP as a source of DPP-IV inhibitory and antioxidant peptides, and it appears that GEP may be an important source of exogenous bioactive peptides (ExBP) in animals. Further work is needed to corroborate these findings *in vivo*.

Chapter 5

5. Gastrointestinal Endogenous Protein-Derived Bioactive Peptides: An *in vitro* Study of Their Gut Modulatory Potential

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Dave, L. A., Hayes, M., Mora, L., Montoya, C. A., Moughan, P. J., & Rutherfurd, S M. (2016). Gastrointestinal endogenous protein-derived bioactive peptides: An *in vitro* study of their gut modulatory potential. International Journal of Molecular Sciences, 17(4), 482.

5.1. Abstract

This study tests the hypothesis that like their dietary counterparts, digestion of gastrointestinal endogenous proteins (GEP) may also produce bioactive peptides by *in vitro* digests of four GEP namely; trypsin (TRYP), lysozyme (LYS), mucin (MUC), serum albumin (SA) and a dietary protein chicken albumin (CA). The peptides from these proteins were screened for their angiotensin-I converting (ACE-I), renin, platelet-activating factor-acetylhydrolase (PAF-AH) and dipeptidyl peptidase-IV inhibitory (DPP-IV) and antioxidant potential following simulated *in vitro* gastrointestinal digestion. Further, the resultant small intestinal digests were enriched to obtain peptides between 3–10 kDa in size. All *in vitro* digests of the four GEP were found to inhibit ACE-I compared to the positive control captopril when assayed at a concentration of 1 mg/mL, while the LYS <3 kDa permeate fraction inhibited renin by 40% (± 1.79%). The LYS <10 kDa fraction inhibited PAF-AH by 39% (± 4.34%), and the SA <3 kDa fraction inhibited DPP-IV by 45% (± 1.24%). The MUC <3 kDa fraction had an ABTS-inhibition antioxidant activity of 150 (± 24.79) µM trolox equivalent and the LYS <10 kDa fraction inhibited 2,2-Diphenyl-1-picrylhydrazyl (DPPH) by 54% (± 1.62%). Moreover, over 190 peptide-sequences were identified from the bioactive GEP fractions. The findings of this chapter indicate that GEP are a significant source of bioactive peptides which may influence gut function.

5.2. Introduction

Gastrointestinal endogenous proteins (GEP) have been proposed (Moughan et al., 2014) and recently identified as a potential source of bioactive peptides based on *in silico* analysis (Chapter 3, Dave et al., 2014). GEP are made up of gastrointestinal tract (GIT) epithelial turnover and gut microflora proteins (Miner-Williams et al., 2009) as well as soluble secreted proteins. The latter include the human mucins, digestive enzymes, and serum albumin (Miner-Williams et al., 2009; Moughan & Rutherfurd, 2012). Other contributors to GEP include the digestive hormones, immunoglobulins, lysozyme, and other gastric and intestinal peptides (Moughan & Rutherfurd, 2012). The results presented in the second chapter identified 26 GEP as potential sources of bioactive peptides possessing a range of biological activities. The aim of the study in this chapter was to investigate if the GEP trypsin (P00761 (TRYP)), human lysozyme (P61626 (LYS)), salivary mucin (P12021 (MUC)), human serum albumin (P02768 (SA)) and the dietary protein chicken albumin (P01012 (CA)) are precursor proteins for bioactive peptides which can be released following gastrointestinal digestion.

The selected proteins were screened for *in vitro* angiotensin-I converting enzyme (ACE-I; EC 3.4.15.1), renin (EC 3.4.23.15), platelet-activating factor-acetylhydrolase (PAF-AH; EC 3.1.1.47) and dipeptidyl peptidase-IV (DPP-IV; EC 3.4.14.5) inhibitory activities and *in vitro* antioxidant activities using the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition assays. The selected proteins were digested using an *in vitro* gastrointestinal digestion model developed previously as part of the EU COST INFOGEST network (Minekus et al., 2014). After sequential *in vitro* gastric

and small intestinal digestion, freeze-dried samples were assessed for their *in vitro* enzyme inhibitory activities.

Inhibition of the enzymes ACE-I, renin, PAF-AH and DPP-IV is known to lower systemic and local blood pressure, and assist in the alleviation of symptoms of several disorders including diabetes mellitus (D'Alessio, 2011), hypercholesterolaemia, inflammatory diseases (Carl-McGrath et al., 2009; Pucar et al., 2012), and fibrosis (Kanasaki et al., 2014). Inhibition of ACE-I prevents the formation of angiotensin-II, a potent vasoconstrictor while renin inhibition prevents the formation of angiotensin-I, the precursor of angiotensin-II (Coates, 2003). It is now known that the GIT also contains a local renin angiotensin aldosterone system (RAAS) (Fandriks, 2011), which plays a role in intestinal fluid and electrolyte balance, and intestinal ischaemia (Garg et al., 2012). PAF-AH catalyzes platelet-activating factor (PAF), a pro-inflammatory phospholipid mediator that is involved in various inflammatory diseases of the GIT (Wang et al., 1997), and elevated levels of PAF-AH are believed to be a risk factor for coronary heart disease (Packard et al., 2000) and systemic inflammation (Shi et al., 2007; Sudhir, 2005). DPP-IV degrades the incretins including glucagon-like peptide-2 (GLP-2). GLP-2 is known to help mucosal epithelial cell proliferation in the small intestine, and thus inhibition of DPP-IV in the GIT may enhance epithelial re-growth in the small intestine (Okawada et al., 2011). DPP-IV inhibition is also known to alleviate the symptoms of hypertension and diabetes mellitus (Salles et al., 2015) and also plays a role in regulation of satiety (Reinehr et al., 2010).

The lumen of the GIT is continually exposed to various pro-oxidants from the diet and the environment, and is thus the site of a significant amount of oxidative

reactions (Halliwell et al., 2000). Natural GEP-derived antioxidants could play a protective role against oxidative damage in the lumen. Therefore, the present study not only investigated the *in vitro* potential of GEP as a source of bioactive peptides with inhibitory activities against ACE-I, renin, DPP-IV and PAF-AH but also the antioxidant potential of these peptides.

5.3. Materials and Methods

5.3.1. Materials and Reagents

Human recombinant LYS (P61626), human recombinant SA (P02768), porcine TRYP (P00761), porcine MUC (Apomucin; (P12021)), CA (P01012), dimethyl sulfoxide, porcine salivary amylase, porcine pepsin and porcine pancreatin, the antioxidant resveratrol and the ACE-I inhibitor captopril, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and all of the tris-tricine SDS-PAGE reagents were supplied by Sigma Aldrich (St. Louis, USA). The ACE-I inhibition assay kit was supplied by Dojindo EU, GmbH (Kumamoto, Japan), the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) inhibition based total antioxidant capacity assay kit was supplied by BioVision Inc. (Milpitas, CA, USA), the total antioxidant capacity assay kit was supplied by Zen-Bio, Inc. (Research Triangle Park, NC, USA) and the renin, PAF-AH and DPP-IV inhibition assay kits were all supplied by the Cayman Chemical Company (Cambridge BioSciences, Cambridge, UK). The molecular weight cut-off filters were obtained from Millipore (Amicon® Ultra, EMD Millipore, MA, USA). The 3.5 kDa dialysis membrane was supplied by the Medical Supply Company (MSC, Dublin, Ireland). All other chemicals used were of analytical grade (≥99% purity). Unless otherwise stated, all reagents were made using Milli-Q deionized water.

5.3.2. Simulated gastrointestinal digestion of selected gastrointestinal endogenous proteins (GEP) and a food protein comparator

Four secreted endogenous gut proteins were selected for the present study, namely, porcine trypsin (TRYP), human recombinant lysozyme (LYS), porcine salivary mucin (MUC) and serum albumin (SA). Chicken albumin (CA) was included in the study for the purpose of comparison, as it is a known source of food-derived bioactive peptides (Fujita et al., 1995; Miguel & Aleixandre, 2006; Nimalaratne et al., 2015; Oda et al., 2012). The molecular weights and the chain lengths of the proteins examined in the present study are presented in Table 5.1.

Table 5.1 *Gastrointestinal endogenous proteins (GEP) and dietary protein examined in the in vitro study.*

Protein	Chain Length	Molecular Weight (*M*w) (KDa)	Uniprot Accession No.
Gut cryptome protein	–	–	–
Trypsin (TRYP)	223	24	P00761
Lysozyme (LYS)	148	17	P61626
Porcine salivary mucin (Apomucin) (MUC)	1150	110	P12021
Serum albumin (SA)	609	69	P02768
Dietary protein	–	–	–
Chicken albumin (CA)	385	43	P01012

All of the studied proteins were subjected to simulated static *in vitro* gastrointestinal digestion using the INFOGEST method described previously (Minekus et al., 2014). Selected proteins were subjected to sequential gastric and small intestinal digestions with the exception of TRYP. TRYP was subjected only to small intestinal digestion as it enters the GIT lumen at the duodenum (Voet et al., 2013). The simulated *in vitro* sequential digestion was performed in triplicate. Samples (3 x 0.5 mL) of the hydrolysates of the chosen GEP at a concentration of 0.5–0.0125 g/mL were collected after the gastric phase (G) and small intestinal phase (G + SI) of digestion, freeze-dried and stored at −30 °C until further analysis. The overall methodology and major steps followed in the present study are shown in Figure 5.1.

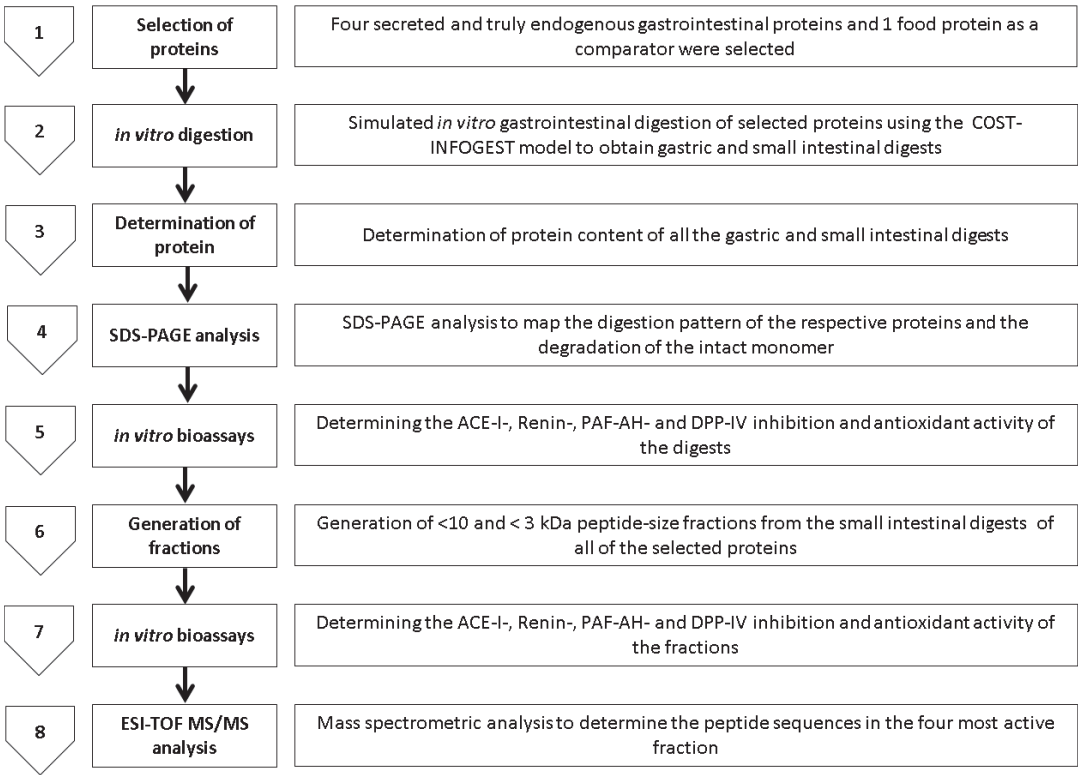


Figure 5.1 *The in vitro experimental design and methodologies used to study the angiotensin-I converting enzyme (ACE-I), renin, Platelet-activating factor-acetylhydrolase (PAF-AH) and dipeptidyl peptidase IV (DPP-IV) inhibition, and Total antioxidant capacity by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl -1-picrylhydrazyl (DPPH) inhibition based antioxidant activity of the gastric and small intestinal hydrolysates and the small intestinal <10 and <3 kDa fractions of gastrointestinal endogenous proteins porcine trypsin, porcine salivary mucin, human recombinant lysozyme and serum albumin, and food protein chicken albumin. SDS-PAGE: Tris-tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis; ESI-TOF MS/MS: Electrospray ionization time of flight mass spectrometry.*

Furthermore, MWCO-based ultra-filtration was applied to all of the small intestinal *in vitro* digests, to obtain <10 and <3 kDa sized peptide-enriched fractions (labelled as TRYP/LYS/MUC/SA/CA <3 and <10 kDa respectively). All of the samples were desalted using a 3.5 kDa dialysis membrane.

5.3.3. Total protein analysis

The total protein content was determined using the Dumas method in accordance with the AOAC method 992.15 (1990) and using a LECO FP628 Protein analyzer (LECO Corp., St. Joseph, MI, USA). A conversion factor of 6.25 was used to estimate the protein content from the nitrogen content.

5.3.4. Tris-tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Tris-tricine SDS-PAGE peptide analysis was carried out using previously described methods (Nicklisch & Waite, 2014; Schagger & von Jagow, 1987). GEP protein samples were diluted 1:10 with reducing PAGE sample buffer (2% or 4% SDS and 100 mM dithiothreitol (DTT)) to obtain a concentration of 1 mg/mL. 5–10 μL of each diluted sample was loaded onto a SDS-PAGE resolving gel (49.5% T, 3% C). The components of the resolving and stacking gels were as per the protocol described by Dave et al. (2013).

The loaded gel was run in a Bio-Rad Laboratories Miniprotean gel apparatus at a voltage of 100 V for 2 h or until the dye front approached the bottom of the gel. Gels were stained using Coomassie brilliant blue R250 (0.3%) for 1 h at 20 °C. Destaining was carried out using 10% *v*/*v* acetic acid and 10% *v*/*v* 2-propanol. Gels were destained for 48 h at 20 °C, with gentle shaking. Gels were scanned on a molecular Gel Doc XR system (Bio-Rad Laboratories, Berkeley, CA, USA) and the gel images were analyzed using QuantityOne and ImageLab software programs (Bio-Rad Laboratories, Berkeley, CA, USA).

5.3.5. ACE-I inhibition assay

This assay was carried out using an ACE-I inhibitor assay kit in accordance with the manufacturer's instructions. All fractions were assayed at a concentration of 1 mg/mL sample dissolved in HPLC grade water in triplicate. The known ACE-I inhibitor Captopril was used as a positive control at a concentration of 1 mg/mL. ACE-I inhibition percentage values for each peptide were calculated using the absorbance values obtained at 450 nm and the equation:

$$\%\ ACE\text{-}I\ inhibition = \left(\frac{Absorbance\ of\ blank\ 1 - Absorbance\ of\ inhibitor}{Absorbance\ of\ blank\ 1 - absorbance\ of\ blank\ 2}\right) X\ 100$$

where, blank 1 is control without the addition of any inhibitor, blank 2 is the reagent blank, and the inhibitor is the positive control or test sample (protein digest/peptide fraction).

5.3.6. Renin inhibition assay

The renin inhibition assay was carried out using a renin inhibitor screening kit in accordance with the manufacturer's instructions. Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-Ome (Sigma Aldrich, St. Louis, USA), a known renin inhibitor, was used as a positive control at a concentration of 10 μM. The % inhibition of renin was calculated using the fluorescence values obtained and the following formula:

$$\%\ Rennin\ inhibition = \left(\frac{100\%\ Initial\ activity\ (AF) - Fluorencence\ of\ test\ sample}{100\%\ Initial\ activity\ (AF)}\right) X\ 100$$

where, test sample is protein digest/peptide fraction.

5.3.7. PAF-AH inhibition assay

This assay was performed using a PAF-AH inhibitor kit supplied by Cambridge BioSciences, Cambridge, UK in accordance with the manufacturer's instructions. All of the digests and enriched fractions were assessed in triplicate. Methyl arachidonyl fluorophosphonate (MAFP), a known PAF-AH inhibitor was used as a positive control at a concentration of 260 ng/mL.

5.3.8. DPP-IV inhibition

The DPP-IV inhibitory activities of the digested protein samples were determined using a DPP-IV inhibitor assay kit (Cambridge BioSciences, Cambridge, UK) in accordance with the manufacturer's instructions. Sitagliptin, a known DPP-IV inhibitor, was used as a positive control at a concentration of 100 μM. The percentage inhibition of DPP-IV was calculated as follows:

$$\% \text{ Inhibition of DPP-IV} = \left(\frac{\text{RFU DPP IV activity - RFU Inhibitor}}{\text{RFU DPP IV activity}}\right) X\ 100$$

where, RFU DPP-IV activity is the fluorescence (measured in relative fluorescence units (RFU)) of the measured without addition of any inhibitor, and RFU Inhibitor is the RFU measured in the presence of the Sitagliptin or the test digests or enriched fractions.

5.3.9. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)-based total antioxidant capacity (ABTS-TAC) assay

The assay was carried out using an ABTS antioxidant assay kit and performed as described by the manufacturer. Trolox was used to obtain a standard curve and the assay buffer was used as a blank. Resveratrol was used as a positive control.

Antioxidant activity was calculated using the trolox standard curve and expressed as μM trolox equivalents (TE).

5.3.10. DPPH inhibition

The DPPH inhibition assay was performed in accordance with previously described methods (Goupy et al., 2003; Nicklisch & Waite, 2014). Briefly, triplicates of the digest or enriched fraction samples (1 mg/mL) and the positive control (resveratrol) were prepared in the assay buffer. The samples and positive control solutions (285 μL) were each combined with a freshly prepared methanolic DPPH solution in a Nunc™ 96-well microWell™ plate to obtain a final DPPH concentration of 100 μM. The plate was incubated in dark at room temperature for 30 min. The absorbance was then read at 515 nm. The control reaction mixture contained DPPH and methanol without sample. The percentage DPPH inhibition was calculated using the following equation:

$$\%\ Inhibition\ of\ DPPH = \left(\frac{Absorbance\ of\ control - Absorbance\ of\ inhibitor}{Absorbance\ of\ control}\right)\ X\ 100$$

where, Absorbance of control is the absorbance of the DPPH solution without any resveratrol or test peptides and Absorbance of inhibitor is the absorbance of the test samples or positive control.

5.3.11. Electrospray ionization time of flight mass spectrometry (ESI-TOF MS) Characterisation of peptides present in the most active fractions

The four fractions which showed the greatest extent of renin, DPP-IV, PAF-AH inhibition and antioxidant activities were selected for peptide characterization. Briefly, the nano LC-MS/MS analysis was performed using an Eksigent Nano-LC

Ultra 1D Plus system (Eksigent of AB Sciex, Framingham, CA, USA) coupled to the quadrupole-time-of-flight (Q-ToF) TripleTOF® 5600 system from AB Sciex Instruments (Framingham, MA, USA) that was equipped with a nano-electrospray ionization source.

Lyophilized samples were re-suspended in 500 µL of H_2O with 0.1% of TFA, and 20 µL of each sample at different times of processing were cleaned and concentrated using Zip-Tip C_{18} with standard bed format (Millipore Corporation, Bedford, MA, USA) according to manufacturer's guidelines, and kept at −20 °C until analysis. Then, 5 µL of the supernatant was injected into the LC-MS/MS system.

After 5 min of pre-concentration, the trap column was automatically switched in-line onto a nano-HPLC capillary column (3 µm, 75 µm × 12.3 cm, C_{18}) (Nikkyo Technos Co, Ltd., Tokyo, Japan). Mobile phase A contained 0.1% v/v formic acid in water, and mobile phase B, contained 0.1% v/v FA in 100% acetonitrile. A linear gradient from 5%–35% of solvent B over 90 min, and 10 min from 35%–65% of solvent B, at a flow rate of 0.3 µL/min and a column temperature of 30 °C was used. The column outlet was directly coupled to a nano-electrospray ion (nESI) source. Operating conditions for the Q/TOF mass spectrometer were positive polarity and information-dependent acquisition (IDA) mode was used. A 0.25-s ToF MS scan from *m*/*z* of 100 to 1200 was performed, followed by 0.05-s product ion scans from *m*/*z* of 100 to 1500 on the 50 most intense 1+ to 5+ charged ions. Automated spectral processing, peak list generation, database search, and *de novo* sequencing for the identification of peptides were performed using Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA, USA) (hppt://www.matrixscience.com). The UniProt protein database was used to identify

the peptides with a significance threshold p <0.1 and a False Discovery Rate of 1.5%. The taxonomy used for identification in the database was *Homo sapiens*. The tolerance on the mass measurement was 0.3 Da in MS mode and MS/MS ions

5.4. Results

5.4.1. Characterization of digests by tris-tricine SDS-PAGE

LYS, MUC, SA, CA were subjected to simulated *in vitro* gastric and small intestinal digestion, and TRYP was subjected to simulated *in vitro* small intestinal digestion alone. The protein content of the gastric and small intestinal digests are given in Table 5.2.

Table 5.2 *The protein content of the in vitro gastric (G) and small intestinal digests (G + SI) of the gastrointestinal endogenous proteins, porcine trypsin (TRYP), human lysozyme (LYS), porcine salivary mucin (MUC) and human serum albumin (SA), and dietary protein chicken albumin (CA).*

Hydrolysate	Protein Content (%)
TRYP G + SI	16.38
LYS G	97.89
LYS G + SI	98.48
MUC G	45.35
MUC G + SI	42.89
SA G	90.12
SA G + SI	87.71
CA G	85.56
CA G + SI	82.26

Tris-tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was used to confirm the digestion of selected proteins (Figure 5.2). All of the proteins except LYS were digested extensively by the end of the simulated small intestinal phase of digestion as shown in Figure 1 lanes 2, 3 and 4 for SA, lanes 2', 3' and 4' for MUC and lanes 5' and 6' for TRYP. Lanes 7', 8' and 9' show the digestion of CA, which was used as a control (a known dietary source of bioactive peptides). In the case of LYS (lanes 5, 6 and 7), a significant amount of LYS (≈15 kDa band) remained intact even after sequential gastric and small intestinal digestion.

