Devenir des protéines dans un système modèle de digestion simulée assisté par spectrométrie de masse

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Introduction – Background

- Gastrointestinal digestion

“Shall I refuse my dinner because I do not fully understand the process of digestion?”

Oliver Heaviside (1850-1925)

- Partial hydrolysis by pepsin (*Stomach*)
- Proteases (trypsin, chymotrypsin, carboxypeptidases) and microorganisms peptidases (*small intestine lumen*)
- Brush border membrane peptidase (*microvilli of epithelial cells*)

**Free amino acids**

**Various molecular weight peptides with potential bioactivities**

**Peptidome**
Sources and roles of bioactive peptides

Sources of bioactive peptides

Animals
All proteins
Plants

→ Cow-milk proteins

benefit / harmful
Hemoglobin (Hb) as a potential source of bioactive peptides?

Hb (α2/β2): a model protein

Meat production food chain

Farm → Transport → Lairage → Slaughter house

Slaughter / bleeding → Removal of hooves → ...

Slaughterhouse blood

30% sold

70% discarded

20% of proteins discarded

Source: NCBI

Objectives

1. Development of an *in vitro* human digestion model to study protein digestion
2. Characterization of peptidomes (GI digestion-derived peptides)

1. Protein digestion and energy homeostasis: impact of generated peptides on intestinal hormones

Bovine hemoglobin
Source: NCBI
**In vitro GI digestion**

- **No heating over 37°C**

- **Mouth**
  - $t = 5 \text{ min}$
  - pH $= 6.5$

- **Stomach**
  - $t = 30 \text{ min}$
  - pH $= 1 - 2$

- **Small intestine**
  - $t = 15 - 60 \text{ min}$
  - pH $= 6.5 - 6.8$

- **Stop digestion (100°C, 15 min)**

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**Analytical strategy**

**Separation method**

- **C18 RP-HPLC**
  - C18 LiChroCART 250-4, LiChrospher 100 column (Merck KGaA, Darmstadt, Germany)

- **C18 NanoLC**
  - Acclaim Pepmap RSLC, 75 μm ID × 50 cm, Thermo Scientific

**Mass spectrometry/Bioinformatics**

- **MALDI-MS/MS**
  - AutoFlex speed, Bruker

- **ESI-HR-MS/MS**
  - Thermo Scientific Orbitrap Elite mass spectrometer

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**Additional Notes**

- **X 3**

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Hemoglobin (Hb): a highly digestible protein

- **Results**

- Hemoglobin (Hb) is highly digestible.

- SDS-PAGE: 16.5% SDS-PAGE indicates the presence of hemoglobin at 14.6 kDa, 6.5 kDa, and 3 kDa.

- SEC-chromatography shows two peaks after 2 hours of digestion:
  - 2 hour gastric digestion
  - 2 hour intestinal digestion

- Normal bore C18-HPLC profiles:
  - Undigested Hb
  - Gastric digestion
  - Intestinal digestion
- Normal bore C18 HPLC-MALDI-MS (off-line)

Digestome samples X3

C18-HPLC separation X3

Manual collect (26 fractions) X3

Automatic MALDI-MS and MS/MS X3

Gathering of all XML files

Combined XML file + renumbering of detected peaks

Enzyme: none
Missed cleavage: 36

Hb-A and Hb-B sequences

Peptides Matching
Peptide heterogeneity (HPLC-MALDI-MS)

HB alpha chain (Hb_A)
- 317 matching peptides (based on MS-data)
  → sequence coverage (MS) = 100 %

HB beta chain (Hb_B)
- 339 matching peptides (based on MS-data)
  → sequence coverage (MS) = 100 %

- 26 peptides unambiguously identified by MS/MS
  → sequence coverage (MS/MS) = 66 %

Digestion resistant sequences?

Ion parent error tolerance 30 ppm and fragment mass error tolerance 0.5 Da.
Results - High resolution approach

- **nanoLC-ESI-HR-MS/MS**

  **Digestome samples** → **zip-tip** → **Acclaim Pepmap RSLC, 75 μm ID × 50 cm on Proxeon Easy-nLC system**

  Orbitrap Elite mass spectrometer

  **MS param.**
  - data-dependent mode
  - RP = 70,000 (FWHM)
  - 300–1600 m/z → 3E6 ions

  **MS/MS param.**
  - 500 counts
  - isolation window of 4 amu
  - normalized collision energy of 35%.
  - 5000 ions and max. inj. time = 60 ms
  - 300–2000 m/z.

**Database:**
- bovine proteins

Peptide identifications

- **Hb_A**
  - 306 peptides unambiguously identified by MS/MS
  - sequence coverage (MS/MS) = 100%
  - False discovery rate (FDR) = 0%

- **Hb_B**
  - 420 peptides unambiguously identified by MS/MS
  - sequence coverage (MS/MS) = 100%

**Total:**
- 726 peptides

- e.g. : intestinal fraction
- Peptide heterogeneity (nanoLC-ESI-HR-MS/MS)

- Ion parent error tolerance 10 ppm and fragment mass error tolerance 0.2 Da.