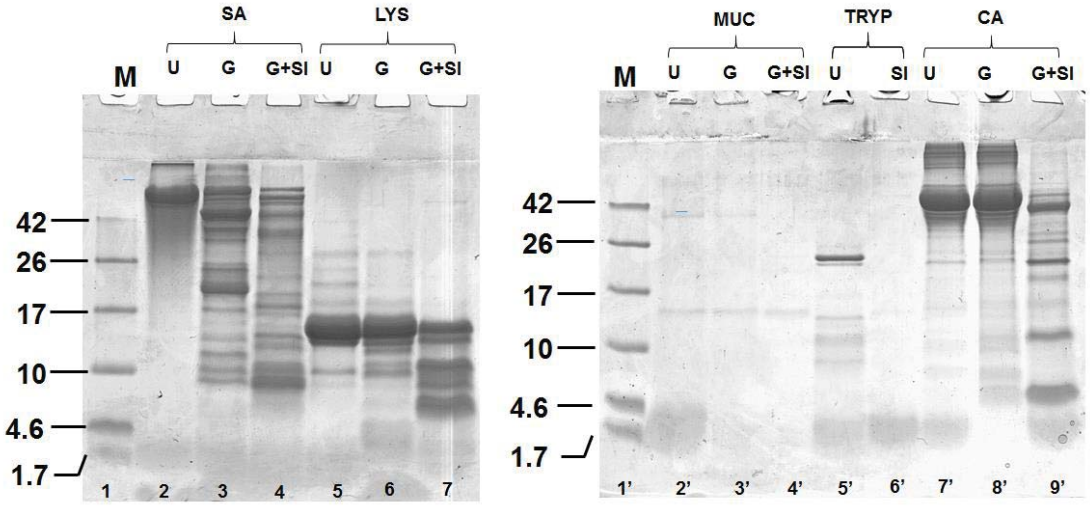


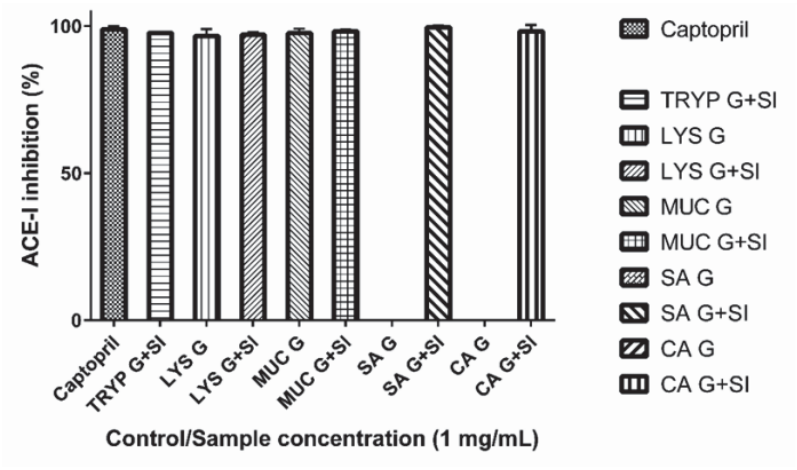
Figure 5.2 *Tris-tricine SDS-PAGE, of the in vitro gastric and small intestinal digests of the gastrointestinal endogenous proteins, porcine trypsin (TRYP), human lysozyme (LYS), porcine salivary mucin (MUC) and human serum albumin (SA), and dietary protein chicken albumin (CA) conducted under reducing conditions. Each protein was analyzed prior to hydrolysis (U) and after gastric (G) and small intestinal digestion (G + SI).*

5.4.2. Angiotensin-I converting enzyme (ACE-I) inhibition by digests and enriched fractions

The *in vitro* small intestinal digest of TRYP, and gastric and small intestinal digests of LYS, MUC, SA and CA are shown in Figure 5.3. These digests were found to inhibit ACE-I significantly (≥ 97% inhibition); while the fractions TRYP <10 kDa, TRYP <3 kDa, LYS <10 kDa, LYS <3 kDa, MUC <10 kDa, MUC <3 kDa, SA <10 kDa, SA <3 kDa, CA <10 kDa, CA <3 kDa, inhibited ACE-I by 99% (± 1.00%), 99% (± 0.57%), 100% (± 0.01%), 98% (± 0.57%), 99% (± 1.15%), 100% (± 0.06%), 100% (± 0.57%), 100% (± 0.05%), 100% (± 0), and 100% (± 0.58%), respectively at a concentration of 1 mg/mL (Figure 5.3). These values were found to be comparable to the ACE-I inhibition values obtained for the positive control

captopril (99% (± 0.57%)) which was also assayed at a concentration of 1 mg/mL. To ensure that the observed ACE-I inhibition was due to the peptides present in the digested GEP fractions, rather than to the presence of the salts or other potentially interfering digestion medium constituents, all samples were desalted using dialysis as described previously. Dialyzed samples showed inhibition comparable to captopril when assayed at 1 mg/mL.

(A)



(B)

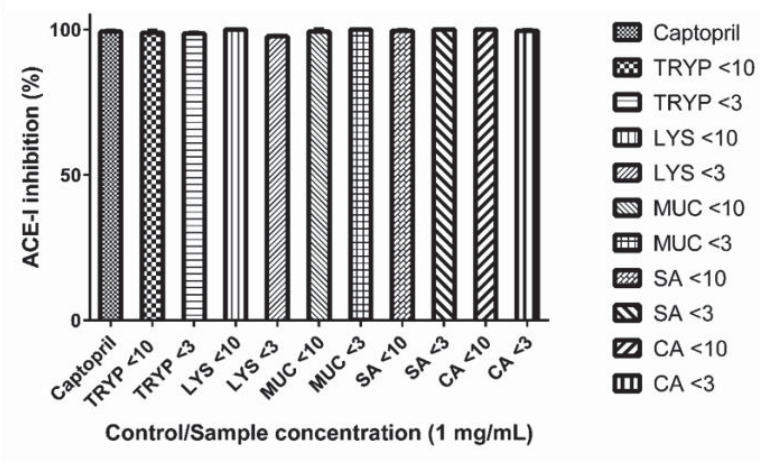


Figure 5.3 *Angiotensin-I converting enzyme (ACE-I) inhibition by (A) gastric (G) and small intestinal digests (G + SI) of the gastrointestinal endogenous proteins, porcine trypsin (TRYP), human lysozyme (LYS), porcine salivary mucin (MUC) and human serum albumin (SA), and dietary protein chicken albumin (CA); and (B) <10 and <3 kDa fractions of the small intestinal digests. All of the samples and the positive control captopril were tested at the concentration of 1 mg/mL.*

5.4.3. Renin inhibition by digests and enriched fractions

Among all the tested digests and fractions, the LYS <3 kDa permeate fraction inhibited renin to the greatest extent (40% ± 1.79 inhibition) (Figure 5.4).

The small intestinal enriched fractions TRYP <10 kDa, TRYP <3 kDa, LYS <10 kDa, MUC <3 kDa, SA <10 kDa, SA <3 kDa, CA <10 kDa and CA <3 kDa inhibited renin by 17% (± 1.19%), 9% (± 1.70%), 25% (± 1.76%), 13% (± 0.93%), 32% (± 1.51%), 24% (± 1.64%), 13% (± 0.82%), and 14% (± 1.41%) respectively at a concentration of 1 mg/mL. TRYP SI, MUC G, MUC G + SI, SA G, and CA G were found to inhibit renin by 16% (± 1.20%), 29% (± .68%), 21% (± 2.39%), 18% (± 3.17%), and 13% (± 0.29%) respectively at a concentration of 1 mg/mL. From the gastric and small intestinal digests tested for renin inhibition LYS G, LYS G + SI, SA G + SI and CA G + SI were found to have negligible renin inhibitory potential. The fraction MUC <10 kDa also showed no renin inhibition. The positive control (Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-Ome), showed 100% (± 0.39%) inhibition when assayed at a concentration of 10 μM.

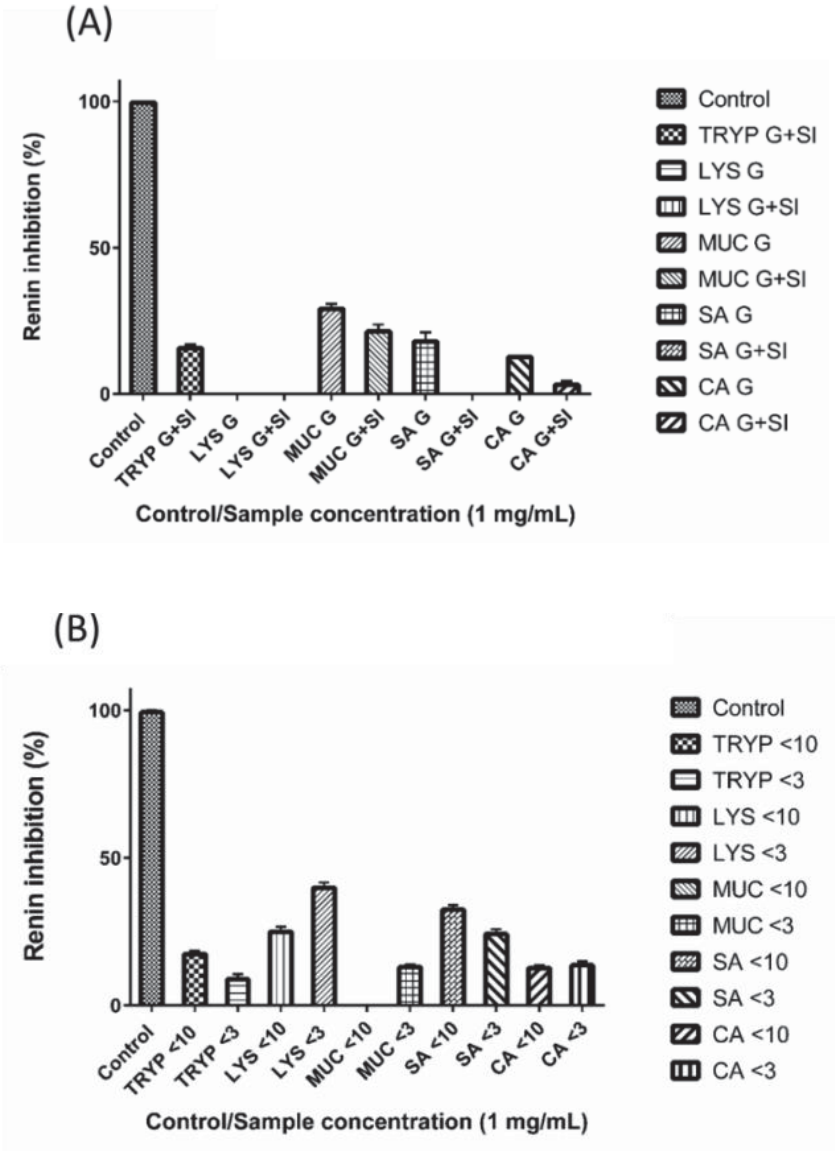


Figure 5.4 *Renin inhibition by (A) gastric (G) and small intestinal digests (G + SI) of the gastrointestinal endogenous proteins, porcine trypsin (TRYP), human lysozyme (LYS), porcine salivary mucin (MUC) and human serum albumin (SA), and dietary protein chicken albumin (CA); and (B) <10 and <3 kDa fractions of the small intestinal digests. All of the samples were tested at the concentration of 1 mg/mL. The positive control Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-Ome was tested at 10 μM.*

5.4.4. Platelet-activating factor-acetylhydrolase (PAF-AH) inhibition by digests and enriched fractions

LYS <10 kDa was found to inhibit PAF-AH by up to 39% (± 4.34%), while the fractions TRYP <10 kDa, TRYP <3 kDa, LYS <3 kDa, SA <10 kDa, SA <3 kDa, CA <10 kDa, CA <3 kDa showed 20% (± 9.93%), 19% (± 6.25%), 21% (± 6.9%), 18% (± 1.99%), 33% (± 5.69%), and 22% (± 4.01%) and 16% (± 2.56%)) inhibition respectively at a concentration of 1 mg/mL (Figure 5.5).

The fractions MUC <10 and MUC <3 showed no PAF-AH inhibition. TRYP SI, LYS G, LYS G + SI, SA G + SI, CA G and CA G + SI inhibited PAF-AH by 22% (± 3.50%), 10% (± 2.50%), 5% (± 2.20%), 9% (± 4.79%), 28% (± 3.88%), and 30% (± 0.81%) respectively at a concentration of 1 mg/mL. The digests MUC G, MUC G + SI and SA G showed no inhibition. The positive control MAFP inhibited PAF-AH by 70% (± 1.31%).

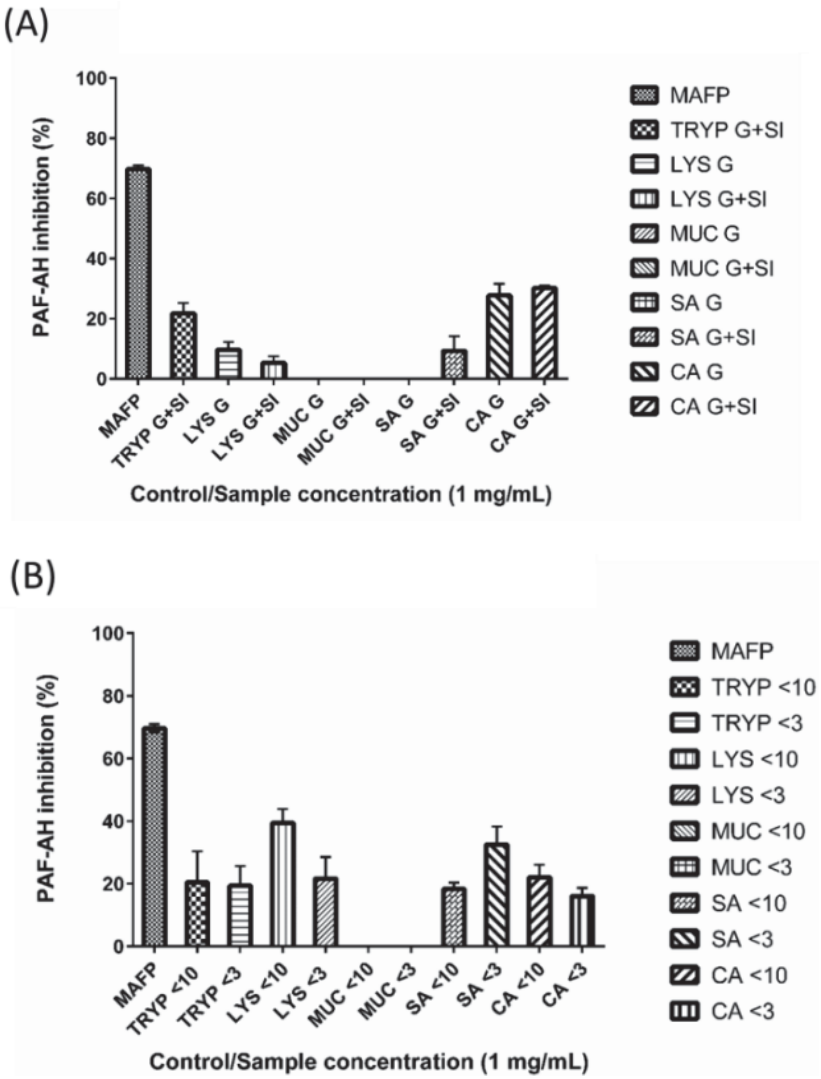


Figure 5.5 *Platelet-activating factor-acetylhydrolase (PAF-AH) inhibition by (A) gastric (G) and small intestinal digests (G + SI) of the gastrointestinal endogenous proteins, porcine trypsin (TRYP), human lysozyme (LYS), porcine salivary mucin (MUC) and human serum albumin (SA), and dietary protein chicken albumin (CA); and (B) <10 and <3 kDa fractions of the small intestinal digests. All of the samples were tested at the concentration of 1 mg/mL. The positive control methyl arachidonyl fluorophosphonate (MAFP) was tested at 260 ng/mL.*

5.4.5. Dipeptidyl peptidase-iv inhibitory (DPP-IV) inhibition by digests and enriched fractions

The fractions LYS <10 kDa, LYS <3 kDa, SA <10 kDa, SA <3 kDa, and CA <10 kDa inhibited DPP-IV by 36% (± 1.52%), 35% (± 1.93%), 36% (± 1.52%), 45% (± 1.24%), and 39% (± 1.54%) respectively when assayed at a concentration of 1 mg/mL (Figure 5.6). The positive control Sitagliptin inhibited DPP-IV by 97% when assayed at the same concentration (± 1.52%).

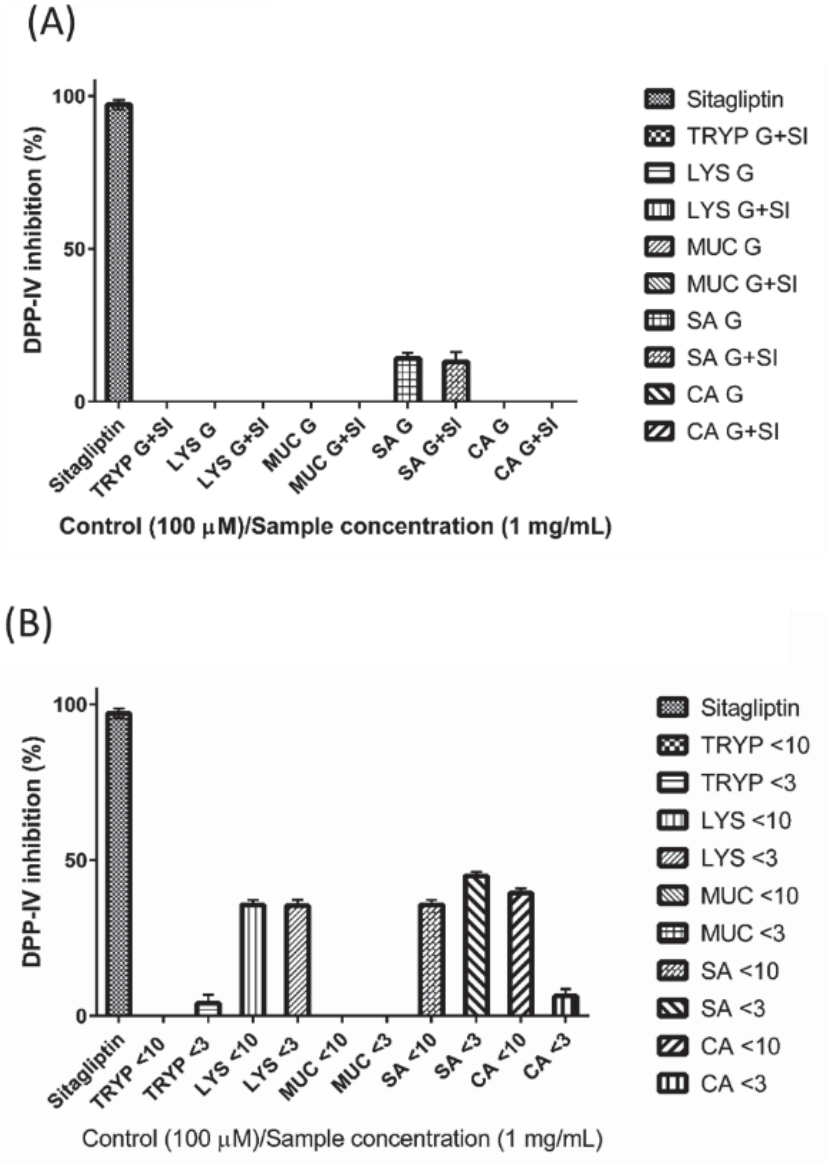


Figure 5.6 *Dipeptidyl peptidase IV (DPP-IV) inhibition by (A) gastric (G) and small intestinal digests (G + SI) of the gastrointestinal endogenous proteins, porcine trypsin (TRYP), human lysozyme (LYS), porcine salivary mucin (MUC) and human serum albumin (SA), and dietary protein chicken albumin (CA); and (B) <10 and <3 kDa fractions of the small intestinal digests. All of the samples were tested at the concentration of 1 mg/mL. The positive control sitagliptin was tested at 100 μM.*

5.4.6. 2,2'-Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid total antioxidant capacity (ABTS-TAC) of digests and enriched fractions

Among all of the tested digests and fractions, the fraction MUC <10 kDa quenched the ABTS radical maximally and had an 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid total antioxidant capacity (ABTS-TAC) value of 150 (± 24.79) μM·TE/mg; while the-fractions TRYP <10 kDa, TYRP <3 kDa, SA <10 kDa, SA <3 kDa AND CA <3 kDa had ABTS-TAC values of 50 (± 4.91), 114 (± 17.81), 131 (± 10.54), 131 (± 28.09) and 134 (± 40.84) μM TE (Figure 5.7).

The *in vitro* digests TRYP SI, MUC G, MUC G + SI, SA G + SI, and CA G + SI were found to have ABTS-TAC of 105 (± 4.62), 58 (± 20.57), 116 (± 19.12), 137 (± 34.27) and 135 (± 29.32) μM·TE/mg. The digests LYS G, LYS G + SI, SA G and CA G were found to have no antioxidant potential using the ABTS-TAC assay. Fractions LYS <10 kDa, LYS <3 kDa, MUC <3 kDa and CA <10 kDa did not inhibit/quench the ABTS radical. The positive control resveratrol was found to have an ABTS-TAC values ranging from 288 to 497 (±12.38–22.11) TE/mg.

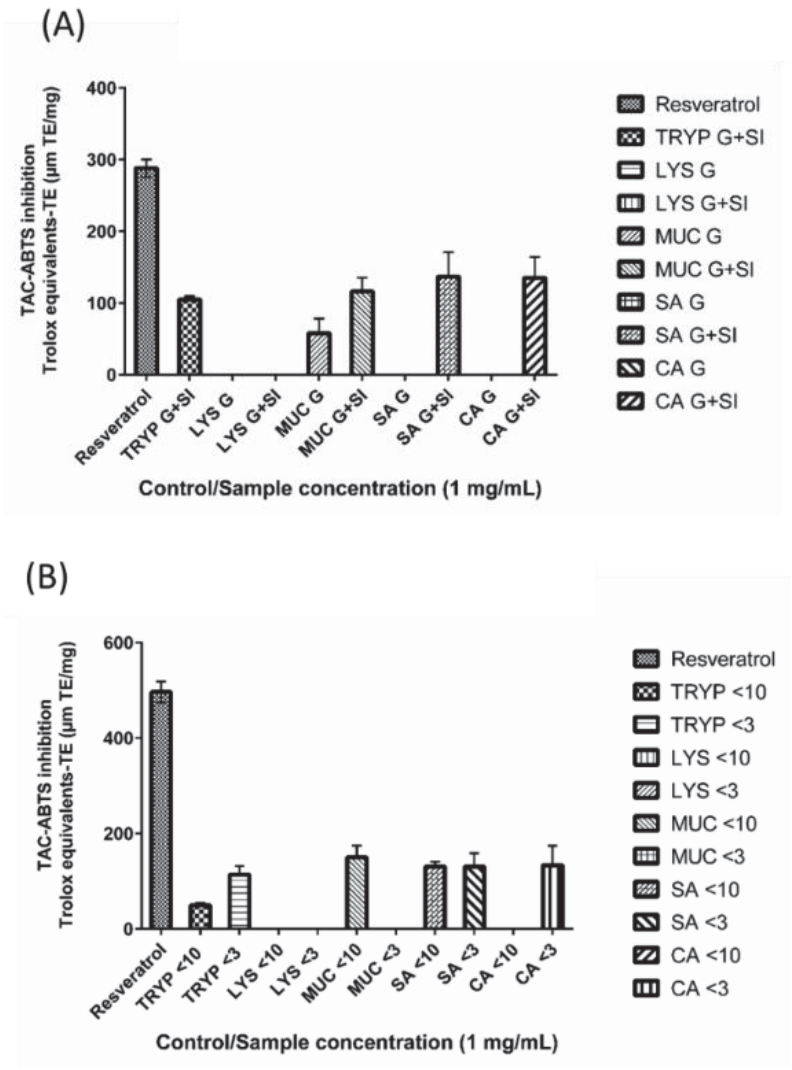


Figure 5.7 *Total antioxidant capacity by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) inhibition by (A) gastric (G) and small intestinal digests (G + SI) of the gastrointestinal endogenous proteins, porcine trypsin (TRYP), human lysozyme (LYS), porcine salivary mucin (MUC) and human serum albumin (SA), and dietary protein chicken albumin (CA); and (B) <10 and <3 kDa fractions of the small intestinal digests. All of the samples and the positive control resveratrol were tested at the concentration of 1 mg/mL and the results are expressed in µM trolox equivalents (TE).*

5.4.7. DPPH inhibition by digests and enriched fractions

The fractions LYS <10 kDa, CA <3 kDa, SA <3 kDa, were found to scavenge the DPPH radical by 54% (± 1.62%), 52% (± 0.89%) and 49% (± 1.58%), respectively at a concentration of 1 mg/mL (Figure 5.8). The remaining enriched fractions (TRYP <10 kDa, TRYP <3 kDa, LYS <3 kDa, MUC <10 kDa, MUC <3 kDa, SA <10 kDa, CA <10 kDa) inhibited the DPPH radical by 18% (± 1.12%), 24% (± 1.17%), 9% (± 0.85%), 2% (± 3.03%), 8% (± 1.32%), 14% (± 0.69%), 28% (± 1.35%) respectively at a concentration of 1 mg/mL.

The un-fractionated digests TRYP SI, LYS G, LYS G + SI, SA G, SA G + SI, CA G, CA G + SI inhibited the DPPH free radical by 13% (± 1.15%), 20% (± 1.68%), 30% (± 1.84%), 41% (± 0.69%), 44% (± 0.24%), 39% (± 1.59%) and 49% (± 1.00%) respectively at a concentration of 1 mg/mL. The MUC G digest showed no DPPH inhibition, while the MUC G + SI digest inhibited DPPH by 7% (± 2.29%). The positive control, resveratrol, inhibited DPPH by 93%–97% when assayed at the same concentration (± 0.95%–0.36%).

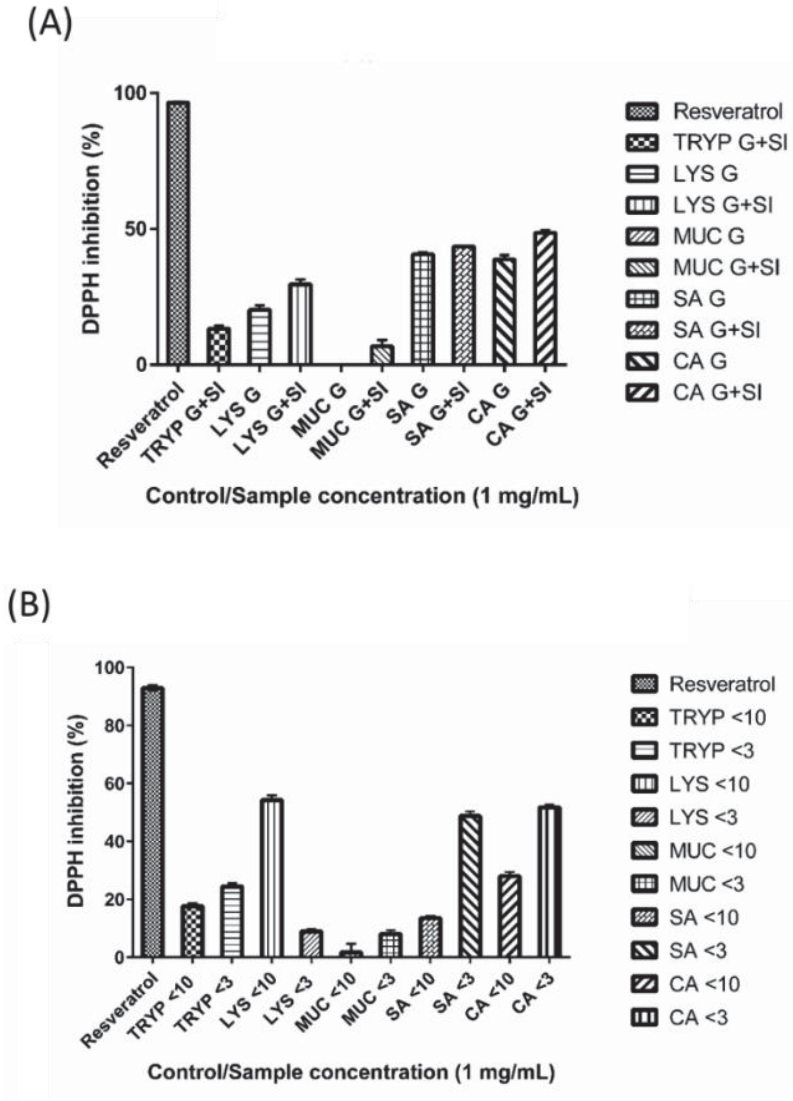


Figure 5.8 *2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition by (A) gastric (G) and small intestinal digests (G + SI) of the gastrointestinal endogenous proteins, porcine trypsin (TRYP), human lysozyme (LYS), porcine salivary mucin (MUC) and human serum albumin (SA), and dietary protein chicken albumin (CA); and (B) <10 and <3 kDa fractions of the small intestinal digests. All of the samples and the positive control resveratrol were tested at the concentration of 1 mg/mL.*

5.4.8. Electrospray ionisation time of flight mass spectrometry (ESI-TOF-MS) characterisation of peptides

A total of 19 and 91 peptides were identified in the <3 kDa fraction of the small intestinal digests of lysozyme and serum albumin respectively and 13 and 70 peptides were identified from the <10 kDa fraction of the small intestinal digests of lysozyme and serum albumin respectively (Table 5.3). The peptides ranged from 7–36 amino acids in chain length.

Table 5.3 *Gastrointestinal endogenous proteins (GEP)-derived peptide sequences found in the <3 and <10 kDa fractions of the in vitro small intestinal digests of lysozyme and serum albumin. The samples were analyzed using electrospray ionization time of flight mass spectrometry (ESI-TOF MS).*

Parent Protein and Hydrolysate Fraction	**Peptide Amino acid Sequence**	**Protein Fragment**	**Obs. Mass (Da)**	**Theor. Mass (Da)**	**Obs. *m/z***	**Theor. *m/z***	**Theor. *Z***
Lysozyme <3 kDa fraction	ALLQDNIADAV	f(101–111)	1141.60	1141.60	571.81	571.81	2
	ALLQDNIADAVA	f(101–112)	1212.64	1212.64	607.33	607.32	2
	ARTLKRLGMDGYRGISL	f(27–43)	1906.06	1906.06	477.52	477.52	4
	DPQGIRAWV	f(120–128)	1040.54	1040.54	521.28	521.28	2
	GIFQINSRYW [a]	f(73–82)	1298.66	1298.64	650.34	650.33	2
	GMDGYRGISLANWM	f(34–47)	1569.71	1569.71	785.86	785.86	2
	LGMDGYRGISL	f(33–43)	1180.59	1180.59	591.30	591.30	2
	LGMDGYRGISLA	f(33–44)	1251.63	1251.63	626.82	626.82	2
	LLQDNIADAV [b]	f(102–111)	1071.54	1071.54	536.78	536.78	2
	NAGDRSTDYG	f(64–73)	1054.43	1054.43	528.22	528.22	2

	NAGDRSTDYGIFQ	f(64–76)	1442.64	1442.64	722.33	722.33	2
	NAGDRSTDYGIFQI	f(64–77)	1555.73	1555.73	778.87	778.87	2
	NAGDRSTDYGIFQIN	f(64–78)	1669.77	1669.77	835.89	835.89	2
	NYNAGDRSTD	f(62–71)	1111.45	1111.45	556.73	556.73	2
	NYNAGDRSTDYGIF	f(62–75)	1591.69	1591.69	796.85	796.85	2
	NYNAGDRSTDYGIFQ	f(62–76)	1719.76	1719.75	860.89	860.88	2
	NYNAGDRSTDYGIFQI	f(62–77)	1832.84	1832.83	917.43	917.42	2
	PQGIRAWVAW	f(121–130)	1182.63	1182.63	592.32	592.32	2
	YNAGDRSTDYGIF	f(63–75)	1477.65	1477.65	739.83	739.83	2
Lysozyme <10 kDa fraction	CNDGKTPGAV	f(83–92)	960.43	960.43	481.22	481.22	2
	CNDGKTPGAVNACHLSCS [c,d]	f(83–100)	1773.73	1773.74	592.25	592.25	3
	GIFQINSRYW	f(73–82)	1282.65	1282.65	642.33	642.33	2
	GMDGYRGISLANWM	f(34–47)	1569.72	1569.71	785.87	785.86	2
	NAGDRSTDYG	f(64–73)	1054.43	1054.43	528.22	528.22	2
	NAGDRSTDYGIFQI	f(64–77)	1555.74	1555.73	778.88	778.87	2

	NYNAGDRSTD	f(62–71)	1111.45	1111.45	556.73	556.73	2
	NYNAGDRSTDY	f(62–72)	1274.52	1274.52	638.27	638.27	2
	NYNAGDRSTDYG	f(62–73)	1331.53	1331.54	666.77	666.78	2
	NYNAGDRSTDYGIFQI	f(62–77)	1832.85	1832.83	917.43	917.42	2
	SALLQDNIADAV	f(100–111)	1228.63	1228.63	615.32	615.32	2
	YNAGDRSTDYG	f(63–73)	1217.49	1217.49	609.75	609.75	2
	ALLQDNIADAVACA [e]	f(101–114)	1434.66	1434.67	718.34	718.34	2
Serum albumin <3 kDa fraction	AEAKDVFLGMFL	f(344–355)	1339.69	1339.68	1339.69	670.85	2
	AEFAEVSKLVTDL	f(250–262)	1420.75	1420.74	1420.75	711.38	2
	AEFAEVSKLVTDLT [f]	f(250–263)	1549.79	1549.79	1549.79	775.90	2
	AEFAEVSKLVTDLTK	f(250–264)	1649.89	1649.89	1649.89	825.95	2
	AEFAEVSKLVTDLTKVHT	f(250–267)	1987.10	1987.06	1987.10	994.54	2
	AEVENDEMPADLPSLA	f(315–330)	1699.76	1699.76	1699.76	850.89	2
	AEVSKLVTDLT	f(253–63)	1174.64	1174.64	1174.64	588.33	2
	AKVFDEFKPL	f(395–404)	1192.65	1192.65	1192.65	597.33	2

AKVFDEFKPLVEEPQ	f(395–409)	1774.91	1774.91	1774.91	888.46	2
ALEVDETYVPK	f(514–524)	1262.64	1262.64	1262.64	632.33	2
ALEVDETYVPKE	f(514–525)	1391.68	1391.68	1391.68	696.85	2
ALEVDETYVPKEF	f(514–526)	1538.75	1538.75	1538.75	770.38	2
ALVLIAFA	f(45–52)	816.51	816.51	816.51	409.26	2
ALVLIAFAQ	f(45–53)	944.57	944.57	944.57	473.29	2
ALVLIAFAQY	f(45–54)	1107.62	1107.63	1107.62	554.82	2
AVMDDFAAFVEK	f(570–581)	1341.63	1341.63	1341.63	671.82	2
DDNPNLPR	f(131–138)	939.44	939.44	939.44	470.73	2
DEFKPLVEEPQNLI	f(399–412)	1669.86	1669.86	1669.86	835.94	2
DEFKPLVEEPQNLIK	f(399–413)	1797.95	1797.95	1797.95	899.98	2
DETYVPKE	f(518–525)	979.45	979.45	979.45	490.73	2
DLGEENFKALV	f(37–47)	1233.63	1233.62	1233.63	617.82	2
DLGEENFKALVL	f(37–48)	1346.71	1346.71	1346.71	674.36	2
DLPSLAADFVES	f(325–336)	1262.60	1262.60	1262.60	632.31	2
DLPSLAADFVESK	f(325–337)	1390.70	1390.70	1390.70	696.36	2

DVFLGMFLYE [g]	f(348–357)	1248.77	1248.57	1248.77	625.29	2
EDHVKLVNEVTEFA	f(61–74)	1628.81	1628.80	1628.81	815.41	2
ENDEMPADLPSL	f(318–329)	1329.58	1329.58	1329.58	665.80	2
ENDEMPADLPSLAADFVES	f(318–336)	2048.90	2048.89	2048.90	1025.45	2
ENDEMPADLPSLAADFVESK	f(318–337)	2177.01	2176.98	2177.01	1089.50	2
EQLKAVMDD	f(566–574)	1047.49	1047.49	1047.49	524.75	2
EQLKAVMDDF [h]	f(566–575)	1176.55	1176.55	1176.55	589.28	2
EQLKAVMDDFA [h]	f(566–576)	1247.59	1247.59	1247.59	624.80	2
EQLKAVMDDFAA [h]	f(566–577)	1318.62	1318.62	1318.62	660.32	2
EVDETYVPK	f(516–524)	1078.52	1078.52	1078.52	540.27	2
EVDETYVPKE	f(516–525)	1207.57	1207.56	1207.57	604.79	2
EVDETYVPKEF	f(516–526)	1354.63	1354.63	1354.63	678.32	2
EVDETYVPKEFN	f(516–527)	1468.67	1468.67	1468.67	735.34	2
EVDETYVPKEFNAET [i]	f(516–530)	1770.79	1770.78	1770.79	886.40	2
FDEFKPLVEEPQNLIK	f(398–413)	1945.02	1945.02	1945.02	973.52	2
FKDLGEEN	f(35–42)	950.44	950.43	950.44	476.22	2

FKDLGEENFKALV	f(35–47)	1508.79	1508.79	1508.79	755.40	2
FKDLGEENFKALVL	f(35–48)	1621.87	1621.87	1621.87	811.94	2
FPKAEFAEVSKLVTDLT	f(247–263)	1894.01	1894.01	1894.01	632.34	3
FYAPELLFFAK	f(173–183)	1344.71	1344.71	1344.71	673.36	2
KPLVEEPQN	f(402–410)	1052.55	1052.55	1052.55	527.28	2
KPLVEEPQNLI	f(402–412)	1278.72	1278.72	1278.72	640.37	2
KVPQVSTPT	f(438–446)	955.53	955.53	955.53	478.77	2
KVPQVSTPTLV	f(438–448)	1167.69	1167.69	1167.69	584.85	2
KVPQVSTPTLVEV	f(438–450)	1395.79	1395.80	1395.79	698.91	2
KVPQVSTPTLVEVS	f(438–451)	1482.83	1482.83	1482.83	742.42	2
LDELRDEG	f(206–213)	945.44	945.44	945.44	473.73	2
LEVDETYVPK	f(515–524)	1191.60	1191.60	1191.60	596.81	2
LEVDETYVPKE	f(515–525)	1320.65	1320.64	1320.65	661.33	2
LQHKDDNPNLPR	f(127–138)	1445.73	1445.74	1445.73	482.92	3
LVAASQAALG	f(599–608)	899.51	899.51	899.51	450.76	2
LVNEVTEFA	f(66–74)	1020.52	1020.51	1020.52	511.26	2

LVNEVTEFAK	f(66–75)	1148.61	1148.61	1148.61	575.31	2
LVNEVTEFAKTCVADESAENCDK [c,d]	f(66–88)	2512.11	2512.13	2512.11	629.04	4
LVRPEVDVM [g]	f(139–147)	1072.56	1072.56	1072.56	537.29	2
NDEMPADLPSLA [i]	f(318–330)	1272.56	1272.55	1272.56	637.28	2
NDEMPADLPSLAADF	f(318–333)	1604.71	1604.70	1604.71	803.36	2
NDEMPADLPSLAADFVES	f(318–336)	1919.86	1919.85	1919.86	960.93	2
NDEMPADLPSLAADFVESK	f(318–337)	2047.95	2047.94	2047.95	1024.98	2
NEVTEFAKTCVADESAENCDK [c,d]	f(68–88)	2299.96	2299.98	2299.96	1150.99	2
NRRPCFSALEVDETYVPKE [d,j]	f(507–525)	2199.96	2200.09	2199.96	734.37	3
NYAEAKDVFLG	f(342–352)	1225.60	1225.60	1225.60	613.81	2
NYAEAKDVFLGM	f(342–353)	1356.64	1356.64	1356.64	679.33	2
NYAEAKDVFLGMFL	f(342–355)	1616.83	1616.79	1616.83	809.40	2
QHKDDNPNLPR [h]	f(128–138)	1315.62	1315.63	1315.62	439.55	3
QYLQQCPFEDHV [d]	f(53–64)	1471.68	1471.67	1471.68	736.84	2
RETYGEM	f(105–111)	884.37	884.37	884.37	443.19	2
RHPDYSVV	f(361–368)	971.48	971.48	971.48	486.75	2

RHPDYSVVL	f(361–369)	1084.57	1084.57	1084.57	543.29	2
SALEVDETYVPK	f(513–524)	1349.67	1349.67	1349.67	675.84	2
SALEVDETYVPKE	f(513–525)	1478.72	1478.71	1478.72	740.36	2
SALEVDETYVPKEF	f(513–526)	1625.79	1625.78	1625.79	813.90	2
TPVSDRVT	f(491–498)	873.45	873.46	873.45	437.74	2
VFDEFKP	f(397–403)	880.43	880.43	880.43	441.22	2
VFDEFKPL	f(397–404)	993.52	993.52	993.52	497.77	2
VFDEFKPLVEEPQ	f(397–409)	1575.78	1575.78	1575.78	788.90	2
VFDEFKPLVEEPQN	f(397–410)	1689.83	1689.82	1689.83	845.92	2
VFDEFKPLVEEPQNLI	f(397–412)	1916.00	1915.99	1916.00	959.00	2
VKLVNEVTEFA	f(64–74)	1247.68	1247.68	1247.68	624.85	2
VLIAFAQYL	f(47–55)	1036.60	1036.60	1036.60	519.31	2
VPQVSTPTLVEVS	f(439–451)	1354.74	1354.73	1354.74	678.37	2
VSTPTLVEVS	f(442–451)	1030.56	1030.55	1030.56	516.28	2
DVFLGMFL [g]	f(348–355)	956.47	956.47	956.47	479.24	2

	TLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPR [d,i]	f(103–138)	4216.84	4216.90	4216.84	844.39	5
	LVNEVTEFAKTC [d]	f(66–77)	1318.71	1318.68	1318.71	660.35	2
	ENDEMPADLPSLA [h]	f(318–330)	1382.60	1382.60	1382.60	692.31	2
	LSVVLNQLCVL [c]	f(477–87)	1231.69	1231.68	1231.69	616.85	2
Serum albumin <10 kDa fraction	AEAKDVFLGMF	f(344–354)	1226.60	1226.60	614.31	614.31	2
	AEAKDVFLGMFL	f(344–355)	1339.70	1339.68	670.86	670.85	2
	AEFAEVSKLVTDL	f(250–262)	1420.76	1420.74	711.39	711.38	2
	AEFAEVSKLVTDLT	f(250–263)	1521.91	1521.79	761.96	761.90	2
	AEFAEVSKLVTDLTK	f(250–264)	1649.88	1649.89	550.97	550.97	3
	AEVSKLVTDLT	f(253–263)	1174.65	1174.64	588.33	588.33	2
	AKVFDEFKPLVEEPQNLIK	f(395–413)	2243.25	2243.22	748.76	748.75	3
	ALVLIAFAQ	f(45–53)	944.56	944.57	473.29	473.29	2
	ALVLIAFAQY	f(45–54)	1107.63	1107.63	554.82	554.82	2
	APELLFFAK	f(175–183)	1034.57	1034.58	518.29	518.30	2
	AVMDDFAAFVE	f(570–580)	1213.54	1213.53	607.78	607.77	2

AVMDDFAAFVEK	f(570–581)	1341.64	1341.63	671.83	671.82	2
DEFKPLVEEPQNL	f(399–411)	1556.80	1556.77	779.41	779.39	2
DEFKPLVEEPQNLI	f(399–412)	1669.89	1669.86	835.95	835.94	2
DEFKPLVEEPQNLIK	f(399–413)	1798.00	1797.95	900.01	899.98	2
DLGEENFKALVLI	f(37–49)	1459.82	1459.79	730.92	730.90	2
DLGEENFKALVLIA	f(37–50)	1530.85	1530.83	766.43	766.42	2
DLGEENFKALVLIAFAQ	f(37–53)	1877.06	1876.99	939.54	939.50	2
DLPSLAADF	f(325–333)	947.45	947.46	474.73	474.74	2
DLPSLAADFVES	f(325–334)	1262.61	1262.60	632.31	632.31	2
DLPSLAADFVESK	f(325–335)	1390.72	1390.70	696.37	696.36	2
DVFLGMFLY [g]	f(348–366)	1119.53	1119.53	560.77	560.77	2
DVFLGMFLYE [g]	f(348–367)	1248.57	1248.57	625.29	625.29	2
DVFLGMFLYEYA [g]	f(348–369)	1482.70	1482.67	742.36	742.34	2
ENDEMPADLPSLA	f(318–330)	1400.63	1400.61	701.32	701.31	2
EQLKAVMDDF	f(566–575)	1176.55	1176.55	589.28	589.28	2
EQLKAVMDDFAA	f(566–577)	1318.63	1318.62	660.32	660.32	2

EQLKAVMDDFAAF	f(566–578)	1483.73	1483.70	742.87	742.86	2
EVDETYVPKE	f(516–525)	1207.56	1207.56	604.79	604.79	2
FDEFKPLVEEPQNLIK	f(398–413)	1945.04	1945.02	649.36	649.35	3
FKDLGEENFKALV	f(35–47)	1508.81	1508.79	755.41	755.40	2
FKDLGEENFKALVL	f(35–48)	1621.91	1621.87	811.96	811.94	2
FKDLGEENFKALVLI	f(35–49)	1735.00	1734.96	868.51	868.49	2
FKDLGEENFKALVLIA	f(35–50)	1806.04	1805.99	904.03	904.00	2
FKDLGEENFKALVLIAFAQ	f(35–53)	2152.24	2152.16	1077.13	1077.09	2
FPKAEFAEVSKLVTDLT	f(247–263)	1894.01	1894.01	632.35	632.34	3
FYAPELLFFA	f(173–182)	1216.62	1216.62	609.32	609.32	2
FYAPELLFFAK	f(173–183)	1344.73	1344.71	673.37	673.36	2
FYAPELLFFAKR	f(173–184)	1500.79	1500.81	501.27	501.28	3
KDLGEENFKALVLIAFAQ	f(36–53)	2005.11	2005.09	669.38	669.37	3
KVPQVSTPT	f(438–446)	955.52	955.53	478.77	478.77	2
KVPQVSTPTLVEV	f(438–450)	1395.81	1395.80	698.91	698.91	2
KVPQVSTPTLVEVS	f(438–451)	1482.85	1482.83	742.43	742.42	2

KVPQVSTPTLVEVSRN [k]	f(438–453)	1710.00	1709.92	856.01	855.97	2
LQHKDDNPNLPR	f(127–138)	1445.71	1445.74	482.91	482.92	3
LVNEVTEFAKTCVADESAENCDKSLHTLF [c,k]	f(66–94)	3210.60	3210.50	1071.21	1071.17	3
NDEMPADLPSLA	f(319–330)	1271.58	1271.57	636.80	636.79	2
NDEMPADLPSLAADFVESK	f(319–337)	2048.01	2047.94	1025.01	1024.98	2
NYAEAKDVFLGMF	f(342–354)	1503.73	1503.71	752.87	752.86	2
NYAEAKDVFLGMFL [g]	f(342–355)	1632.81	1632.79	817.41	817.40	2
RHPDYSVVLLL	f(361–371)	1310.74	1310.73	656.38	656.37	2
SALEVDETYVPK	f(513–524)	1349.68	1349.67	675.85	675.84	2
TKKVPQVSTPTLVEVS[f]	f(436–451)	1739.99	1739.97	871.00	870.99	2
TYETTLEK	f(376–383)	983.47	983.48	492.74	492.75	2
VFDEFKPL	f(397–404)	993.50	993.52	497.76	497.77	2
VFDEFKPLVEEPQ	f(397–409)	1575.81	1575.78	788.91	788.90	2
VFDEFKPLVEEPQN	f(397–410)	1689.86	1689.82	845.94	845.92	2
VFDEFKPLVEEPQNL	f(397–411)	1802.96	1802.91	902.49	902.46	2
VFDEFKPLVEEPQNLI	f(397–412)	1916.05	1915.99	959.03	959.00	2

VFDEFKPLVEEPQNLIK	f(397–413)	2044.11	2044.09	682.38	682.37	3
VFDEFKPLVEEPQNLIKQ	f(397–414)	2172.21	2172.15	725.08	725.06	3
VLIAFAQYL	f(47–55)	1036.58	1036.60	519.30	519.31	2
VPQVSTPTLVEV	f(439–450)	1267.71	1267.70	634.86	634.86	2
VPQVSTPTLVEVS	f(439–451)	1354.75	1354.73	678.38	678.37	2
EQLKAVMDDFA	f(566–576)	1265.60	1265.60	633.81	633.81	2
YAPELLFFAK	f(174–183)	1197.65	1197.64	599.83	599.83	2
LVRPEVDV	f(139–146)	925.51	925.52	463.76	463.77	2
EVDETYVPK	f(516–524)	1078.51	1078.52	540.26	540.27	2
LSQRFPKAEFAEVSKLVTDLT	f(243–263)	2379.28	2378.28	595.83	595.58	4
VLIAFAQY	f(47–54)	923.49	923.51	462.75	462.76	2

[a] Deamidated W; [b] Deamidated Q; [c] Dioxidation(C); [d] Cys– >Dha(C); [e] Trioxidation(C); [f] Formyl(K); [g] Oxidation(M); [h] Glu– >pyro-Glu@N-term; [i] Deamidated(N);

[j] Dehydrated(S); [k] Arg– >GluSA(R).