Results - High resolution approach

GI digestion-resistant sequences

e.g. : intestinal fraction
**Results - The GI digestion-resistant sequences**

**Hb_A – intestinal maps**

**Hb_B – intestinal maps**

**HB-A: heat map of AA occurrence frequency**

**HB-B: heat map of AA occurrence frequency**

K/Preferential cleavage site for trypsin
Objectives

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2. Protein digestion and energy homeostasis: impact of generated peptides on intestinal hormones

Bovine hemoglobin

*Source: NCBI*
● Energy Homeostasis:

Energy expenditure vs caloric intake: need to ensure a balance

● Regulation mechanisms

Long term - adiposity signal: To maintain body weight « adiposity negative feedback » (Leptin)

Short term - satiation signal: (gut hormones, gastric distension)
Background - Intestinal hormones

- **Cholecystokinin (CCK)**

  Produced by **I cells** (duodenum) in response to lipids and **proteins**.
  Promotes **satiation**: increase gastric secretion, decrease gastric emptying, induces satiety feeling by vagal afferents.

- **Glucagon-like Peptide 1 (GLP-1)**

  Produced by **L cells** (ileum and colon)
  One of the proglucagon products
  **Promotes satiation** by various pathways
  **Incretin**: stimulates glucose-dependant insulin secretion

  **GLP-1 inactivation** by dipeptidyl peptidase IV (DPP-IV). Only 10-20% plasmatic GLP-1 remains
DPP-IV – GLP-1:

Inhibiting DPP-IV extends GLP-1 incretin activity

New target for type-2 diabetes therapy

Ex: Gliptins (e.g. Vildagliptin and saxagliptin)

- Dipeptidyl peptidase 4 (DPP-IV)

DPP-IV rapidly degrades GLP-1 → decrease in plasma

DPP-IV inhibition → indirect increase of GLP-1 activity
→ indirect impact on food intake

Dietary proteins: promising sources as “natural”

DPP-IV inhibitors
In vitro digestion

- Mouth
  - $t = 5\ \text{min}$
  - $pH = 6.5$
- Stomach
  - $t = 30\ \text{min}$
  - $p = 2 - 3$
- Small intestine
  - $t = 15 - 60\ \text{min}$
  - $pH = 6.5 - 6.8$

Bioactivity assays
- Hormone secretion & gene expression
- DPPIV activity

Bioactive sequence identification

Experimental design
Results - Intestinal hormone regulation

Hormones secretion

STC-1
Enteroendocrine cells

CCK &
GLP-1
secretion
RIA detection

Significant increase of both CCK and GLP-1 secretion in presence of intestinal samples

→ Beneficial effect of intestinal enzymes on peptide potential bioactivity
Regulation of hormone gene expression

Intestinal hydrolysate (I4) significantly induces both CCK and proglucagon gene expression
Results - DPP-IV activity inhibition

- DPP-IV activity assay

Intestinal DPP-IV inhibition activity is enhanced during GI digestion

Final intestinal hydrolysate: best bioactivity like for intestinal hormone

Physiological relevance, same peptides involved?

Need to identify resistant active sequences
**Results – peptide purification**

- **Size exclusion chromatography**

- **CCK and GLP-1 different peptide groups involved**

  ![SEC profile](image)

  - 4h digestion
  - intestinal digest

- **DPP-IV and GLP-1**
  - Same peptides involved?
  - MW range 500 – 1500 Da

- **MW < 1000 Da**
  - MW > 500 Da

- **GLP-1 secretion**
  - MW > 500 Da

- **DPP-IV inhibition**
  - MW range 500 – 1500 Da

- **CCK secretion**
  - MW < 1000 Da

<table>
<thead>
<tr>
<th>Control</th>
<th>1A 1%</th>
<th>1A 0.5%</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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<tbody>
<tr>
<td>CCK (pM)</td>
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<td>Absorbance at 214 nm (mAU)</td>
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</table>

  *bioactivities*
C18 RP-HPLC

Results – peptide purification

Peptides contained in F4 both stimulate GLP-1 secretion and inhibit DPP-IV activity
## Results – peptide purification

- **Peptide identification and passage across intestinal wall**

![Diagram showing peptide purification process]

### LC-MSMS

- **60 peptides**
- **LC-MSMS**
- **F4 intestinal sub-fraction**
- **Caco-2 cell monolayer**
- **Apical**
- **Basolateral**
- **18 peptides**

### Table: Protein Sequence Mass (Da)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
<th>Mass (Da)</th>
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</tbody>
</table>
Conclusion and Perspectives

- **GLP-1 / DPP4**

  - Significant increase of both GLP-1 secretion and proglucagon gene expression
  - Inhibition of DPP-IV activity
  - Extending GLP-1 actions (food intake regulation and incretin effect)
Hemoglobin peptidomes

- More than **700 sequences unambiguously identified** in gastric and intestinal peptidomes
- **Specific cleavage sites** identified
- Resistant sequences identified – **recurrent patterns**

- These recurring patterns were made of amino acids that **could be potential preferential cleavage sites** with regard to enzyme specificity.
- **No particular link** between enzyme resistivity and isoelectric point or hydrophobicity index has been found out so far.
- **Peptide conformations** could prevent or slower enzyme activity. **Secondary structure** implicated.

- New tool for screening dietary protein bioactivities

**Proteases and peptidases (GI environnement)**

**Emergence of resistant bioactive sequences**

**Peptidofoodomics**

**New tool for the study of this complexe phenomenon**

**New tool for screening dietary protein bioactivities**
Thank you for your attention!