5.5. Discussion

To date several studies have investigated the quantity of GEP found in the digestive contents of animals (Hodgkinson et al., 2000; Miner-Williams et al., 2009; Montagne et al., 2001) and humans (Baglieri et al., 1995; Miner-Williams et al., 2012, 2014a), and the effect of dietary constituents such as protein (and its structure), fiber and anti-nutritional factors on the GEP (Deglaire et al., 2007; Gilani et al., 2005; Ouellet et al., 2002; Rutherfurd et al., 2015a). It is now widely accepted that GEP contribute significantly to the amino acid pool in the GIT, and consequently are now recognized as important in the estimation of the true amino acid requirements of humans (Moughan & Rutherfurd, 2012; Rutherfurd et al., 2015b). However, in spite of the knowledge that up to 80% of the GEP secreted into the gut lumen are digested and reabsorbed by the end of the terminal ileum, the possibility that they may also give rise to bioactive peptides has only recently been considered (Moughan et al., 2014). Quantitatively, in mammals, GEP are secreted in an equal or greater amount as dietary proteins ingested per day (Miner-Williams et al., 2014a). However, while an extensive range of dietary proteins have been investigated for their ability to generate bioactive peptides during food processing, fermentation and gastrointestinal digestion *in vitro* and *in vivo*, GEP have not been widely investigated. Therefore, analogous to enzymatically-derived dietary bioactive peptides, peptides derived from GEP in the gut lumen may also possess gut-specific bioactivities (Chapter 2, Dave et al., 2015)

The present study used an *in vitro* digestion model to examine GEP as a source of peptides with physiological effects including heart-health regulatory, GIT health beneficial, anti-inflammatory and weight gain prevention. Four GEP and one dietary

protein (chicken albumin) were selected to examine their potential to generation bioactive peptides following simulated GIT digestion. This work demonstrates that *in vitro* gastric and small intestinal digests of GEP possess biological activity relevant to the GIT and overall human health. These biological activities include potential renin, ACE-I, DPP-IV inhibitory and antioxidant activities.

GEP used in this work included TRYP, LYS, MUC, and SA. These were subjected to simulated, sequential gastric and small intestinal digestion using pepsin and pancreatin (containing proteases from the exocrine cells of the porcine pancreas), while TRYP was only subjected to small intestinal digestion. Pepsin and proteolytic enzymes present in pancreatin digested all of the four GEP as shown using SDS-PAGE (Figure 5.2), and the extent of digestion generally compared favorably to the protein CA (Figure 5.2). LYS was not completely digested and this finding is in agreement with a previous study that demonstrated that hen egg lysozyme, which is 57.5% homologous (BLAST identity match) to human lysozyme (Altschul et al., 1990), was only partially digested in the GIT (Jimenez-Saiz et al., 2014).

All of the gastric and small intestinal digests, and the enriched fractions containing peptides <10 and <3 kDa in size inhibited ACE-I when assayed at a concentration of 1 mg/mL and results obtained were comparable to ACE-I inhibition values obtained for captopril and for food derived ACE-I inhibitory peptides identified previously. In comparison, dietary bioactive peptides, from various sources including cereals, legumes, meat, fish and poultry, have been documented to show a wide range of ACE-I inhibition ranging from 1% to ≥90% inhibition (Iwaniak et al., 2014; Minkiewicz et al., 2008b). The ACE-I inhibition activity observed suggests that the enzyme may be inhibited by a broad-spectrum of peptidic inhibitors. From a gut

perspective, inhibition of ACE-I is important since it is now known that an extensive RAAS exists within the GIT. While the GIT-RAAS is known to balance the fluid and electrolyte dynamics in the gut, it is not entirely clear as to what the exact role of ACE-I inhibitors may be in the context of the gut. It is important to note that, postprandial or functional hyperemia (an increased blood flow to the gut after a meal: postprandial state) is a known phenomenon that helps in the digestion of food (Kvietys, 2010; Kvietys et al., 1981), and it may be that the dietary protein- and GEP-derived ACE-I inhibitory peptides play a protective role against a severe rise in local hypertension in the GIT.

Only a few GEP digests including TRYP SI, MUC G, MUC G + SI, SA G and CA G inhibited renin. While the LYS G + SI digest did not inhibit renin, the fractions LYS <10 kDa and <3 kDa were found to inhibit renin by 25% and 40% each, indicating that low molecular weight peptides derived from LYS may be responsible for the observed inhibition. In comparison to previously reported values for renin and PAF-AH inhibition by peptides from dietary proteins, inhibition values obtained in the present work were comparable. For example, a previous study investigated the renin inhibition of a papain digest of the red seaweed *Palmaria palmata* and reported that a reverse phase high pressure liquid chromatography (RP-HPLC) fraction of the latter hydrolysate inhibited renin by 58.97% (± 1.26%) when assayed at a concentration of 1 mg/mL (Fitzgerald et al., 2012).

Although the small intestinal digest of LYS and SA inhibited PAF-AH by 5% and 9%, their enriched fractions (LYS <10 kDa and SA <3 kDa) inhibited the PAF-AH by 39% and 33% respectively. This suggests that smaller peptides derived from LYS and SA may be more effective inhibitors of PAF-AH. The PAF-AH inhibitory

values obtained in this work were comparable to those obtained previously for a papain hydrolysate of *Palmaria palmata* which inhibited PAF-AH by 30.37% (Fitzgerald et al., 2013).

A similar trend was observed in the case of DPP-IV inhibition by GEP subjected to GI digestion. While the gastric and small intestinal digests of all of the proteins showed either no inhibition or low inhibition values (SA G and SA G + SI), following enrichment of the small intestinal digests a substantial increase in the DPP-IV inhibitory activity was observed. For example, the SA <3 kDa, SA <10 kDa, LYS <10 kDa and LYS <3 kDa were found to inhibit DPP-IV by greater than 35% at a concentration of 1 mg/mL. In particular, the DPP-IV inhibition exerted by SA <3 kDa (45% (± 1.24%)), is comparable to the DPP-IV inhibition reported for the gastrointestinal digests of oat flour, alcalase digest of oat glutelin and a tryptic digest of highland barley glutelin that were found to have IC_{50} values of 0.99, 0.13 and 1.83 mg/mL respectively (Wang et al., 2015b). In relation to gut health, the optimal inhibition of DPP-IV may aid in remediation of non-steroidal anti-inflammatory drug-induced intestinal ulcers (Inoue et al., 2014), by possibly enhancing the beneficial amino acid- and bile acid-induced bicarbonate secretion in the small intestine (Inoue et al., 2012), besides promoting epithelial re-growth (Okawada et al., 2011) and stimulating intestinal growth (Sueyoshi et al., 2014).

The extent of inhibition of the ACE-I, renin, DPP-IV and PAF-AH enzymes observed in the current work is comparable to that of various food protein digests (Darewicz et al., 2014; Fitzgerald et al., 2013; Jin et al., 2015; Lafarga et al., 2015b). The inhibition of all of the above mentioned enzymes by peptides is associated with a positive impact on cardiovascular health and reduction of high blood pressure

(Atlas, 2007; Salles et al., 2015; Samsamshariat et al., 2011). However, ACE-I, renin, DPP-IV and PAF-AH are enzymes that are also present in the gut, and recent evidence suggests that inhibition of these enzymes can impact positively on gut health and may help to prevent necrosis, ulcerative colitis and other inflammatory disorders of the human gut (Carl-McGrath et al., 2009; Garg et al., 2012; Pucar et al., 2012; Shi et al., 2007).

Furthermore, the digested GEP and enriched fractions were also found to have considerable antioxidant activity *in vitro*. Among the digests that were able to inhibit ABTS ion (TRYP SI, MUC G, MUC G + SI, SA G + SI, and CA G + SI), SA G + SI had the highest total antioxidant capacity value (137 μM·TE/mg), which is much higher than the previously reported values (0.47 μM·TE/mg protein) for the 3 kDa fraction of *in vitro* digests of human milk (Hernandez-Ledesma et al., 2007). However, the highest TAC-ABTS values obtained for GEP in the present study are significantly lower than the values for human plasma (430–569 μM·TE) which was analyzed for TAC-ABTS using ABTS and hydrogen peroxide (Kambayashi et al., 2009) previously. In addition, a small increase in the ABTS scavenging was observed after fraction enrichment, particularly for MUC protein. However, in the antioxidant potential measurement using the DPPH radical assay, a greater difference was observed between the un-fractionated GEP digest fractions and the enriched fraction of the same GEP. For example, LYS G + SI inhibited DPPH by only 30% (± 1.84%) but the <10 kDa fraction of the same sample inhibited DPPH by 54% (± 1.62), followed by the SA <3 kDa that also inhibited DPPH by 49% (± 1.58%). The antioxidant activity of GEP-derived peptides may be of great importance to gut health as an exclusive dependence on diet-derived antioxidants

may not be entirely possible given that our diet varies considerably (Moughan et al., 2014). The gut undergoes a significant turnover of the epithelial lining and is constantly exposed to the external environment and various oxidants on a daily basis, thus GEP may form an intrinsic source of potential antioxidant peptides that can offset this regular damage.

In this work, the GEP serum albumin and lysozyme were found to inhibit enzymes in all of the *in vitro* bioassays carried out and the results were comparable to enzyme inhibition values obtained for the dietary protein chicken albumin (CA). In particular, the <10 and <3 kDa fractions of SA and LYS were found to be the most active. Consequently, the latter two fractions were examined further using ESI-TOF MS to characterize the peptides present. Thirty-two peptides were identified in the enriched fractions (< 10 and 3 kDa) of LYS, and 161 different peptides in the SA fractions. Most of the identified peptides were within the general range of the chain length reported for bioactive peptides (3–30 amino acids) (Moller et al., 2008; Shahidi & Zhong, 2008), but tending towards the longer chain length (7–36 amino acids). Overall, among all of the 22 amino acids, LYS has the highest amounts of the amino acids A (10.8%), R (10.8%) and N (7.7%). In terms of the most commonly found amino acids in bioactive peptide sequences, LYS sequences contained significant amounts of A (10.8%), R (10.8%), G (8.5%), L (6.2%), V (6.9%), Q (4.6%) and Y (4.6%). In the <3 and <10 kDa fractions of LYS, the major amino acids observed were A, V, N, Q and Y. Most of the peptide fractions identified were from the f(62–130) region of the protein, and a few from the f(27–47) region of the protein. In particular, from the LYS fractions that showed the highest DPPH inhibition, 10 peptide sequences that have an N residue at the N-terminal of the

peptide have been identified. These results are in agreement with the findings of Rajapakse et al. (2005), who identified the two peptides NADFGLNGLEGLA and NGLEGLK, containing an N-terminal N residue. These peptides, generated from squid, showed the greatest antioxidant effect in an *in vitro* analysis based on lipid peroxidation studies previously. Overall, the SA protein derived peptides contained the highest amounts (up to 10%) of the amino acids A, E, L, T and K. In terms of the most commonly found amino acids in bioactive peptide sequences, SA was found to be rich in L (10.4%), V (7%), F (5.3%), T (4.8%), P (4.1%) and R (4.1%). The highest frequency of occurrence of peptides with D, E, K, F, L, V, A, R, T and S in the peptides were identified in the <3 and <10 kDa fraction of SA. Within the <3 and <10 kDa fraction of SA, over 80 peptide fragments with a hydrophobic or aromatic amino acid residues of A, V, L and F were identified, and the presence of these peptides is known to have a positive impact on the renin inhibition (Girgih et al., 2014) and antioxidant potential of peptides (Chen et al., 1995; Chi et al., 2015; Kitts & Weiler, 2003). Thus, while both the LYS and SA fractions were found to contain peptides with previously identified bioactive amino acids such as A, L, V, R, T and F (Rajapakse et al., 2005; Udenigwe & Aluko, 2011; Wu et al., 2006), the observed range of bioactivity in this study suggests that apart from the constituting amino acids, the peptide structure and certain other amino acids may also play a role in the bioactivity of various hydrolysates.

A majority of the peptides identified in the present study are novel sequences that have not been reported from these GEP previously. However, two peptides in the LYS <3 kDa fractions (DPQGIRWV (f(120–128)), and PQGIRAWVAW (f(121–130))) that are subsequences (Minkiewicz et al., 2015) of a previously identified

antimicrobial sequence reported from human lysozyme DNIADAVACAKRVVRDPQGIRAWVAWRNR (f(87–115)) (BIOPEP, 2015; Ibrahim et al., 2001) were also found. Future work will specifically aim to chemically synthesize the identified peptides and examine their individual biological activities, which will allow us to determine which particular GEP-derived peptide sequences result in ACE-I, renin, DPP-IV, PAF-AH and antioxidant activity.

The present study, although limited in its scope, due to its *in vitro* nature, is the first to illustrate that GEP are a source bioactive peptides with *in vitro* ACE-I, renin, PAF-AH and DPP-IV inhibitory and antioxidant activities. The known amino acid composition of the peptides identified in the present work may have contributed to the observed biological activities *in vitro*. However, the peptide structure and certain lesser studied amino acids that were also found to be present in abundance may also have positively contributed to the observed *in vitro* bioactivities. Indeed, some of the food derived bioactive peptides with IC_{50} values in the range of 100 to 500 μM can be of nutritive/ physiological importance in that they can be active after oral administration (FitzGerald & Meisel, 2000). It is known that peptides greater than 4 amino acids in size are usually not absorbed into the blood. However, the bioavailability of the peptides in this work should be further studied using *in vitro* transcytosis assays using cell models with epithelial cell lines including the human epithelial cororectal adenocarcinoma cells (Caco-2) or human brain microvascular endothelial cell lines (hCMEC/D3) as previously reported (Hayes et al., 2016). Further *in vivo* work is essential to determine if GEP can indeed give rise to bioactive peptides during their digestion in the GIT. However, the present study points towards the possibility that the GEP may have the potential to constitute a

feedback-like peptidergic system within the gut lumen, particularly considering that the GEP are constantly present in the gut and can be modulated by dietary constituents.

Chapter 6

6 Identification of Bioactive Peptides Derived from the Gastrointestinal Endogenous Proteins of Growing Pigs

6.1 Abstract

Recent *in silico* and *in vitro* digestion studies have shown that, like dietary proteins, mammalian gastrointestinal endogenous proteins (GEP) are a source of bioactive peptides. However, identification of such bioactive peptides following *in vivo* digestion has not been undertaken. The present work examined the generation of bioactive peptides from GEP secreted into the lumen of the GIT of growing pigs that had been fed a protein-free diet for 3 days. The stomach chyme (SC) and jejunal digesta (JD) samples from 6 pigs (<6 months of age) were collected and fractionated, and the resultant protein and peptide containing fractions analysed using tris-tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis. Bioactivity of these fractions was examined using *in vitro* bioassays including the angiotensin-I-converting enzyme (ACE-I) inhibition, 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition, ferric reducing antioxidant power (FRAP) and microsomal lipid peroxidation (MLP) inhibition assays. Potential bioactive peptides were subsequently identified using mass spectrometry. Porcine JD samples isolated from two pigs were found to inhibit ACE-I and DPPH by 81 (± 2.8)% and 94 (± 0.66)%, respectively. Pig SC samples were found to inhibit MLP by between 15-39 (±2.46)%. The study identified over 180 novel peptide sequences, and one pig serum albumin-derived peptide (FAKTCVADESAENCDKS) sequence that was found to match a human serum albumin-derived peptide reported in the previous *in vitro* study of Chapter 5. While, there was considerable variation between individual animals for the determined bioactivity and for the peptide profiles found, which was likely due to sampling effects and natural variations in the gut contents, the present study gives the first *in vivo* evidence for GEP as a source of bioactive peptides.

6.2 Introduction

It has been proposed that the non-dietary, gastrointestinal endogenous proteins (GEP) may be a source of bioactive peptides in the gastrointestinal tract (GIT) (Moughan et al., 2014). Indeed, recently conducted *in silico* studies have shown that the GEP contain numerous previously known as well as novel bioactive peptide sequences within their intact protein sequences that show a range of bioactivities *in vitro* (Chapter 5, Dave et al., 2016b; Chapter 3, Dave et al., 2014). While *in vivo* studies have established that GEP (proteins arising directly from GIT tissue) are largely digested and (re-) absorbed by the end of the small intestine (Souffrant et al., 1993), bioactivities associated with these breakdown products during their digestion in the GIT lumen have not been reported to date. The aim of the present work was to investigate the release of bioactive peptides from the GEP during gastric and small intestinal digestion in the GIT of the growing pig.

Growing pigs (<6 months of age) were fed a protein-free diet for three days. The animals were subsequently euthanised and gastric (stomach chyme) and small intestinal (jejunal digesta) samples were collected from the animals. Samples were enriched for protein content using ammonium sulphate-based salting out, and the obtained proteins and peptides were purified and examined using Tris-tricine SDS-PAGE and several *in vitro* bioassays for potential antihypertension and antioxidant activity. These assays included the angiotensin-I converting enzyme (ACE-I; EC 3.4.15.1) inhibition assay, and antioxidant assays including the 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition, ferric reducing antioxidant potential (FRAP) and microsomal lipid peroxidation (MLP) inhibition bioassays. These bioactivities were selected as they are important in the prevention and amelioration of diseases

associated with the gut and systemically in cardiovascular health (Chapter 2, Dave et al., 2015; Shahidi & Zhong, 2008). ACE-I is a key enzyme in the renin angiotensin aldosterone system (RAAS), the receptors for which are also known to be expressed in the GIT (Fandriks, 2011). Inhibition of ACE-I may prevent local blood pressure rise in the GIT (Kvietys, 2010), as well as prevent the ulceration and inflammation of the GIT wall (Wegman-Ostrosky et al., 2013). Furthermore, since the lumen of the GIT is continuously exposed to various oxidants, irritants and toxins ingested along with food (Halliwell et al., 2000), the action of antioxidant compounds is thought to play an important role in the prevention of oxidation-mediated damage of the gut organ system (Halliwell, 1996; Halliwell et al., 2000). In addition, the peptide sequences found in stomach chyme and jejunal digesta samples, that were thought to be responsible for the observed bioactivities *in vitro*, were characterised using mass spectrometry. This paper is the first to examine the bioactivity of peptides obtained from the digestion of porcine GEP *in vivo.*

6.3 Materials and methods

6.3.1 Materials and Reagents

The components for the anaesthetic cocktail, and intra-cardial injection for euthanasia were supplied by Provet (New Zealand), and prepared before use as described previously (Montoya et al., 2014). The antioxidant resveratrol and the ACE-I inhibitor captopril (positive control), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and all of the FRAP reagents were supplied by Sigma Aldrich (St. Louis, USA). The tris-tricine SDS-PAGE reagents were supplied by Fisher Scientific (Dublin, Ireland) and Bio-Rad Life Science Research (Dublin, Ireland). The ACE-I inhibition assay kit was supplied by NBS Biologicals, (Cambridge, England). The 3.5 kDa snakeskin dialysis membrane was supplied by the Medical Supply Company (MSC, Dublin, Ireland). All other chemicals used were of analytical grade (≥99% purity). Unless otherwise stated, all reagents were made using Milli-Q deionised water (18.2 ohms).

6.3.2 Test diet

Prior to the feeding of the experimental diet, a basal diet (Table 6.1) was used during the acclimatisation period of the animals. A single experimental diet (Table 6.1) with a protein-free formulation was used. A diet devoid of protein enabled the collection of porcine gut endogenous proteins and peptide fractions, including potential bioactive peptides, from the porcine stomach and small intestine.

Table 6.1 *Ingredient composition of the basal and experimental diet (g/kg dry matter)*

Ingredient	Basal diet	Experimental diet
	Amount (g/kg dry matter)	Amount (g/kg dry matter)
Casein	156	0
Soyabean oil	50	50
Sucrose	106	106
Vitamin-mineral premix	5	5
Purified cellulose	51	51
Sodium chloride	3	3
Calcium bicarbonate	1	1
Dicalcium phosphate	20	20
Wheat starch	605	761
Titanium dioxide	3	3

6.3.3 Experimental design and sample collection

Ethics approval for the animal trials was obtained from the Animal Ethics Committee, Massey University, Palmerston North, New Zealand. The study was conducted as described in detail previously (Montoya et al., 2014). Briefly, a total of 6 entire male pigs of <6 months of age (PIC Camborough 46 ×PICboar 356L, 28 (SD 2·9) kg BW), were used. The animals were housed singly in metabolism crates in a room maintained at 21 ± 2 °C with a 10 h light and 14 h dark cycle. Pigs were allowed to adapt to this environment for 10 d during which the basal diet (Table 6.1), containing bovine casein as a protein source, was fed to them. From the 11th day onwards, the animals were fed twice-daily with a protein-free diet for 3 days. On the 13th day, water was withheld from the pigs for 2 h before each pig received a single meal of the protein-free diet (i.e., one half of its daily ration or 45 g DM/kg $BW^{0.75}$, where, $BW^{0.75}$ = determined metabolic body weight) at 9:00 hours. At 3 h and 5 h (2 time points, 3 pigs per time point) after the meal was given, the animals were sedated using an intramuscular injection containing an anaesthetic cocktail of 0·04 mL/kg BW of Zoletil 100 (50 mg/mL), Ketamine (50 mg/mL) and Xylazine (50 mg/mL). Immediately after the animals were sedated, they were euthanised by an intra-cardial injection of sodium pentobarbitone (0·3 mL/kg BW of Pentobarb 300). The stomach and jejunum section were isolated and immediately dissected out, and the respective contents collected, weighed and thoroughly mixed. Samples of stomach chyme were collected from the three pigs killed at the 3 h time point, and the jejunal digesta samples were collected from the three pigs killed at 5 h. The samples were freeze-dried, homogenised and stored at -80 °C until further analysis.

6.3.4 Ammonium sulphate precipitation of proteins

Protein and peptide fractions were precipitated from the gastric and small intestinal samples using saturated ammonium sulphate solution (80% w/v) as described previously (Fitzgerald et al., 2012). Briefly, weighed samples were mixed with saturated (80%) ammonium sulphate solution, and stirred for an hour at 4 °C, before being centrifuged at 20 000*g* for 1 h. The procedure was repeated a minimum of three times or until no pellet formation was observed. The precipitates were then dialysed overnight at 4 °C, against ultrapure water, using a 3.5 kDa MWCO dialysis tubing (Fisher Scientific, New Hampshire State, USA). The dialysed samples were freeze-dried and stored at −80 °C until further use. Henceforth, the three dialysed stomach chyme (SC) and three jejunal digesta (JD) samples obtained from six different animals are referred to as SC1, SC2, SC3 and JD1, JD2, JD3 respectively.

6.3.5 Total protein analysis

The total protein content was determined using the Dumas method in accordance with the AOAC method 992.15 (1990) and using a LECO FP628 Protein analyzer (LECO Corp., St. Joseph, MI, USA). A conversion factor of 6.25 was used to determine the total protein content from the determined nitrogen concentration.

6.3.6 Tris-tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE analysis was performed using a Bio-Rad Mini-PROTEAN 3 cell System (Bio-Rad Laboratories Ltd., Hertfordshire, UK) and a 10-well Mini-PROTEAN Tris-Tricine gel according to the manufacturer's instructions. The dialysed,

precipitated protein and peptide fractions obtained from SC and JD were loaded onto the gels at a concentration of 1 mg/mL. Both a low molecular weight (LMW (2-250 kDa)) and a high molecular weight (HMW (10-250 kDa) marker were used.

6.3.7 ACE-I inhibition assay

This assay was carried out using an ACE-I inhibitor assay kit in accordance with the manufacturer's instructions. All fractions were assayed at a concentration of 1 mg/mL sample dissolved in HPLC grade water in triplicate. The known ACE-I inhibitor, captopril, was used as a positive control at a concentration of 1 mg/mL. ACE-I inhibition percentage values for each sample were calculated using the equation:

$$\%\ Inhibition\ ACE\text{-}I = \left[\frac{[Blank\ 1 - Abs\ Inhibitor]}{Blank\ 1 - Blank\ 2}\right] \times 100$$

Where, blank 1 is control without the addition of any inhibitor, blank 2 is the reagent blank, and the inhibitor is the positive control or test sample.

6.3.8 FRAP value

The ferric reducing antioxidant potential of peptides was studied using the method of ferric reducing ability of plasma previously described by Benzie and Strain (1996) with modifications suggested by Bolanos de la Torre et al. (2015).

6.3.9 DPPH inhibition

The DPPH inhibition assay was performed in accordance with previously described methods (Goupy et al., 2003; Nicklisch & Waite, 2014). Briefly, the stomach chyme

or jejunal digesta samples and the positive control (resveratrol) were prepared in the assay buffer (1 mg/mL). The samples and positive control solutions (285 µL) were each combined with a freshly prepared methanolic DPPH solution in a Nunc™ 96-well microWell™ plate to obtain a final DPPH concentration of 100 µM. The plate was incubated in the dark at ambient room temperature for 30 min. The absorbance was then read at 515 nm. A (negative) control reaction mixture contained DPPH and methanol but without any sample. The percentage DPPH inhibition was calculated using the following equation:

$$\% \text{ Inhibition DPPH} = \frac{[\text{Abs Control} - \text{Abs Inhibitor}]}{\text{Abs Control}} \times 100$$

Where, Abs Control is the absorbance of the DPPH solution without any resveratrol or test samples and Abs Inhibitor is the absorbance of the test samples or positive control.

6.3.10 MLP inhibition

MLP inhibition by the pig SC and JD samples was studied using the method described by van der Sluis et al. (2000). Briefly, rat liver microsomes were stored at -80 °C until use. For the assay, microsomes were made up in Tris-HCl buffer (50 mM, pH 7.4), containing 150 mM KCl at a concentration of 5 mg protein/mL. The microsomes were then centrifuged at 10,000 g for 60 min at 4 °C to obtain the pellet that was resuspended in the Tris buffer and diluted for further use (0.5 mg/mL of protein). Microsomes (240 µL) were then pre-incubated in a 96-well plate at 37 °C for 5 min, followed by the addition of 30 µL of blank (methanol), antioxidant resveratrol, and inhibitor (SC and JD). Lipid peroxidation (LPO) was induced by the

addition of 15 µL of 4 mM ascorbic acid and 15 µL of 0.2 mM $FeSO_4$. The plate was then incubated again at 37 °C for 60 min. The reaction was stopped by the addition of 500 µL of 0.83% thiobarbituric acid made up in Trichloroacetic acid (TCA)-HCl (16.8% w/v TCA in 0.125 N HCl). The well-plate was then heated at 80 °C for 15 min and 250 µL of each of the chyme or digesta sample/control/blank was transferred to another 96-well plate and the absorption was read at 540 nm. Essentially, the method involved the measurement of the extent of LPO by measuring the thiobarbituric acid reactive species (TBARS). The % of MLP inhibition was calculated as follows:

$$\% \, Inhibition \, MLP = \frac{[Abs \, Blank - Abs \, Inhibitor]}{Abs \, Blank} \times 100$$

Where, Abs blank is the absorbance of the blank solution without any resveratrol or test samples and Abs Inhibitor is the absorbance related to the test samples or positive control.

6.3.11 Electrospray ionization time-of-flight liquid chromatography tandem mass spectrometry (ESI-TOF LC-MS/MS)-based characterisation of peptides

The identification of peptides was carried out in a high resolution mode using an LC Ultra 1D Plus system (Eksigent) with a quadrupole/Time-of-Flight (Q/ToF) TripleTOF® 5600+ detector from ABSciex Instruments (Framingham, MA, USA) that allowed the identification and subsequent relative quantitation of the peptides generated during the processing, using a label-free approach. The chromatographic conditions were established according to (Mora et al., 2015). Briefly, 5 µL of each

sample was loaded onto a trap column (NanoLC Column, 3 µm C_{18}-CL, 75 µm × 15 cm (Eksigent)) and desalted with 0.1% TFA at the rate of 3 µL/min during a period of 5 min. The peptides were then loaded onto an analytical column (LC Column, 3 µm C_{18}-CL, 75 µm × 12 cm (Nikkyo)) that was equilibrated in 5% of acetonitrile (ACN) and 0.1% of formic acid (FA). Peptide elution was carried out with a linear gradient from 5% to 35% of B over a period of 120 min (A: 0.1% FA; B: 0.1% FA in ACN), and at a flow rate of 0.3 µL/min, with a running temperature of 30 °C.

Samples were ionized using an electrospray ionization source (ESI) applying 2.8 kV to the spray emitter. Analysis was carried out in a data-dependent mode. Survey MS1 scans were acquired from 350–1250 m/z for 250 ms. The quadrupole resolution was set to 'UNIT' for MS2 experiments, which were acquired at100–1500 m/z for 50 ms in 'high sensitivity' mode. The following switch criteria were used: charge: 2+ to 5+; minimum intensity; 70 counts per second (cps). Up to 50 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15s. The identification of peptides was performed using the Protein-Pilot v4.5 (AB Sciex) search program. Protein-Pilot default parameters were used to generate the peak list directly from 5600 Triple TOF wiff files. The Paragon algorithm of the Protein-Pilot was used to search the NCBI protein database with the following parameters: no enzyme specificity, no Cys alquilation, and the search effort set to Thorough. To avoid using the same spectral evidence for more than one protein, the identified proteins were grouped based on the MS/MS spectra by using the Protein-Pilot Progroup algorithm. Thus, proteins sharing MS/MS spectra were grouped, regardless of the peptide sequence assigned.

6.3.12 Data analysis

For each activity (ACE-I inhibition, FRAP activity, DPPH- and MLP inhibition), and for each of the SC and JD samples three independent replicates (n=3) were analysed. The results for each of the activity and gastrointestinal location were compared among three individual samples. Where appropriate, a mean value (n=3) and standard deviation were calculated.

The methodology and steps followed to establish whether GEP is a source of bioactive peptides are shown in Figure 6.1.

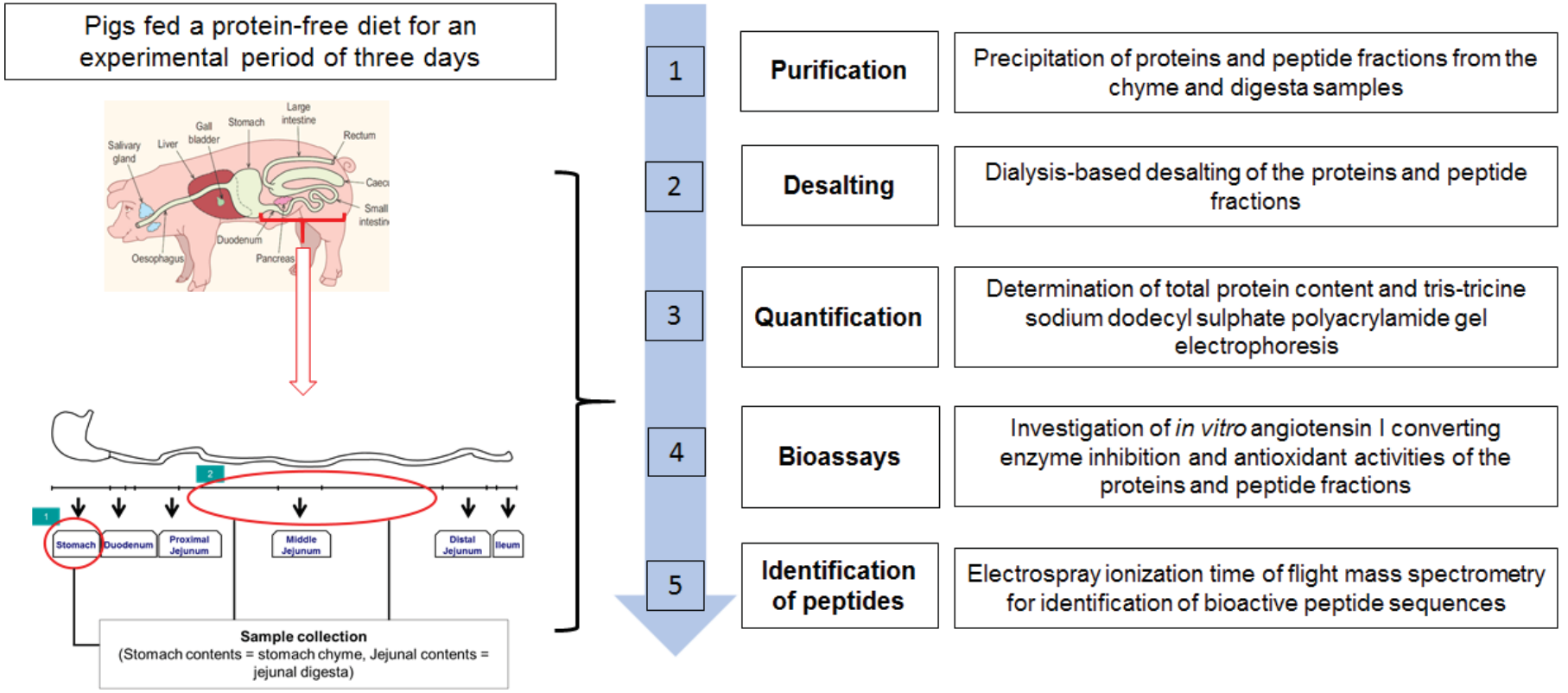


Figure 6.1 *The in vivo and in vitro experimental design and methodologies used to study the generation of porcine gastrointestinal endogenous protein (GEP)-derived bioactive peptides in the stomach chyme and jejunal digesta of pigs, following gastrointestinal (GIT) digestion of a protein free diet.*

6.4 Results

6.4.1 SDS-PAGE analysis

The SDS-PAGE analysis showed the presence of a diverse range of proteins and peptides in both the stomach chyme and jejunal digesta samples. The molecular weight distribution of the extracted proteins and peptides characterised using SDS-PAGE is shown in Figure 6.2, and the respective protein content of the analysed samples is given in Table 6.2.

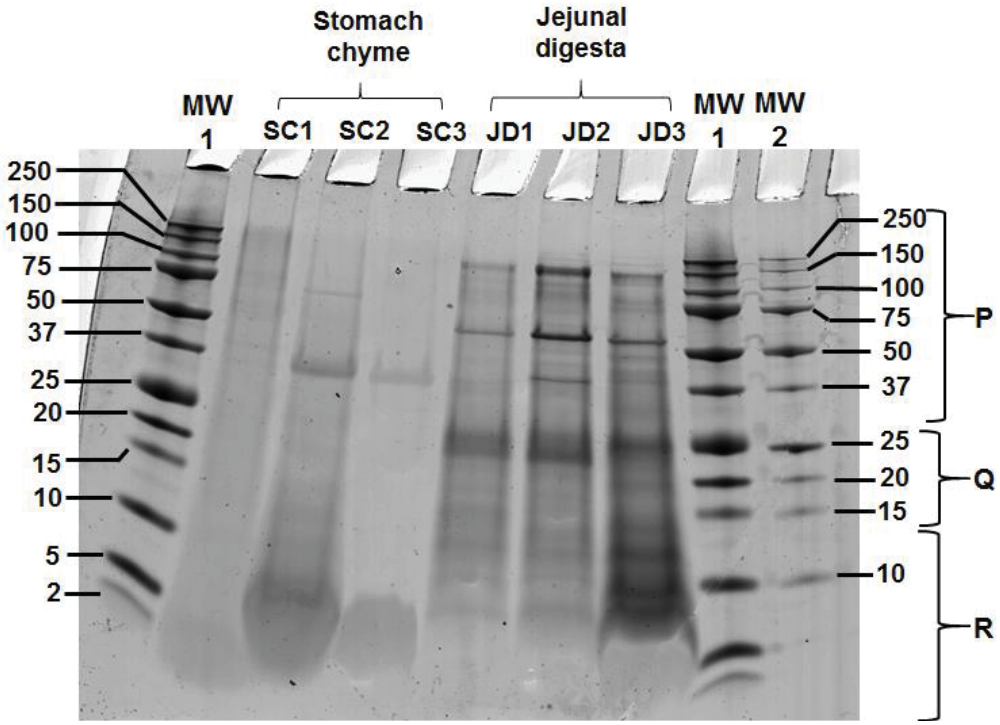


Figure 6.2 *Tris-tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the dialysed protein and peptide fractions precipitated from stomach chyme (SC1, SC2, and SC3) and jejunal digesta (JD1, JD2 and JD3) samples obtained from the growing pigs.*

Table 6.2 *The protein content of the dialysed protein and peptide fractions precipitated from stomach chyme (SC1, SC2, and SC3) and jejunal digesta (JD1, JD2 and JD3).*

Sample	Pig No.	Protein content (%)	Mean (n=3 ± S.D) protein content (%)
Gastric chyme	SC1	10.5	24.0 ± 12.3
	SC2	21.0	
	SC3	40.0	
Jejunal digesta	JD1	36.0	45.0 ± 6.2
	JD2	49.0	
	JD3	49.0	

The SC samples SC2 and SC3 showed similar patterns, while the sample SC1 was observed to have a higher proportion of peptides >37 kDa in size (regions Q and R). The SC sample (SC2) showed an additional band at approximately 75 kDa (region P) which was absent in SC1 and SC3. A higher proportion of peptides <25 kDa (region Q) were identified in SC2, while SC3 showed a small proportion of bands around 2 kDa in size (region R).

The JD samples had a comparatively higher quantity of proteins and/or peptides than the SC samples. The polypeptide composition of all of the JD samples appeared different. In region P, for the sample JD2 strong bands were observed at 150 kDa and 50 kDa; while JD3 showed faint bands between 50 and 37 kDa. In region Q, the

peptide bands of the samples JD1 and JD2 were different from those found in JD3. In region R, all of the three JD samples showed bands that were different from each other, in particular in the size range between 15-10 kDa.

6.4.2 ACE-I inhibition

The ACE-I inhibitory activity of the SC and JD samples is shown in Figure 6.3. ACE-I inhibitory bioactivity of the chyme samples ranged from 96.5% ± 1.2 for SC3 to 38.0% ± 11.2 for SC2. In comparison, the digesta samples JD2 and JD3 showed activity of >80% (80.7% ± 2.8 and 93.7% ± 1.7 respectively).

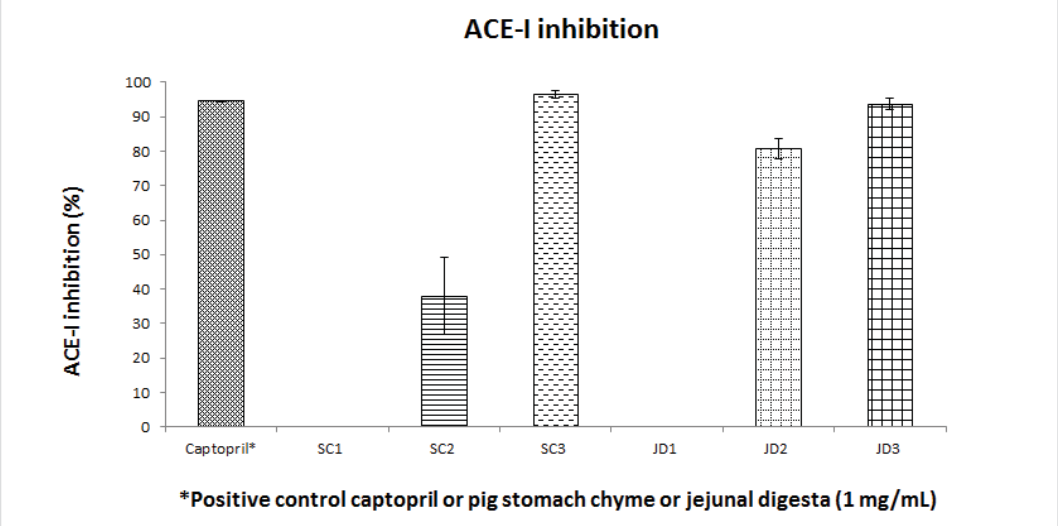


Figure 6.3 *ACE-I (Angiotensin-I converting enzyme) inhibition for the dialysed protein and peptide fractions precipitated from stomach chyme (SC1, SC2, and SC3) and jejunal digesta (JD1, JD2 and JD3) samples each obtained from three growing pigs and for the positive control captopril.*

The ACE-I inhibitory activities of SC3 and JD3 were comparable to the positive control captopril when assayed at a concentration of 1 mg/mL. The activities in the samples SC1 and JD1 were not detectable which may have been due to the relatively lower protein content observed or due to interference from other components that may have been co-extracted along with the polypeptides from these samples.

6.4.3 Antioxidant potential

6.4.3.1 FRAP values

The FRAP assay measures the antioxidant potential of polypeptides based on their ferric reducing ability and the colorimetric detection of the resultant ferrous-tripyridyltriazine complex (Benzie & Strain, 1996). All of the SC samples with the exception of SC2, and all of the JD samples showed ferric reducing potential. The maxima of FRAP values (Figure 6.4) amongst JD samples (JD2 = 56.65 ± 0.07 μM trolox equivalents/g) was double that of the most active SC sample (SC3 = 28.68 ± 1.58 μM trolox equivalents/g). The activities of samples SC1 and JD3 were less than 10 μM trolox equivalents/g.

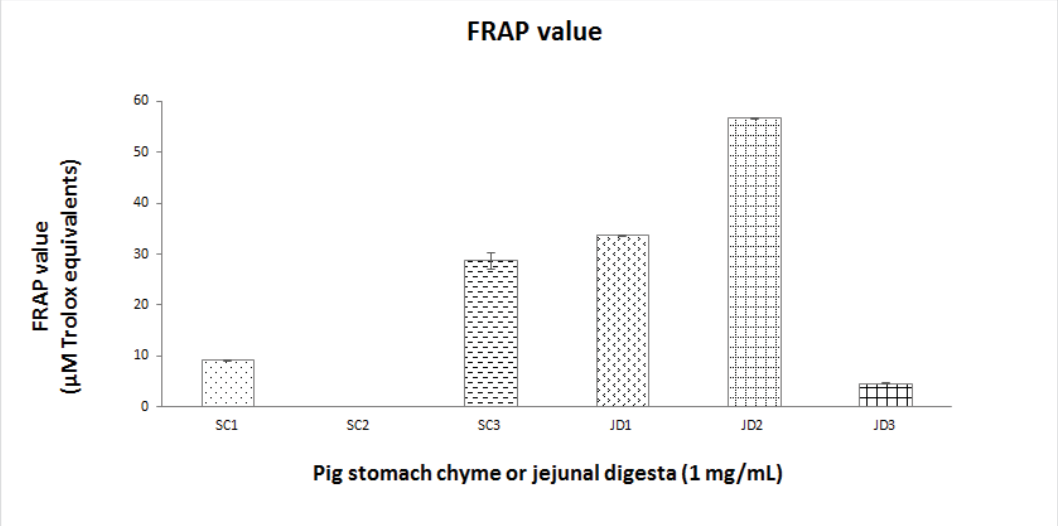


Figure 6.4 *FRAP (Ferric reducing antioxidant potential) for the dialysed protein and peptide fractions precipitated from stomach chyme (SC1, SC2, and SC3) and jejunal digesta (JD1, JD2 and JD3) samples each obtained from three growing pigs.*

6.4.3.2 DPPH inhibition

DPPH is a stable free radical, and this bioassay tests the antioxidant potential of polypeptides by hydrophobic interaction-based scavenging and/or reducing capacity (Kedare & Singh, 2011; Udenigwe & Aluko, 2011). Among the SC samples, only SC2 showed DPPH inhibition (45.22% ± 2.93, Figure 6.5). All of the JD samples were found to have DPPH inhibition potential with the activity of JD1 (94.01% ± 0.66) being comparable to that of the control resveratrol (94.77% ± 0.75) when assayed at 1 mg/mL. The DPPH inhibition potential of polypeptides is known to be affected by stearic hindrance arising from side chain residues (Udenigwe & Aluko,

2011), and the heterogeneous composition of the samples (Figure 6.2) may be one of the reasons for the observed variations in bioactivities.

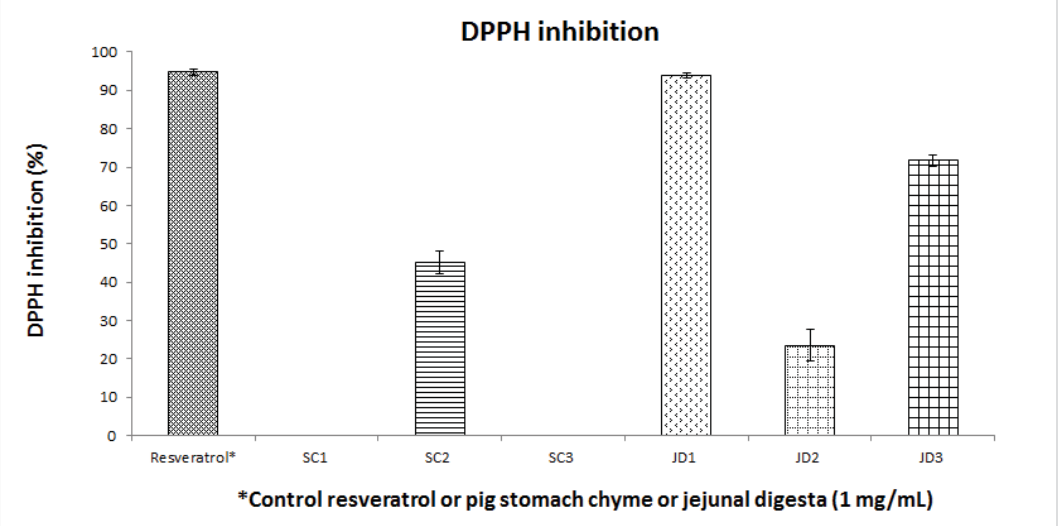


Figure 6.5 *DPPH (2,2-diphenyl-1-picrylhydrazyl) inhibition for the dialysed protein and peptide fractions precipitated from stomach chyme (SC1, SC2, and SC3) and jejunal digesta (JD1, JD2 and JD3) samples each obtained from three growing pigs and for the positive control (resveratrol).*

6.4.3.3 MLP inhibition

MLP inhibition utilises rat liver microsomes to measure the inhibition of chemically induced (radical-based) lipid peroxidation (LPO) that can be estimated using thiobarbituric acid reactive species (TBARS). All of the SC samples (Figure 6.6) showed MLP inhibition ranging from 15.43% ± 3.52 to 38.81% ± 1.40 for SC2 and SC3 respectively. In comparison, although JD1 showed an activity of 59.90% ± 0.64, no activity was detected in JD2 and JD3.

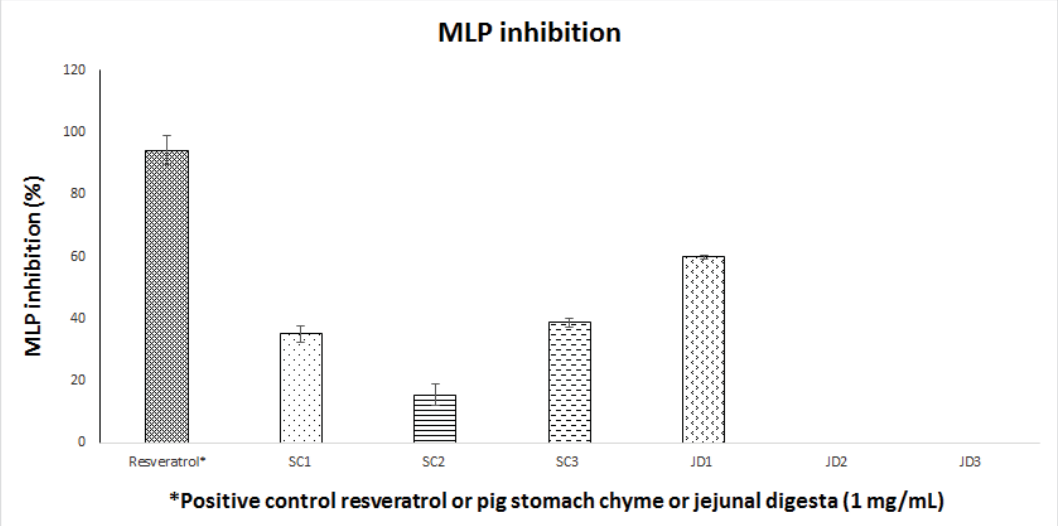


Figure 6.6 *MLP (Microsomal lipid peroxidation) inhibition for the dialysed protein and peptide fractions precipitated from stomach chyme (SC1, SC2, and SC3) and jejunal digesta (JD1, JD2 and JD3) samples each obtained from three growing pigs and from the positive control resveratrol.*

6.4.4 ESI-TOF LC-MS/MS characterisation of peptide sequences

Over 1100 peptide sequences (with different cut-off scores) were identified from SC1, SC2, S3, JD1, JD2 and JD3 samples. The various parent proteins (GEP) from which these peptide sequences were released in the GIT are shown in Table 6.3. Table 6.4 lists the sequences of the identified peptides together with their m/z obtained in the detector and their molecular masses calculated based on the charge of ionisation are also given. The data reported in Table 6.4 are for the peptide sequences that were identified with a 90% or greater confidence level and for the three most active fractions i.e. SC3, JD1 and JD3.

The peptides identified were released from over 45 different pig GEP (Table 6.3), including the important contributors to GEP such as gastric mucin (identified in SC2), serum albumin (identified in SC1, SC2, S3), and lysozyme (identified in SC1), and GIT enzymes (identified in all of the samples). Among all of the GEP shown in Table 6.3, a basic proline-rich protein (PRP) precursor, singly, was found to release the highest number of identified peptide sequences in the majority of samples. The PRP as the highest source was followed by the GEP pepsin and/or pepsinogen. Other parent GEP from which peptide sequences were identified, are likely to be secreted in small quantities and these include immunoglobulins, GIT hormones, and proteins derived from epithelial cells.

A majority of the peptide sequences identified from the GEP were found to be rich in proline residues. The amino acid chain length of the identified peptides ranged from 10 to >50 amino acid residues. All of the 181 peptide motifs reported in Table 6.4 were further screened against previously reported bioactive peptides documented in BIOPEP (Minkiewicz et al., 2008b), and it was found that these motifs may be potentially novel bioactive peptides.

Table 6.3 *Parent gastrointestinal endogenous proteins (GEP) that released the peptide sequences that were identified from the dialysed protein and peptide fractions precipitated from stomach chyme (SC) and jejunal digesta (JD) of growing pigs fed a protein-free diet. The samples were analysed using electrospray ionization time-of-flight liquid chromatography tandem mass spectrometry (ESI-TOF LC-MS/MS).*

Parent GEP that released the peptide sequences identified from stomach chyme and jejunal digesta
Basic proline-rich protein precursor
Cystatin-C precursor
Calcium-activated chloride channel regulator 1 precursor
Transferrin/serotransferrin precursor
Pancreatic alpha-amylase precursor
Carbonic anhydrase 6 precursor
Antibacterial protein precursor
Albumin/serum albumin precursor
Cytoskeletal beta actin
Complement C3 precursor
Lactotransferrin precursor
Protein S100-A8
Gastrokine-1 precursor
Gastrokine-3 precursor

Lysozyme C-3

RNA-binding region containing protein 2-like

Pancreatic lipase-related protein 2 precursor

Pepsin A preproprotein

Pepsinogen A precursor

Albumin/Serum Albumin

Pancreatic lipase-related protein 2 precursor

Protegrin-4 precursor

Serotransferrin precursor

Galectin-3

Hemopexin precursor

Apolipoprotein A-I

Chitinase, acidic precursor

Gastric mucin

Tetraspanin-1

Hemoglobin subunit beta

Gastrotropin

Aminopeptidase N

Glutamyl aminopeptidase

Chymotrypsin-like elastase family member 2A preproprotein

Trypsin-2 precursor

Liver fatty acid binding protein

Chymotrypsin-C precursor

Dipeptidase 1 precursor

Cyclophilin

Proline-rich protein 13 isoform 1

Protein S100-A12

Angiotensin-I converting enzyme isoform 2 precursor

Angiotensin-I converting enzyme 2

Protein S100-G

Pancreatic elastase 2 precursor

Dipeptidyl peptidase 4

Prostaglandin reductase 1

Retinol-binding protein 2

Cytoplasmic Aspartate aminotransferase

Table 6.4 *Gastrointestinal endogenous protein (GEP)-derived peptide sequences that were identified from the dialysed protein and peptide fractions precipitated from stomach chyme (SC3) and jejunal digesta (JD1 and JD3) of growing pigs fed a protein-free diet. The samples were analysed using electrospray ionization time-of-flight liquid chromatography tandem mass spectrometry (ESI-TOF LC-MS/MS).*

Sample	Identified protein	Sequence	Obs. Mass (Da)	Theor. Mass (Da)	Obs. m/z	Theor. m/z	Theor. z
SC3	Basic proline-rich protein precursor	APPGARPPPGPPPPAGGL[a]	1601.89	1601.87	801.95	801.94	2
		APPGARPPPGPPPPGPPPPGPAPPGARPPPGPPPPAGG[a]	3373.86	3373.78	844.47	844.45	4
		APPGARPPPGPPPPGPPPPGPAPPGARPPPGPPPPGP[a]	3342.95	3342.78	1115.32	1115.27	3
		APPGARPPPGPPPPPPGPSPPRPPPGPPPQ[a]	2856.52	2856.52	715.14	715.14	4
		APPGARPPPPPPPPADE	1658.87	1658.84	830.44	830.43	2
		APPGARPPPPPPPPADEPQQGP[a]	2166.10	2166.09	723.04	723.04	3

ARPPPGPPPPGPPPPGP	1580.87	1580.85	791.44	791.43	2
DKPKKKPPPPAGPPPPPPPPPGPPPPGPAPPGARPPPGPPPPGPPPPGP[a]	4616.58	4616.52	770.44	770.43	6
FLRPPPGGGPPRPPPPEE	1892.97	1892.99	632.00	632.00	3
GPPPPGPAPPGARPPPGPPPPGPPPPGP[a]	2502.37	2502.32	835.13	835.11	3
GPPPPPPPPPGPPPPGP	1547.75	1547.81	516.93	516.95	3
KPPPPAGPPPPPPPPPGPPPPGP	2135.17	2135.16	712.73	712.73	3
LRPPPGGGPPRPPPPEESQGEG[a]	2204.11	2204.10	735.71	735.71	3
PEQGPAPPGARPPPGPPPPGPPPPGP	2411.29	2411.24	804.77	804.75	3
PGPAPPGARPPPGPPPPPPGPSPPRPPPGPPPQ	3107.69	3107.65	777.93	777.92	4
PPGPAPPGARPPPGPPPPGPPPPGP	2251.25	2251.19	751.42	751.40	3
RPPPGPPPPGPPPPGP	1509.82	1509.81	755.92	755.91	2

SGDKPKKKPPPPAGPPPPPPPPPGPPPPGPAPPGARPPPGPPPPGPPPPGP[a]	4760.65	4760.57	794.45	794.44	6
SHVGPKKKPPPPGAG	1452.75	1452.82	485.26	485.28	3
SHVGPKKKPPPPGAGHPPRPPPPANE[a]	2642.41	2642.42	661.61	661.61	4
SQEGSPPSADGPQQGP	1537.68	1537.66	769.85	769.84	2
SQGEGHQKRPRPPGDGPEQGP	2210.08	2210.06	737.70	737.69	3
EQGPAPPGARPPPGPPPPGPPPPGP	2314.32	2314.19	1158.17	1158.10	2
QGEGHQKRPRPPGDGPEQGPAPPGARPPPGPPPPPGPPPPGP	3991.06	3991.00	799.22	799.21	5
QGPAPPGARPPPGPPPPGPPPPGP	2313.26	2313.24	772.09	772.09	3
APPGARPPPGPPPPGPPPPGPAPPGARPPPPPPPPADE[a]	3543.94	3543.84	886.99	886.97	4
DKPKKKPPPPAGPPPPGPPSPGP	2236.26	2236.24	746.43	746.42	3

	PQQGPAPSGDKPKKKPPPPAGPPPPGPPSPGP	3055.66	3055.62	764.92	764.91	4
	PAPPGAEPPPGPPPPGPPPPGP	2011.03	2010.96	671.35	671.33	3
Pepsinogen A precursor	DTEYFGTIGIGTPAQDF	1830.90	1830.83	916.46	916.42	2
	DTVQVGGISDTNQIFGLS	1849.97	1849.91	925.99	925.96	2
	EYFGTIGIGTPAQDF	1614.78	1614.76	808.40	808.39	2
	FTVIFDTGSSNL	1299.63	1299.63	650.82	650.82	2
	GLAYPSISASGATPVFDNL	1879.00	1878.94	940.51	940.48	2
	IGDEPLENYLDTEY	1669.77	1669.74	835.89	835.88	2
	LAYPSISASGATPVFDNL	1821.97	1821.91	911.99	911.96	2
	LDTEYFGTIGIGTPAQDF	1943.99	1943.92	973.00	972.96	2
	TVQVGGISDTNQIFGLSETEPGSF[a]	2482.25	2482.19	828.42	828.40	3
	VGGISDTNQIFGLSETEPGSFL[a]	2267.21	2267.10	1134.61	1134.56	2

	FLKTHKHNPASKY	1569.78	1569.84	524.27	524.29	3
	KTHKHNPASKYFPEA	1753.85	1753.89	585.63	585.64	3
	LKTHKHNPASKYFPEA[a]	1866.95	1866.97	623.32	623.33	3
	CSSLACSDHNQFNPDDSST	2024.83	2024.75	1013.42	1013.38	2
Albumin/serum albumin precursor	LQHKNDNPDIPKLKPDPVA[a]	2138.15	2138.15	713.73	713.72	3
	FAKTCVADESAENCDKS[‡]	1814.80	1814.76	908.41	908.39	2
Chitinase,	DIGAPTSGAGPAGPYTKE	1687.84	1687.81	844.93	844.91	2

	acidic precursor	IGAPTSGAGPAGPYTKE	1572.80	1572.78	787.41	787.40	2
JD1	basic proline-rich protein precursor	APPGARPPPGPPPPAGG	1488.79	1488.78	745.40	745.40	2
		APPGARPPPGPPPPGPPP[a]	1651.88	1651.88	826.95	826.95	2
		APPGARPPPGPPPPPPGP	1651.88	1651.88	826.95	826.95	2
		ARPPPGPPPPGPPPPGPAPPGARPPPGPPPPPPGP[a]	3213.72	3214.72	804.44	804.69	4
		ARPPPGPPPPPPGPSPPRPPPGPPP	2406.30	2406.30	803.11	803.11	3
		GARPPPGPPPPGPPPPGPAPPGARPPPGPPPPGPPPPGP[a]	3522.87	3522.87	881.73	881.72	4
		GPPPPGPAPPGARPPPGPPPPGPPPPGP[a]	2502.32	2502.32	835.11	835.11	3
		GPPPPGPPPPGPAPPGARPPPGPPPPGPPPPGP[a]	2947.56	2947.55	737.90	737.89	4
		GPPPPPPPPPGPPPP	1391.66	1393.74	696.84	697.88	2

GPPPPPPPPPGPPPPGPAPPGARPPPGPPPPGPPPPGP[a]	3432.82	3432.81	859.21	859.21	4
LRPPPGGGPPRPPPPEES	1832.96	1832.95	611.99	611.99	3
PGPPPPGPAPHGARPPPGPPPPGPPPPGP[a,b]	2682.41	2682.38	895.14	895.13	3
PGPPPPGPPPPGPAPPGARPPPGPPPPGPPPPGP	3035.60	3044.60	759.91	762.16	4
PLPGPPPPGPPPPGPAPPGARPPPPPPPP[a]	2695.47	2695.46	674.87	674.87	4
PPGARPPPGPPPPGPPPPGP	1831.98	1831.97	917.00	916.99	2
PPGPPPPGPAPPG	1126.59	1133.59	564.30	567.80	2
PPGPPPPGPPPPGPAPPGARPPPGPPPPGPPPPGP	3141.48	3141.66	629.30	629.34	5
PPPGPPPPGPPPPGPAPPGARPPPGPPPPGPPPPGP[a]	3239.72	3238.71	1080.91	1080.58	3
PPPGPPPPPPGPSPPRPPPGPPP	2179.16	2179.16	727.39	727.39	3

PPPPPPPPPGPPPPGP	1490.79	1490.79	497.94	497.94	3
RPPPGGGPPRPPPPEE	1632.84	1632.84	545.29	545.29	3
RPPPGPPPPGPPPPGPAPPGARPPPGPPPPGPPPPGP	3394.81	3394.81	849.71	849.71	4
PAGPPPPPPPPPGPPPPGPAPPGARPPPGPPPPGPPPPGP[a]	3599.85	3600.90	900.97	901.23	4
PPPGPPPPGPPPPGPAPPGA[a]	1742.83	1746.91	581.95	583.31	3
GARPPPGPPPPGPPPPGP	1633.76	1637.87	545.59	546.96	3
PGPPPPGPPPPGPAPPGARPPPGPPPPPPGPSPPRPPPG[a]	3560.89	3560.88	891.23	891.23	4
RPPPGPPPPGPPPPGPAPHGARPPPGPPPPGPPPPGP	3429.81	3434.82	858.46	859.71	4
PPPPGPPPPGPAPHGARPPPGPPPPGPP[b]	2625.39	2625.36	657.36	657.35	4

PGARPPPGPPPPGPPPPGP	1731.94	1734.92	578.32	579.31	3
PGPPPPGPAPPGARPPPGPPPPGPPPPGP	2590.97	2599.37	648.75	650.85	4
ARPPPGPPPPGPPPPGPAPPGARPPPPPPPP	2906.58	2906.57	969.87	969.86	3
AEPPPGPPPPGPPPPGPAPPG	1901.98	1901.97	635.00	635.00	3
PPGPPPLGPPPPGPAPPGARPPPGPPPPGPPPPGP	3147.69	3157.69	787.93	790.43	4
PPPGPPPPGPPPPGPAPPGARPPPPPPPP	2679.43	2679.43	894.15	894.15	3
AEPPPGPPPPGPPPPGP	1579.82	1579.80	790.92	790.91	2
PPPGPPPPGPPPPGPAPPGARPPPGPPPLGPPPPGPAPPGARPPPGPPPPGPPPPGP	5126.47	5139.74	855.42	857.63	6
PAGPPPPGPPSPGPAPPGARPPPGPPPPGPPPPGP[c]	3147.69	3147.63	787.93	787.91	4
PPPGPPPPGPPPPGPAPPGARPPPPPPPPA[c]	2766.50	2766.46	923.18	923.16	3

	TWSQQYPGGHSITNT[c]	1691.76	1691.75	846.89	846.88	2
	IISEVKQDGQNFTWSQQYPGGHSITNT	3034.45	3034.44	1012.49	1012.49	3
	KQDGQNFTWSQQYPGGHSITNT	2493.13	2493.13	832.05	832.05	3
	EIESEKNYDEFMKR	1816.84	1816.83	606.62	606.62	3
Gastrotropin	FTWSQQYPGGHSITNT	1822.83	1822.83	912.42	912.42	2
	NLKIISEVKQDGQNFTWSQQYPGGHSITNT[a]	3389.67	3389.66	848.43	848.42	4
	SEVKQDGQNFTWSQQYPGGHSITNT	2808.28	2808.27	937.10	937.10	3
	VDGKLVEVSTVGGVS	1444.78	1444.78	723.40	723.40	2
	ISEVKQDGQNFTWSQQYPGGHSITNT[d]	2970.33	2970.33	991.12	991.12	3
Aminopeptidase N	DATSTINSIASNVIGQPL	1799.99	1799.93	901.00	900.97	2
	DALASSHPLTTPAEEVNTPAQI	2261.12	2261.12	1131.57	1131.57	2

	DFAWGQLQQAQLVNEADKLR[a]	2329.23	2329.18	777.42	777.40	3
	GGGSFSFSNLIQGVTR	1624.85	1625.82	813.43	813.92	2
Glutamyl aminopeptidase	ITQLPVLWRPSGEQVQV	1949.16	1949.07	975.59	975.54	2
	DDSLISSHPIVVTV	1480.79	1480.78	741.40	741.40	2
Chymotrypsin -like elastase family member 2A preproprotein	TRVSNYIDWINSVIANN	1977.06	1977.99	989.54	990.00	2
	GRLQTNGASPDILQQGQLLV	2107.25	2107.14	1054.63	1054.58	2
	VVGGEDARPNSWPWQVS[a]	1882.90	1882.90	942.46	942.46	2
	GRLQTNGASPDILQQGQLL	2008.08	2008.07	670.37	670.36	3
Trypsin-2 precursor	TLDNDILLIKLSSPAV	1711.02	1710.98	856.52	856.50	2
Liver fatty acid binding protein	TVVQLEGDNKLVTT	1515.82	1515.81	758.92	758.91	2

	Chymotrypsin -C precursor	VVGGENAVPHSWPWQIS	1861.99	1861.91	932.00	931.96	2
	Dipeptidase 1 precursor	GFGGDYDGVSRVPSGL	1581.75	1581.74	791.88	791.88	2
	Cyclophilin	DIAVDGEPLGRVSF	1473.76	1473.75	737.89	737.88	2
JD3	Basic proline-rich protein precursor	APPGARPPPGPPPPAGG	1488.80	1488.78	745.41	745.40	2
		APPGARPPPGPPPPGPPPPGP[a]	1902.99	1903.01	635.34	635.34	3
		APPGARPPPGPPPPPPGP	1651.91	1651.88	826.96	826.95	2
		GARPPPGPPPPGPPPPGP	1637.90	1637.87	819.96	819.94	2
		GPPPPPPPPPGPPPPGPAPPG[a]	1869.95	1869.98	624.32	624.33	3
		GPPRPPPPEES[a]	1158.54	1158.57	580.28	580.29	2
		PGPPPPGPPPPGP	1159.58	1159.60	580.80	580.81	2
		PLPGPPPPGPPPPGP[a]	1369.66	1369.74	457.56	457.59	3

PPGARPPPGPPPPGPPPPGP	1832.03	1831.97	917.02	916.99	2
PPGARPPPGPPPPPPGP	1580.87	1580.85	791.44	791.43	2
PPPGPPPPGPPPPGPAPPGA[a]	1746.87	1746.91	583.30	583.31	3
PPPGPPPPPPGP	1102.55	1102.58	552.28	552.30	2
PPPPPPPGPPPPGP	1296.60	1296.69	433.21	433.24	3
PPPPPPPPPGPPPPGP	1490.73	1490.79	497.92	497.94	3
PRPGPPPPGPPPPGP	1412.76	1412.76	707.39	707.39	2
RPPPGGGPPRPPPPE	1503.73	1503.79	502.25	502.27	3
RPPPGGGPPRPPPPEES[a]	1719.83	1719.87	574.28	574.30	3
RPPPGPPPPAGG	1095.55	1095.58	548.78	548.80	2
RPPPGPPPPGPPPPGP[a]	1509.82	1509.81	755.92	755.91	2
RPPPGPPPPPPGP	1258.60	1258.68	420.54	420.57	3

	SQPGPRPPPGPPSPPA	1534.80	1534.79	768.41	768.40	2
	SPPRPPPGPPP	1094.56	1094.59	548.29	548.30	2
	EIESEKNYDEFMKRL[a]	1929.90	1929.91	644.31	644.31	3
	IISEVKQDGQNFTWSQQYPGGHSITNT	3034.57	3034.44	1012.53	1012.49	3
	NLKIISEVKQDGQNFTWSQQYPGGHSITNT[a]	3389.73	3389.66	848.44	848.42	4
	RLALPSDAIDKAR	1424.74	1424.81	475.92	475.94	3
	SEVKQDGQNFTWSQQYPGGHSITNT[a]	2808.36	2808.27	937.13	937.10	3
Gastrotropin	SQQYPGGHSITNT	1388.63	1388.63	695.32	695.32	2
	TWSQQYPGGHSITNT[a,c]	1707.79	1707.75	854.90	854.88	2
	VDGKLVEVSTVGGVS	1444.80	1444.78	723.41	723.40	2
	LALPSDAIDKAR	1268.62	1268.71	423.88	423.91	3
	KQDGQNFTWSQQYPGGHSITNT	2509.19	2509.13	837.40	837.38	3

	EAFMKAVGLPDEL	1418.71	1418.71	710.36	710.36	2
	IQKGKDIKGTSEIVQNGKH	2079.07	2079.14	520.77	520.79	4
liver fatty acid binding protein	KLTITTGSKVVQNE[a]	1516.78	1516.85	506.60	506.62	3
	NGDIITSTMTL[a]	1164.55	1164.57	583.28	583.29	2
	QVQSQENFEAFMKAVGLPDEL[c]	2395.18	2395.14	799.40	799.39	3
	SVTELNGDIITSTM[a]	1479.72	1479.71	740.87	740.86	2
	ITQLPVLWRPSGE	1494.83	1494.82	748.42	748.42	2
	KALSNMPVEKEESVDDIWTQTTFQK	2923.45	2923.43	731.87	731.86	4
Glutamyl aminopeptidase	SGKPLTIYVQPEQKHTAE	2025.05	2025.05	676.02	676.02	3
	SLPKLDKIAIPDFGTGAMENW[c]	2318.18	2318.16	773.73	773.73	3
	SVDDIWTQTTFQK	1567.77	1567.75	784.89	784.88	2

	TSDFWEALEEASNLPVK	1935.00	1934.93	968.51	968.47	2
Glutamyl aminopeptidase	TNQMGYPVLNVEDMRIISQKR[c]	2523.23	2523.26	631.81	631.82	4
Angiotensin-I converting enzyme isoform 2 precursor	DLVVAHHEMGHIQ	1484.65	1484.72	495.89	495.91	3
	KQDGFSDTGAYWR[a]	1529.63	1529.69	510.88	510.90	3
	REGANPGFHEAIGDV	1567.68	1567.74	523.57	523.59	3
	SLDPELTNILATSRS	1615.87	1615.84	808.94	808.93	2
	SNIYDLVVPFPSASK	1635.88	1635.85	818.95	818.93	2
Angiotensin-I converting enzyme 2	QSTTEELAKTFLEKF[a]	1753.92	1753.88	877.97	877.95	2
	TLALENIVGVKT	1256.72	1256.73	629.37	629.37	2
Pancreatic triacylglycerol lipase	RLDPSDAKFVDVIHTDAAPIIPNLG[a]	2673.40	2673.41	669.36	669.36	4
	SQIVDIDGIWEGTRD	1702.82	1702.82	852.42	852.42	2

precursor	VDVIHTDAAPIIPNLG[a]	1643.91	1643.89	822.96	822.95	2
	VADPSTITDSNF	1265.56	1265.58	633.79	633.80	2
	YLNTGDASNF	1100.45	1100.48	551.23	551.25	2
	SEVCFPRLGCFS	1341.58	1341.60	671.80	671.81	2
Protein S100-G	EGDPNQLSKEELKQLIQAEFPS	2499.30	2499.25	834.11	834.09	3
	TLDDLFQELDKNGDGEVSFE[a]	2270.14	2270.02	1136.08	1136.02	2
Pepsin A preproprotein	DTVQVGGISDTNQIFGLS[a]	1849.96	1849.91	925.99	925.96	2
	TVQVGGISDTNQIFGLS	1734.91	1734.88	868.46	868.45	2
Pancreatic elastase 2 precursor	IDWINSVIANN	1257.62	1257.64	629.82	629.82	2
	VVGGEDARPNSWPWQVS	1882.96	1882.90	942.49	942.46	2
	VVHQDWNSNQLSKGNDIAL	2137.03	2137.06	713.35	713.36	3
Dipeptidyl	TGKENVIYNGVTDW	1594.79	1594.76	798.40	798.39	2

peptidase 4						
Prostaglandin reductase 1	TGPPPPGPPPEVV[a]	1239.64	1239.65	620.83	620.83	2
Chymotrypsin -C precursor	VVGGENAVPHSWPWQISLQ[a]	2103.12	2103.05	1052.57	1052.53	2
Dipeptidase 1 precursor	AAVGFGGDYDGVSRVPSGL	1822.94	1822.89	912.48	912.45	2
Retinol-binding protein 2	SNDNFEGYMKAL	1387.61	1387.61	694.81	694.81	2
Hemopexin precursor	SLQEEFPGVPSPL	1398.70	1398.70	700.36	700.36	2
Cytoplasmic Aspartate aminotransfe-rase	AEVPQAQPVLVF	1296.70	1296.71	649.36	649.36	2

[a] Longest identified peptide from the respective GEP; [b] Carbamylation; [c] Oxidation (W, P, M); [d] Deamidation N; [e] Amidation; [ǂ] Peptide reported in a previous *in vitro* study (Chapter 5) on gastrointestinal endogenous proteins (Chapter 5, Dave et al., 2016a)

6.5 Discussion

Mammalian GEP are an important and consistent source of protein delivered to the GIT lumen that aid the processes of digestion and absorption of nutrients in the GIT. The human atlas project suggests that up to 71% of all of the proteins in the human body may be expressed in at least one region of the GIT (Gremel et al., 2015; Uhlen et al., 2015). This indicates that a wide range of proteins can be secreted into the gut lumen. This myriad of proteins may be a potentially significant source of bioactive peptides in the gut, and may in turn contribute to gut and systemic health.

Recent *in silico* studies (Chapter 3, Dave et al., 2014) have reported that GEP, upon digestion by the major gut proteases, may potentially release numerous bioactive peptides that are found to be active *in vitro* (Chapter 5, Dave et al., 2016a; Chapter 4, Dave et al., 2016b). Although *in silico* and *in vitro* methods are a logical first step in determining whether GEP-derived peptides are an important source of exogenous bioactive peptides (ExBP) in the gut (Chapter 2, Dave et al., 2015), the larger question of whether GEP can release bioactive peptides *in vivo* has until now remained unanswered. There are three sources of ExBP in the lumen of the GIT; dietary proteins, GEP and bacterial proteins. Several studies have examined gut contents to identify bioactive peptides released during the GIT digestion of dietary proteins in rats (Trompette et al., 2003), pigs (Bauchart et al., 2007) and humans (Boutrou et al., 2013; Boutrou et al., 2015), but bioactive peptides specifically from the GEP have not been investigated.

In the presently reported work, a protein-free diet was fed to pigs and digesta (containing digested GEP) sampled from the stomach and small intestine. Although, feeding a protein-free diet is known to underestimate the amount of GEP secreted

into the GIT, it is a suitable method for obtaining GEP proteins for the purpose of qualitative analysis (Moughan et al., 1998). Gut proteins and peptides obtained upon feeding a protein-free diet are unequivocally of gut endogenous origin.

The SDS-PAGE results showed that the proportion of polypeptides extracted from the JD was comparatively higher than that found in pig SC, and these results are in agreement with the determined protein content (Table 6.2) of the samples. This difference may partly result from the time of the sample (chyme) collection for the gastric phase. The gastric chyme samples were collected from pigs sacrificed at 3 hours after the test meal, by which time a significant amount of stomach contents may have been emptied into the duodenum (Montoya et al., 2014). Moreover, the proportion of proteins secreted in the intestinal lumen is much higher (Jackson, 2000) and potentially more diverse (Gremel et al., 2015; Miner-Williams et al., 2012) as found in the SDS-PAGE analysis of the JD samples.

The range of the sizes of polypeptides in the SC and JD samples were different, which may also indicate the diversity of proteins secreted at various stages of digestion in the GIT. All of the individual samples for SC and JD showed heterogeneity in their compositions (Figure 6.2; regions P, Q and R) indicating that the way proteins are digested may vary from animal to animal and over time. Also, there is likely to be an effect of time of sampling, and this effect would likely vary between animals.

The SC and JD samples from different pigs showed variation in the ACE-I inhibitory activity. Nevertheless, both groups showed bioactivity. The observed difference in bioactivities within the same group could arise from the possibility that

the protein and peptide compositions of SC1 and JD1 respectively could be different from SC2, SC3 and JD2, JD3 respectively, which may be due to the effect of sampling at one point of time for each location. Although the SDS-PAGE did not show any significant changes in the polypeptide patterns to this effect, the activities in SC1 and JD1 remained undetectable. This may be attributed to the lower protein concentration in these samples. It is possible that the concentration of the "active" peptides present in these samples was not high enough to inhibit ACE-I.

The relative antioxidant potential across pigs for both the SC and JD samples differed depending on the assay used. Regardless all samples recorded antioxidant potential with at least one of the three bioassays employed in the present work. (i.e. DPPH inhibition, FRAP value or MLP inhibition). Given that each of the assays used reflects a different mechanism of action, it is likely that the antioxidant compounds present in the SC and JD samples do not all act via the same mechanisms. Further, it is not known whether single peptides or proteins present in the SC and JD samples may act via multiple mechanisms. Further investigations are necessary to study this aspect of peptidic antioxidants.

The highest (57 μM trolox equivalents/g) and the lowest (<10 μM trolox equivalents/g) FRAP values obtained for the JD and SC samples in the present work are comparable to previously reported FRAP values for known dietary protein sources. For example, the FRAP values of Alaska Pollack skin collagen after simulated *in vitro* gastrointestinal digestion have been reported to be in the range of 0.87 ± 0.10 and 1.27 ± 0.03 μmol trolox equivalent/g (Guo et al., 2015), while *Palmaria palmata* protein hydrolysates obtained using food-grade proteolytic

enzymes were found to have FRAP values ranging from 1.06 to 21.59 μmol trolox equivalents/g (Harnedy & FitzGerald, 2013).

In the DPPH inhibition based antioxidant assay, the percentage inhibition by the SC and JD samples analysed ranged from 24 – 94% at a concentration of 1 mg/mL. This level of inhibition is comparable to the DPPH inhibition values of up to 31% observed for egg yolk gel filtration fractions that were obtained after *in vitro* simulated pepsin and pancreatin digestion at a concentration of 20 mg/mL (Yousr & Howell, 2015). In general, various dietary proteins, their hydrolysates and peptides, are known to inhibit the DPPH radical by anywhere between <5 to >90% (Di Bernardini et al., 2012; Esteve et al., 2015; Siow & Gan, 2016).

All of the three SC samples showed MLP inhibition (15-39%), indicating that the peptides generated in the gastric phase of digestion of GEP may be effective in the prevention of lipid peroxidation. One, but not all of the JD samples also showed significant MLP inhibition (JD1=60%). This may be due to heterogeneity arising from the sampling procedure where one digestion sample per animal was taken at one location and point of time. The percentages of LPO inhibition observed in our samples are comparable to those exerted by fractionated egg yolk protein hydrolysates (Yousr & Howell, 2015). In the latter study, LPO was studied using a linoleic acid model system and measurement of TBARS, and it was found that the egg yolk protein hydrolysates inhibited LPO by 39-48%. Similarly, in another study, the peptides from the water soluble extracts of various varieties of ovine cheese samples were found to inhibit LPO by 25-51% (Meira et al., 2012).

It is interesting to note that the digesta samples JD2 and JD3, despite having similar protein extraction yields (≈ 49%), had different FRAP values and DPPH inhibition potential. Similarly, although JD2 and JD3 were found to have antioxidant properties by both FRAP and DPPH inhibition analysis, they showed no activity in the MLP inhibition based antioxidant assay.

Gastrointestinal proteins and peptides derived from gut microbes may also be a source of bioactivity. Porcine GEP is reported to contain up to 45% of nitrogen of bacterial-protein origin (Miner-Williams et al., 2009). The microorganisms themselves contribute to the protein pool in the lumen and may secrete exo-peptidases that may result in hydrolysis of GEP or GEP-derived peptides.

Because the fractionation cut-off point here was 3.5 kDa, it is possible that small bioactive peptides were excluded from our samples. If a lower cut-off membrane was used even higher GEP-derived bioactive peptide bioactivity would be expected to be found. Also, it is possible that large proteins contributed to the effects seen here, but particularly for the JD samples, considerable proteolysis would have occurred. SDS-PAGE showed that a high proportion of the polypeptides had molecular weights less than those of proteins typically found in the GIT. The study clearly shows that peptides must have contributed to the observed, relatively high bioactivity.

Further, the role of (bioactive) peptides in the bioactivities observed here is also demonstrated by the numerous peptides identified in this study. The LC-MS/MS data show a wide range of different and novel potential bioactive peptide sequences from over 45 different GEP from the six animals. This finding is indicative of the

vast range of diverse endogenous proteins secreted into the gut (Miner-Williams et al., 2009). Some of the key parent GEP that released the identified peptide sequences were also found to be potential sources of bioactive peptides in our previous *in vitro* study wherein it was found that the porcine GEP trypsin and mucin and the human GEP lysozyme and serum albumin were a significant source of bioactive peptides in the GIT (Chapter 5, Dave et al., 2016a). Interestingly, in the present study, the pig GEP serum albumin-derived peptide sequences were detected in all of the SC samples, which is in agreement with previous studies that have reported that serum albumin undergoes gastric clearance (Brassinne, 1974; Glass & Ishimori, 1961). The highest number of peptide sequences identified were released from the GEP basic PRP precursor, a protein that forms a major component of the mammalian saliva (Bennick, 1982), the peptide sequences that were released from PRP, as expected, contained a large number of proline residues, and it is known that many of the known bioactive peptides contain proline. Therefore, some of these proline-rich peptides identified here might have contributed to a part of the observed ACE-I inhibition and antioxidant effect (Foltz et al., 2007; Vitali, 2015).

While none of the 181 peptide sequences reported in Table 6.4 have been reported previously as bioactive (BIOPEP, 2015; Minkiewicz et al., 2008b), most of the identified peptides were found to be precursors of previously known bioactive peptide sequences (BIOPEP, 2015). For example, the pig GEP trypsin-2 precursor-derived peptide sequence, TLDNDILLIKLSSPAV, identified in the sample JD1, is a precursor for fifteen 2-3 amino acid residue long bioactive peptides including KL, AV, IKL, AV and TL that have ACE-I- or DPP-IV inhibitory or antioxidant activity (BIOPEP, 2015). Further, the pig GEP serum albumin-derived peptide sequence

FAKTCVADESAENCDKS, identified in the sample SC3, was also found to be present as a subsequence of a larger peptide (LVNEVTEFAKTCVADESAE-NCDKSLHTLF) identified in our previous *in vitro* study on human serum albumin as a source of bioactive peptides (Chapter 5, Dave et al., 2016a). This is likely due to the fact that the porcine and human serum albumins have a significant amino acid sequence homology (Chruszcz et al., 2013). Further, it is noteable that, in both the aforementioned *in vitro* study and the present *in vivo* study, the sequence FAKTCVADESAENCDKS was identified in samples that showed relatively meaningful levels of ACE-I inhibition and antioxidant activity. This suggests that the above sequence might be a potentially bioactive peptide. However, further studies are required to chemically synthesise the peptide sequences identified in this study to confirm their bioactivity and mechanisms of action.

The *in vivo* digestive system is extremely complex with a heterogenous mixture of proteins from many different sources. Systems containing both dietary and endogenous proteins may pose a significant challenge in the identification of bioactive peptides. Unless the dietary proteins are themselves labelled, it is difficult to distinguish between dietary protein-derived or GEP-derived bioactive peptides (Moughan et al., 2014; Nongonierma & FitzGerald, 2015b). During digestion, the amount of GEP secreted into the gut is equivalent to the amount of dietary protein consumed per day (Moughan & Rutherfurd, 2012). In addition, the GEP consist of a wide range of proteins including numerous mucins, digestive enzymes, hormones, and proteins from sloughed-off epithelial cells and bacterial cells. It is therefore not straightforward to distinguish between the smaller bioactive peptides which are between 3-20 amino acids in size, (Shahidi & Zhong, 2008) and to discern which

parent protein they are derived from. This methodological problem is further complicated by the fact that numerous proteins may contain similar and/or the same bioactive peptide motifs or sub-sequences (Minkiewicz et al., 2015).

In summary, this study has identified (*in vivo*) the existence of peptides exhibiting ACE-I inhibition and antioxidant potential in jejunal digesta and gastric chyme samples obtained from pigs given a protein-free diet. Although, similar bioactivities may be observed from the peptide fractions released during gastric and small intestinal phases of digestion, the extent of activity and the underlying mechanisms may be different. Thus, there is a need for an in depth study of these aspects related to both dietary and GEP-derived bioactive peptides.

Thus, the study presented in this chapter is the first to utilise an *in vivo* porcine model to show that like dietary proteins, GEP also release bioactive peptides into the lumen of the digestive tract upon digestion. The GEP-derived bioactive peptides studied here exhibited ACE-I inhibitory and antioxidant potential.

Chapter 7

7. Overall Conclusions and Avenues for Future Work

7.1. Introduction

The primary objective of the work described herein was to investigate the mammalian gastrointestinal endogenous proteins (GEP) in a hitherto unconsidered role by investigating their potential as a source of bioactive peptides. For this investigation, sequential *in silico*, *in vitro* and *in vivo* models were used to identify the bioactive potential of GEP-derived peptides in animals and humans. For the *in vitro* and *in vivo* digestion studies, the potential bioactive peptide sequences were characterised using mass spectrometry. The results of the work which are presented in chapters 3 to 6 of this dissertation are summarised as follows:

7.2. Summary of results

7.2.1. *In silico* approach to identify potential bioactive sequences

The study described in Chapter 3 utilised the major web-based bioinformatics databases and tools as a first step to understanding the potential of a wide range of GEP as a source of bioactive peptides. An *in silico* model was employed to determine whether the GEP contain any previously reported bioactive peptides within their mature protein sequences, and whether these peptide sequences may potentially be released during gastrointestinal digestion. Such *in silico* models have been used extensively to identify and document bioactive peptides from a vast range of dietary proteins, however, the results presented in this dissertation (Chapter 3) are the first to use an *in silico* model to mine a wide range of human GEP as a source of bioactive peptides.

In the model, the protein sequences of twenty six individual GEP were screened using a bioactive peptide database (BIOPEP) that allowed an estimation of the

number of bioactive peptide sequences in each of the proteins. Seven known dietary protein sources of bioactive peptides were also included in the study as comparators for the unexplored GEP. The selected proteins in both groups (i.e. dietary proteins and GEP), had a wide variation in size and their chain lengths ranged from <100 to >5000 amino acid residues. This study is one of the first to compare the bioactive potential of GEP with that of dietary proteins known to generate bioactive peptides upon GIT digestion.

Further, the sequences of GEP were subjected to an *in silico* simulated gastrointestinal digestion using an online tool (PeptideCutter) to understand the potential of GEP to release bioactive peptides during gastrointestinal digestion. The three major known gut proteases (pepsin, trypsin and chymotrypsin) were used to simulate protein digestion *in silico*.

The key finding was that the total numbers of bioactive peptide sequences found in intact dietary proteins and intact GEP were comparable. On average, both the groups of proteins contained a high number of bioactive peptides encrypted in their primary structure, ranging from ≈ 60 to 2500 bioactive peptide sequences per molecule. Moreover, the results indicated that GIT digestion of the GEP would likely release numerous peptides and several peptides with more than one potential bioactivity.

This study also compared the amounts of bioactive peptides that would likely be released at different stages of gastrointestinal digestion. Only a very small fraction (≈1% of both GEP and dietary proteins) of these bioactive peptide sequences was predicted to be released after simulated (*in silico*) gastric digestion. The number of bioactive peptides predicted to be released after the simulated (*in silico*) small

intestinal digestion was higher ((≈3.3%). Further, *in silico* digestion predicted that GEP released in the small intestine would also give rise to bioactive peptides.

7.2.2. Identification of novel bioactive peptides using in silico digestion, de novo chemical synthesis and in vitro bioactivity assays

In silico digestion and *in vitro* bioactivity assays were combined to investigate the potential release of bioactive peptides from GEP in this work (Chapter 4). Eleven representative GEP were first investigated using an *in silico* digestion model as described in Chapter 3. Eight of the eleven studied GEP (serum albumin, mucin-7, pepsin, salivary amylase, trypsin, cholecystokinin, somatostatin, and lysozyme) were selected based on the findings of the first study (Chapter 3). In addition, the GEP ghrelin, an important gut hormone, keratin and CRISPR-associated endoribonuclease Cas2 that represent epithelial and microbial proteins respectively were also included in the study.

All of the selected 11 proteins were subjected to simulated (*in silico*) gastrointestinal digestion (SIGIT), and of all the resultant peptides only those peptides which were predicted to be released after SIGIT, with no previously attributed biological activity, were selected based on previously reported findings of quantitative structure–activity relationship (QSAR) and their PeptideRanker scores (Chapter 4).

Consequently nineteen potential peptides were selected and chemically synthesised using microwave assisted peptide synthesis, and were screened for either their DPP-IV inhibitory and, or their antioxidant potential using *in vitro* bioactivity assays.

Several novel bioactive peptides such as CK, MIM, FPQW, MPSDR, RHPY, RPCF were found to inhibit human DPP-IV *in vitro* with peptides MIM and RPCF being the most potent. Further, the peptides SHF, RHPY, RPCF, MSY, CRPK, CCK, DCR, VAW, and QQCP were found to possess DPPH inhibitory potential with CCK, RPCF and CRPK showing inhibition of >90%. In addition, the peptide CCK was also found to be an effective antioxidant when tested using the ferric reducing antioxidant power (FRAP) bioassay.

7.2.3. GEP-derived bioactive peptides: an in vitro digestion study

The work described in chapter 5 used a simulated *in vitro* gastrointestinal digestion model to corroborate the generation of bioactive peptides (based on the *in silico* observation) from four common GEP namely, trypsin, lysozyme, mucin and serum albumin. Chicken albumin, a known dietary source of bioactive peptides, was included for comparison. The proteins were sequentially digested and the resultant gastric and small intestinal digests were analysed for angiotensin-I converting enzyme (ACE-I-), renin, platelet-activating factor acetylhydrolase (PAF-AH) and dipeptidyl peptidase-IV (DPP-IV) inhibitory activity, and a range of antioxidant bioactivities.

The results from this study, showed that the GEP undergo sequential digestion by the major GIT proteases employed in conventional *in vitro* digestion models. Further the digests for these proteins were shown, for the first time, to possess bioactivities that were comparable to those found for dietary proteins.

The study also monitored the bioactivity levels in the selected proteins at different stages of GIT digestion. The extent of bioactivity depended on the type of protein

and the stage of digestion, indicating an influence of progressive digestion of peptides on their respective bioactivities.

To identify the possible peptides responsible for the observed bioactivities, selected digests were filtered through membranes of <3 kDa and <10 kDa and the resultant filtrates were re-analysed for bioactivities. Among the four GEP studied, the <3 and <10 kDa fractions of lysozyme and serum albumin digests were found to be the most potent antioxidants and inhibitors of renin, PAF-AH and DPP-IV. Therefore, these fractions (that showed maximum bioactivities) were further characterized by mass spectrometry. Several new, potential bioactive peptides (and their sequences) with chain-lengths of 7 to 36 were identified from these fractions.

7.2.4. Identifying potential bioactive peptides from porcine GEP: an in vivo study

The work done in chapter 6 used a pig model to obtain samples of stomach chyme (SC) and jejunal digesta (JD) containing partially digested GEP and GEP-derived peptides. The experimental pigs were fed a protein-free diet and were euthanised for the collection of SC and JD. The samples were purified extensively to obtain the GEP and its digested fractions. The potential of the fractions, for ACE-I inhibitory and antioxidant activity, was then tested using *in vitro* bioassays. The analysis of SC and JD fractions revealed that while the general pattern of GEP digestion across samples of a GIT section (i.e. stomach or small intestinal) appeared to be similar between animals, the samples from each animal, perhaps unsurprisingly given variations in time of sampling, were different.

This study is the first to reveal that protein fragments and peptides isolated from the *in vivo* digestion of GEP indeed possess measurable bioactivities, although the SC

and JD samples showed a wide variation in the type and the extent of the observed bioactivites. In both the SC and JD samples, two out of three samples were found to have significant ACE-I inhibitory activity that was comparable to the positive control captopril. Further, three different bioassays (FRAP, DPPH inhibition and MLP inhibition) were used for determining the antioxidant potential of the peptides. All of the SC and JD samples showed meaningful bioactivities for at least one of these three assays.

This study also identified the several potentially bioactive peptide sequences from the most active SC and JD fractions using mass spectrometry. Several of the peptides identified in this study were found to be released by GEP commonly present in the GIT, including serum albumin, digestive enzymes, mucin and salivary proteins. A particular peptide sequence, FAKTCVADESAENCDKS, from the porcine serum albumin (from SC fraction) was also identified, as a subsequence, of a peptide identified from the <10 kDa fraction of the small intestinal *in vitro* digest of human serum albumin (Chapter 5).

The main objective of this dissertation was to test the hypothesis that GEP contain potentially gut modulatory bioactive peptide sequences that may be released during gastrointestinal digestion of GEP. Based on the advice of statistics experts, a further detailed statistical analysis of the results was not considered due to limiting factors such as the limitations of the selected digestion protocol and low sample recovery from digests, and the limited number of animals used in case of the *in vivo* work. Nevertheless, the study has shown the major qualitative differences in the bioactive potential of different GEP and their potential when compared to selected dietary proteins that are a known source of ExBP.

7.3. General discussion of results

7.3.1. Classification of bioactive peptides

A considerable amount of literature has been published on biologically active peptides of various kinds and from a wide range of sources, including the ones in the body derived from classical post-translational modification of precursor proteins to peptides from cryptomic proteins with their own primary functions. However, from the point of view of the gut, to date, no systematic classification of all of these varied bioactive peptides has been proposed. The present dissertation provides a unique classification of the various bioactive peptides, based on their source and how they are derived.

As discussed in Chapter 2 (review of literature), broadly there can be two different sources of bioactive peptides in the human body, namely, truly endogenous bioactive peptides (TEnBP) and exogenous bioactive peptides (ExBP). While TEnBP are formed biosynthetically, or directly encoded or derived from cryptomic proteins within the body, the ExBP are peptides that are formed outside the body such as during food processing, or via digestion of dietary proteins or GEP in the GIT lumen (Chapter 2, Dave et al., 2015). The study described here, systematically explored the potential of GEP as a source of bioactive peptides using a sequential *in silico*, *in vitro* and *in vivo* strategy.

The work described supports the larger view that every protein, from cellular to organ system level, is a potential cryptomic source of EnBP or ExBP (Pimenta & Lebrun, 2007). The discovery of this vast range of EnBP and ExBP indicates the significance that that body attaches to the recycling and reuse of macromolecules,

especially proteins. Proteolytic activity, a ubiquitous process in the body, is a key step in various physiological processes such as digestion of dietary proteins, proteolytic cleavage of N-terminal signalling sequences, and activation of zymogens. Thus, it follows naturally, that there is scope for the body to utilise proteins beyond their primary functions, as cryptomic sources of bioactive peptides relevant to the dynamic local and systemic environments.

7.3.2. Bioactive potential of GEP is comparable to that of dietary proteins

In chapter 3, the predicted amounts (as percentages) of bioactive peptides released after simulated *in silico* gastric and small intestinal digestion was low, nevertheless when compared to the total number of bioactive peptides found in the two protein groups (GEP and dietary), the numbers are biologically significant as it is known that relatively low amounts of bioactive peptides may exert physiological effects (Boutrou et al., 2013).

It was interesting to see that some of the GEP secreted both in the upper GIT (stomach and above, and the first section of the small intestine) and in the small intestine at different phases of digestion, such as serum albumin and somatostatin, were predicted to have different profiles of bioactive peptides that varied in the number of peptides, bioactive peptide sequences and types of bioactivities.

Although, among all of the studied GEP, larger proteins such as mucins were predicted to contain a large number of bioactive peptide motifs, when normalised against the chain length, among all of the GEP, serum albumin was predicted to be the richest source of bioactive peptides, including ACE-I-, dipeptidyl peptidase IV-, dipeptidyl-aminopeptidase IV, dipeptidyl carboxypeptidase-inhibitor, antioxidant,

and glucose uptake-, -vasoactive substance release stimulating peptides. Serum albumin as a source of bioactive peptides was comparable to the dietary protein *β*-casein, considered to be a particularly potent dietary source of bioactive peptides (Shahidi & Zhong, 2008).

However, the findings of the *in silico* work have to be interpreted with caution as a pure *in silico* approach to simulating protein digestion is limited in several ways. Firstly, only the primary structure of a protein is taken into consideration, and the actions of only some of all of the proteolytic enzymes of the GIT can be applied. Also, *in silico* digestion models assume that the selected enzymes will break every peptide bond for which they have a known cleavage specificity, (i.e. no intermediate peptides would be predicted to be present), but in the *in vivo* situation, this may not necessarily be the case. It is possible, therefore, that not all of the bioactive peptides predicted to be released after simulated *in silico* GIT digestion may be released *in vivo*. It may also be the case that when exposed to the numerous proteases in the GIT, the unfolding of the proteins due to destabilizing conditions in the GIT (e.g. the presence of acid, bile and salts) might generate different profiles of bioactive peptides *in vivo*. Regardless, the use of the *in silico* model can be useful in providing a "first look" at what the bioactive peptide profile might look like after GIT digestion.

Using the *in silico* model in this study, it was predicted that an average diet for a healthy human adult may give around 1842 mg/d of bioactive peptides, and the daily secretion of GEP may give a comparable amount of 2689 mg/d bioactive peptides. The findings of this *in silico* study support the initial hypothesis of this research, particularly since while dietary bioactive proteins remain in the GIT for only limited

periods of time, several of the GEP are continually present in the GIT. In addition, this study also paved the way for further experiments, by helping decide which of the GEP might prove to be the most promising source of bioactive peptides.

7.3.3. Digestion of GEP may generate numerous peptides with multiple bioactivities and, or novel sequences

The work detailed in Chapter 6 is the first to demonstrate that GEP release bioactive peptide fragments after *in vitro* simulated gastrointestinal digestion (SGID), and that these peptides have a wide range of bioactivities relevant to gut physiology. It was observed that SGID of GEP releases peptides with a diverse range of bioactivities ranging from ACE-I-, renin-, PAF-AH-, DPP-IV inhibition and antioxidant activity. The *in vitro* SGID and subsequent bioactivity assays of the digests indicated that for a given protein digest the bioactivity varied with the stage of digestion. It is likely that the peptides released during the early part of digestion retain a part of their secondary structure and the bioactive patches in the peptide sequences might, therefore, remain embedded within this structure. These bioactive patches are then released as digestion progresses.

Indeed, the small intestinal digests tended to show a higher quantum of activity than their respective gastric digests. Characterization studies on the digests indicated that the bioactive peptides were largely <10 kDa while the fraction >10 kDa had comparatively little bioactivity. Further, numerous potentially bioactive peptide sequences were identified from the most active fractions (lysozyme and serum albumin <10 kDa and <3 kDa fractions). The peptide sequences identified were generally higher in chain length (7-36 residues). Similarly, in the subsequent *in vivo*

study (Chapter 6) also, the peptides identified in the SC and JD fractions of pigs, were higher in chain length (10 - >50 amino acid residues).

It is intriguing to enquire, that for a given protein why were these peptides found after *in vitro* and *in vivo* digestion not identified in the *in silico* study. This question can be addressed by considering the evolution of the databases used in the *in silico* study, and the mass spectrometry method used to characterize the peptides here. Most studies and online databases concerning bioactive peptides have reported bioactive peptides with short length (2-8 amino acid) since it is known that peptides of these chain lengths may be transported across the intestinal epithelium into the systemic circulation (Aluko, 2012a; BIOPEP, 2015; Miner-Williams et al., 2014b; Minkiewicz et al., 2008b; Moller et al., 2008). This may have resulted in larger peptides being overlooked in the *in silico* study.

The absence of small molecular weight peptides in the mass spectrometry data of the *in vitro and in vivo* digests could also be explained by their limited ability to be ionized in the mass spectrometer. It is possible that the small molecular weight peptides were indeed present in the digests but they were difficult to identify due to their low probability scores (Dave et al., 2013).

The question that arises then is, would these relatively larger peptides (7-50 residues long) have any systemic physiological effect given that they are bound to undergo further digestion in the GIT, brush border membrane and the plasma? Indeed the larger bioactive peptides may not escape the GIT digestion and enter the systemic circulation (Miner-Williams et al., 2014b) in small quantities, but, there also exists the possibility that they exert their effect within the gut lumen. The gut is known to

contain numerous receptors including those involved in the RAAS (Fandriks, 2011), opioid (Sobczak et al., 2014; Yoshikawa, 2015), neurotensin, chemotaxis, anaphylatoxis, anorexia (Yoshikawa, 2015), taste recognition (Depoortere, 2014), mucosal immune function (Mantis et al., 2011), gut hormones (Moran-Ramos et al., 2012); and other functions that depend on enzymes such as ACE-I and DPP-IV (Chapter 2, Dave et al., 2015; Chapter 5, Dave et al., 2016a). Thus, the larger bioactive peptides may exert physiologically relevant bioactivity before undergoing further digestion. Further, their progressive digestion may result in other peptides which may exhibit the same or different bioactivities as noted in the *in silico, in vitro and in vivo* studies of the current work.

This implies that a given peptide may undergo changes in its bioactivity profile with advancing digestion and in turn regulate gut function accordingly. Nevertheless, there is also some experimental evidence that suggests that some relatively larger peptides ranging between 10 and 51 amino acids in chain length may also be absorbed intact from the intestines to be able to exert an effect elsewhere in the body (Roberts et al., 1999).

Another significant finding from the work summarized in Chapter 4 is that GEP sequences may contain numerous novel bioactive peptide sequences with bioactivities that are particularly relevant to the GIT. This study discovered two previously unreported DPP-IV inhibitory peptides (RPCF and MIM), and five potent antioxidant peptides (CCK, RPCF, CRPK, QQCP and DCR) from the GEP serum albumin, microbial protein GGCAE, mucin 7, and salivary amylase.

The *in silico*, chemical synthesis, *in vitro* digestion and *in vivo* digestion work (Chapters 3-6), showed that serum albumin (SALB) was consistently found to be the most potent GEP for bioactive delivery, and was comparable to the respective dietary proteins used as a control. Likewise, in all of the studies that were carried out, lysozyme (LYS) was also predicted to be a significant source of ExBP in the gut. These findings are significant since both SALB and LYS in their unhydrolyzed form have previously been classed as bioactive proteins or proteins with bioactive peptide components or domains (Goncharov et al., 2015; Lonnerdal, 2013; Roche et al., 2008; Sitar et al., 2013), while SALB is a known antioxidant (Roche et al., 2008) and chicken egg lysozyme has been reported to possess antibacterial properties (Masschalck & Michiels, 2003). Similarly, a 17 amino acid domain of LYS, CNDGRIPGSRNLCNIPC (Li et al., 1995) is known to prevent acute and chronic oxidant injury by suppressing genes involved in response to exogenous oxidant stress (such as that induced by paraquat and H_2O_2) and reduces the generation of reactive oxygen species induced by advanced glycation end products (Liu et al., 2006). In the same way, in the present work, LYS digests and fractions (<10 kDa and <3 KDa) were found to act as antioxidants by being effective quenchers of the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and also by way of possessing ferric reducing ability (FRAP). Also, numerous peptide sequences, named as albutensins, that are derived from the tryptic digests of bovine serum albumin have been reported to possess ileum-contracting or agonist activities in porcine ileum preparations (Takahashi et al., 1998). Further some of the albutensin sequences are also found to correspond to sequences found in porcine and human serum albumins (Takahashi et al., 1998; Yoshikawa, 2015).

7.3.4. Generation of bioactive peptides from GEP in in vivo systems

Whether the GEP indeed release ExBP during GIT digestion could only be confirmed using an *in vivo* model. Therefore, and as described in chapter 6, an attempt was made to corroborate the findings of the work conducted in the *in silico* and *in vitro* studies (Chapters 3, 4 and 5).

In the work described in chapter 6, it was asked whether porcine GEP give rise to bioactive peptides *in vivo,* during actual GIT digestion in growing pigs. The traditional protein-free diet method was used to study the *in vivo* digestion of GEP alone without any interference from dietary protein-derived bioactive peptides (Moughan et al., 1998). The digested GEP fractions (including various peptides) were collected and isolated from both stomach chyme (SC) and jejunal digesta (JD) samples and were tested for different bioactivities after purification. It also calls into question variation among samples due to the sampling and variation in composition of materials over time.

Significant ACE-I inhibition and antioxidant activities were observed in samples from both the SC and JD fractions. This suggests that a significant amount of ACE-I inhibition and antioxidant activity in the gut lumen may be due to the GEP-derived peptides.

From these findings, it can be assumed that during the normal course of GEP digestion in the GIT of mammals, the generation of bioactive peptides is a natural occurrence. These findings raise further questions regarding the nature and physiological concentration of bioactive peptides that may be generated from GEP

on a daily basis, particularly considering that basal levels of GEP are always present in the GIT.

These data must be interpreted with some caution, as the observed bioactivities may in part also be due to other constituents that might have been present in the digestive contents. However, much care was taken to isolate proteins and peptides from the samples prior to the step of bioactivity determination. It is not known as to what proportion of peptides in digesta showing bioactivities came from which specific proteins, and further studies are necessary to ascertain this. However, it is interesting to observe that although the *in vivo* samples showed measurable bioactivities, there was no consistent pattern that emerged with regards to the magnitude of observed bioactivities. This may be explained by the heterogeneity of the peptide composition of extracts resulting from the wide variation in the GIT conditions in each of the animals and by heterogeneity inherent to the sampling procedure. The individual variations include differences in the degree of hydrolysis by gut proteases, differences in secretion of digestive aids (acid, protease composition, salts, bile, etc.), and differences in the contribution of microbial proteases, and the dry matter (DM) intake.

7.3.5. Gut microbiota influences generation of bioactive peptides

The GIT is an ecosystem of a wide variety of bacteria and the balance of these bacteria has been known to play an important role in regulation of important metabolic functions (Oriach et al., 2016; Zhang et al., 2015b). The gut microbiota-origin protein makes up over 45% of total GEP and given their significant numbers in the GIT lumen bacterial protein is likely to be one of the major constituents of

GEP that would undergo digestion. However identification of such proteins and their peptides upon digestion by gut proteases poses a challenge due to the variability in the type of microflora in the gut and the numerous proteins associated with them, and their minute quantities individually.

It is not known whether bioactive peptides identified in the *in vivo* samples contained bacterial protein-derived peptides. Nevertheless, the possibility of bacterial proteins being a contributory source for bioactive peptides in the gut was considered in the *in silico* study of Chapter 4. This study demonstrated that the microbial protein GGCAE was a source of a novel peptide MIM that exhibited significant DPP-IV inhibition *in vitro*.

Another possibility for the generation of bioactive peptides (ExBP) from various dietary and GEP proteins in the gut is through the action of microbial proteases. It is possible that one of the mechanisms by which the microflora in the gut exerts an effect in modulating physiological functions is by secreting proteases which in turn hydrolyse the GEP, resulting in peptides which are bioactive. Other mechanisms of microbiota-mediated physiological effects in the gut could be the physiological modulation resulting from certain ExBP acting as receptors for bacterial attachment (Lu & Walker, 2001). Similarly, certain species of bacteria may secrete gut-modulatory peptides and proteins, a phenomenon observed in certain probiotic strains such as Bifidobacterium, Lactobacillus and Escherichia (Ewaschuk et al., 2008; O'Shea et al., 2012; Sánchez et al., 2008; Sanchez et al., 2010). This underlines the complexity in interpretation of data from *in vivo* studies like this and highlights the challenges in planning such studies.

7.3.6. Epithelial cells as a source of bioactive peptides in the GIT

The mucosal turnover, depending on the health of the individual, may contribute from 5 to 40% of GEP per day (Chapter 2, Dave et al., 2015; Leblond & Walker, 1956; Miner-Williams et al., 2014a). Some of the epithelial-cell derived proteins have already been reported to be bioactive in other systems. By way of example, proteolytically-derived peptides from the protein ubiquitin (76 residues), that is found in all eukaryotic cells including epithelial cells, have been shown to act as enzyme substrates for peptidylglycine α-amidating monooxygenase (Chew et al., 2005). Similarly, the peptides derived from actin protein analogue, that forms an integral part of the intestinal microvilli (McConnell et al., 2011), have been found to be bioactive *in vitro* (Ryan et al., 2011). The present study also found that the epithelial cell protein keratin may release the precursor (with the amino acid sequence GSR) of an ACE-I inhibitory peptide sequence (GS) upon simulated *in silico* gastrointestinal digestion (Chapter 4).

7.3.7. Structural aspects of proteins/peptides governing digestion and subsequent bioactivity

An important aspect that is likely to affect the release and action of ExBP is the structural attributes of the parent proteins and the physicochemical interactions occurring during and after their hydrolysis. The amino acid composition and peptide sequences with reference to the structure-activity relationship of bioactive peptides have been discussed in detail in Chapter 2 (Review of Literature), and in Chapters 4 and 5 with respect to the specific bioactivities studied therein. The (higher order structure including secondary and tertiary-) structure mediated bioactivity may assume greater significance in peptides with relatively larger chain-lengths such as

those from 7 to 36 residues long that were identified in the work described in Chapter 5.

It is interesting that the *in vivo* samples showed the presence of peptides that were rich in proline residues. This is in agreement with previous studies that indicate that the proline residues, especially when flanking the amino- or carboxyl-end of peptides, may have an important role in the bioactivity of the peptides (Manjunatha Kini & Evans, 1995). It is also well known that the presence of proline in a protein sequence opposes formation of ordered structure (Rauscher et al., 2006). These two findings are notable since they suggest that the proline residues may either be involved in the interactions with the receptor sites for bioactive peptides, or that their presence might result in an open structure exposing the sequences responsible for the exhibited bioactivity. Further experiments are necessary to test this hypothesis.

Since the contents of the gut lumen represent extremely heterogeneous mixtures consisting of numerous endogenous proteins at different stages of digestion, it follows that there exists a strong possibility that one or more of the peptides from the same or different proteins may interact with each other. Further the nature of the interactions or their degree of association may vary with changes in the aqueous conditions prevailing in the GIT. The different protein-protein interactions known to mediate bioactivity of peptides include disulphide bonds (Patil et al., 2015a), electrostatic (Wang et al.) and hydrophobic interactions (Mozsolits et al., 1999).

Similarly the proteins and peptides may also undergo modifications such as the Maillard reaction which might alter their ultimate structure and, or bioactive

potential (Roscic & Horvat, 2006), and also potentially making them less accessible to gut proteases such as trypsin (Tuohy et al., 2006) The latter phenomenon might in turn alter the degree of digestion of glycated proteins and peptides which will affect the type of bioactive peptides produced from such precursors. With respect to glycation induced modification of proteins, it is important to note that, while dietary protein-derived ExBP might not undergo glycation due to their short half-lives of <2 h and rapid digestion (Ichikawa et al., 2010; Iwai et al., 2005), certain GEP that are secreted into the gut are susceptible to glycation due to their longer half-lives of 1-2 weeks (Liumbruno et al., 2009). One such example of GEP are plasma proteins including serum albumin that enters the GIT for the purpose of clearance (Wetterfors et al., 1960), and that been shown to be a major source of bioactive peptides in the present work.

Furthermore, it has previously been reported that the activity of peptides might be enhanced in the presence of certain amino acids or act synergistically with the bioactivity of amino acids (Davalos et al., 2004), this is not surprising considering the fact that individually, amino acids are also known to have biological activities such as the antioxidant properties of tyrosine, tryptophan, cysteine and methionine (Del Angel-Meza et al., 2011; Gülçin, 2006; Kedare & Singh, 2011; Levine et al., 1996). Thus, the digestion of the cryptome (dietary/GEP) protein and the bioactivity exhibited by the resultant peptides *in vivo* would be the net effect arising from all of these inter-proteins/inter-peptide/inter-amino acid interactions and polypeptide modifications.

Some of the variabilities observed in the present study may perhaps be due to the abovementioned factors and therefore, the actual effect of these interactions and

modifications needs to be understood in greater detail and represents an interesting area for future studies.

7.3.8. Safety and toxicity of GEP-derived bioactive peptides

Having defined some of the key factors that might have an impact on the ExBP generated in the GIT, it is important to mention that there are a few reports that indicate that some of these bioactive peptides might not necessarily have a beneficial effect on the host or the consumer. Some bovine casein-derived bioactive peptides from the A1 variant of the protein have been implicated in inflammation, diabetes and heart disease (Laugesen & Elliott, 2003). However, only a few studies in animal models have reported these adverse effects, and more studies are required to substantiate these findings in humans.

Further investigations concerning whether any of the GEP derived bioactive peptides exhibit adverse effects would therefore be important, and would pave the way for a greater understanding regarding the safety of GEP-derived bioactive peptides. However, given that GEP are a significant and constant source of protein in the gut, and therefore, peptides released from them form a natural part of the gut contents, it is highly unlikely that under normal conditions, GEP-derived peptides have a negative impact on the host.

7.3.9. Some important considerations in the study of GEP- and dietary protein-derived bioactive peptides

Unlike dietary proteins, the quantity of consumption of which will vary from meal to meal, day to day and from population to population, a certain basal amount of GEP

will always remain present in the GIT. In addition to the major dietary factors that may influence GEP flows that have been highlighted in Chapter 2, a few other factors that might influence GEP secretion are listed here:

i. Age, sex and overall health of the individual including the type of lifestyle led (i.e. sedentary, moderate or heavy work) (Duska et al., 2007; Paddon-Jones et al., 2008; Phillips, 2004; Tessari, 2006). Other conditions that might potentially impact GEP secretion include growth spurts during infancy and adolescence, pregnancy, lactation, and fasting.
ii. Dietary intake and composition; and type of processing applied to the food (Deglaire et al., 2016; Moughan et al., 2005; Moughan et al., 2007; Rutherfurd et al., 2015a), and in particular the protein intake (both quantitative and qualitative) of the individual, since it is know that malnutrition can effect protein synthesis (Costarelli & Emery, 2009; Millward et al., 2008; Tomé & Bos, 2000)
iii. Medication (Matsui et al., 2011; Roberton et al., 1996; Sostres & Lanas, 2011)
iv. Stress levels of the individual (Stress is known to impact general health, digestion, gut motility and the gut microbiota) (Bailey et al., 2011; Bercik et al., 2011; Konturek et al., 2011).
v. Natural, inter-individual variations in gut microflora (Conlon & Bird, 2015; Human Microbiome Project Consortium, 2012), and dysbiosis (Oriach et al., 2016).

Variations arising from all of the above factors may be responsible for differences in GEP-derived ExBP in the gut of different individuals, and may perhaps to some extent make GEP-derived peptides host-specific.

7.4. Conclusions

Taken together, the findings of this work demonstrate for the first time that GEP are an important cryptomic source of exogenously-derived bioactive peptides in the gut lumen, augmenting the dietary supply of bioactive peptides. Further, given that basal amounts of GEP are always present in the GIT and that the GIT is a large extracorporeal proteolytic system, GEP-derived peptides will be a much more consistent supply of ExBP to the gut than their dietary counterparts. These peptides have a wide range of biological activities that are highly relevant to the GIT. This study is also the first to discuss the aspect that both of these exogenous sources of bioactive peptides in the GIT (dietary and GEP) might constitute a host-specific peptidergic system of regulation of the gut. The two most important implications of this work are that, firstly, when studying the role of dietary bioactive peptides, the inherent potential of GEP to generate similar peptide sequences needs to be taken into consideration, as also acknowledged in a recent review (Nongonierma & FitzGerald, 2015b). Secondly, the diet of an individual may modulate the flows of GEP, to obtain the desirable GEP-derived bioactive peptides, in specific health conditions providing an exquisitely regulated system of homeostatic control. In the light of this knowledge, much more research is required to investigate in depth the web of physiological effects that may be mediated through GEP-derived bioactive peptides individually or in conjunction with their dietary counterparts.

7.5. Avenues for further work

In the light of the observations made in the present study, the following areas of investigation are recommended:

- A fuller characterisation of the GEP-derived bioactive peptides, and their biological functions and role in regulation.
- Development of methods for distinguishing between GEP and dietary protein derived bioactive peptides (the two natural types of ExBP) in the gut lumen.
- Studying the impact of the major dietary components (including proteins, peptides, polysaccharides, and non-starch polysaccharides (fibre)) on GEP flows (Butts et al., 1993b; Claustre et al., 2002; Cowieson et al., 2004; Deglaire, 2008; Deglaire et al., 2006; Han et al., 2008; Miner-Williams et al., 2014a; Moughan et al., 2005; Moughan et al., 2007; Moughan & Rutherfurd, 1990; Ouellet et al., 2002; Rutherfurd et al., 2015a) and therefore, the subsequent impact on the generation of bioactive peptides, and profiling of the GEP-derived bioactive peptides of healthy populations.
- Studying the differences in the ExBP types and profiles in healthy and diseased states. This is particularly important since it is known that in certain diseases, the gut protease activity is significantly altered, which suggests that the type of bioactive peptides generated may also vary.
- Studying the type of bioactive peptides generated due to the action of microbial proteases of the microbiota inhabiting the upper GIT and investigation of the differences in bioactive peptide pools in the gut of healthy subjects versus subjects with dysbiosis.

- Investigating the interactive effects of simultaneously generated amino acids on the activity of bioactive peptides released during GIT digestion.
- Development of an advanced model for the detailed study of the *in vitro* digestion of dietary proteins and, or GEP. Building on the current INFOGEST *in vitro* digestion model, the new model could include basic bacterial proteases, post small intestinal digestion simulation by including brush border digestion, followed by the study of peptide biotransformation under the effect of plasma proteases.
- Some bioactive peptides are known to possess multiple bioactivities, it would be interesting to study how this phenomenon manifests itself *in vivo* and if such peptides demonstrate any "preferential" biological action depending on the physiological state of the host.

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9. Appendices

9.1. Statements of contribution to doctoral thesis containing publications

9.2. Copyright permissions for adaptations of publications into thesis chapters

9.3. Copies of publications