From (waste) protein to bioactive functional food ingredient

Bioactive Food & Feed Ingredients (BFFI)

Heleen van den Bosch

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Functional foods, nothing new!!!!
Functional foods, nothing new!!!

- Hippocrates
  - ±460 to ±370 BC
  - the father of modern medicine

"Let your food be your medicine and let your medicine be your food"

Wageningen University & Research

- Combination of university departments and application-directed research institutes
- Five Sciences Groups (several departments and one institute):
  - Animal
  - Agrotechnology & Food
  - Plant
  - Environment
  - Social
Wageningen Food & Biobased Research

- Not-for-profit contract research organisation (CRO) of the Agrotechnology and Food Sciences Group
- Activities/Fields:
  - Sustainable innovation in the areas of healthy food, fresh produce chains and biobased materials, chemicals and energy
- > 70% of overall budget is result of acquisition of projects
- 'Customers': SMEs, multinationals, governments, EU, ...
- Business units: Fresh, Food & Chains Biobased Products

Bioactive Food & Feed Ingredients

- Activities:
  - Development of bioactive peptides / hydrolysates for the prevention or reduction of cardiovascular and diabetes complications
  - Antimicrobial compounds (peptides and other ingredients)
  - Antioxidant compounds (low-grade inflammation)
  - Identification and purification of added value compounds from waste organs / materials
Bioactive Food & Feed Ingredients

- **Expertise:**
  - *In vitro* hydrolysis of high-potential proteins
    - Over 20 different proteases and protease mixtures have been used in optimizing the bioactivity of proteins.
  - Development/Application of miniaturized *in vitro* bioassays for selected enzymes (drug targets):
  - Antioxidant assays such as the DPPH and ORAC tests; prevention of 'low grade inflammation'.
  - *In vitro* cell assays to test uptake, bioavailability and/or biofunctionality

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**functional foods**
Market of functional foods / nutraceuticals

- Trends in pharma and food industries

- **Pharmaceutical** example:
  - captopril; ACE-inhibiting drug; blood pressure reduction

- **Nutraceutical** example (application in a clinical setting):
  - IgA in whey powder; neutralising *Clostridium difficile*

- **Functional food** example:
  - drink with antihypertensive peptides, protein-derived

Market of functional foods / nutraceuticals

- Average age is increasing
- Increased risk of diseases such as CVD, CHD, obesity (MBS), cancer, .......
- Some foods play a role in the onset of diseases
- However, functional foods can be applied in the prevention of diseases
  - The global functional foods market is growing at a compound annual growth rate (CAGR) of roughly 6% (Ref: MarketsandMarket)
- Challenge: Development of functional foods that contribute to a better and healthier life
Disease (prevention) targets

- (Food) protein hydrolysates (processed or via \textit{in vivo} digestion) contain bioactive peptides

- High potential application areas:
  - Antihypertensive activity (drop of blood pressure)
  - Cholesterol / lipid reduction
  - Improving insulin resistance (diabetes)
  - Reduction of inflammation markers
  - Circumference of waist (obesity)
  - Anti-oxidant activity
  - Cognitive function and stress

Functional foods - Considerations

- Bioactivity foreseen in GI-tract or in systemic circulation?
  - Length of bioactive peptides relevant criterion
- Mild activity; pharmaceutical application not intended
- Availability of good biomarker(s)
  - Relevant to the \textit{in vivo} situation
- Expected toxicological implications
  - Which parameters should be studied
Functional foods - Considerations

- For systemic application:
  - 'The smaller, the better'
  - Bioactivity in GIT allows larger peptides to be applicable

- Smaller: higher chance of presence in source protein
  - Chance decreases with \( n^{20} \)

- Smaller: better oral availability
  - >98% arrives in blood as mono-/di-/tri-peptides

- Why peptides?
  - Many endogenous bioactives are peptides: neurotransmitters, hormones, enzyme inhibitors
  - Broad range of activities
  - No (?) accumulation-related toxicity

- Which peptides are relevant?
  - Active in gastro-intestinal tract (GIT)
  - Or: small enough to be taken up actively in systemic circulation
  - Or: sufficient passive uptake

- Systemic activity: The smaller, the better!
Functional foods - Considerations

- Many bioactive peptides in proteins are there by coincidence (ACE inhibitors, bile acids/cholesterol binders)
- However: Concentration sufficient for *in vivo* activity?
  - Occurrence increases exponentially with decreasing peptide length
  - Chance to exactly match a bioactive sequence is much higher for smaller peptides
  - Release of bioactive peptides by normal, endogenous digestion (pepsin, (chymo)trypsin, etc.) is far from optimal
  - Normal digestion should deliver free amino acids; building blocks for the body
- Therefore, *in vivo* activity is often not to be expected

Strategy
Strategy

- **Strategy:**
  - Search for high-potential protein sources by an *in silico* approach
  - *In vitro* hydrolysis of high-potential proteins
  - Development/Application of miniaturized *in vitro* bioassays for selected enzymes/proteins
  - Testing/Optimizing hydrolysates for enzyme-inhibiting activity
  - If necessary: partial purification of active peptides

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Strategy continued:

- *In vivo* experiments with hydrolysates that show significant inhibiting activity; animal and, if successful, human studies (in collaboration)
- Design and formulation of functional food products
  - Peptides have a bitter taste, addition of aroma and or masking compounds is needed
Strateg

- NB: targets of drugs; chosen by the pharmaceutical industry
- Examples:
  - Angiotensin-Converting-Enzyme (ACE) inhibitors such as Captopril
  - Dipeptidyl Peptidase-4 (DPP4) inhibitors such as Vildagliptin
  - α-glucosidase inhibitors such as Voglibose
From protein to functional food ingredient

Potential protein sources

- Sources (bulk, economically feasible):
  - **Animal**: structure and mobility proteins (milk, egg, collagen, keratin, muscle, ...)
  - **Plant**: storage and structure proteins (soy, wheat, pea, maize, oat, rice, ....)
  - **Waste streams** (animal, plant, ....)
Amino acids: Building blocks of proteins

- Amino acid characteristics:
  - 20 naturally occurring
  - Some derivatives occurring
  - Identical basic structure
  - Different R-groups (neutral, hydrophobic, positive, negative)

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Hydrolysis of proteins with proteases

Degree of Hydrolysis (DH)
Level of cutting
$\text{DH} = 1$: intact chain
$\text{DH} = 25$: average peptide length is 4

ACE – inhibiting peptide
Peptide preparation routes

- Isolation from natural resources
  - ‘Anabolic’ route -> Antimicrobials
    - Biotechnological production
    - Chemical synthesis
  - ‘Catabolic’ route -> Functional ingredients / Nutraceuticals
    - Proteolysis of bulk proteins accepted way of processing in food industry
      - At least nutritional value
      - Added value: based on particular bioactivity

Suitable proteins: rich in bioactive peptides

- Classical method: empirical
  - Labour-intensive, ‘trial and error’

- Rational method: application of bio-informatics
  - Rapid *in silico* screening
  - Requires sequences & software
WFBR bioinformatics screening software

- Procedure to screen protein (and DNA) databases against bioactive peptides (1995-2005):
  - Possible searches:

  1. Hydrolysis of proteins with proteases
     - Example in silico screening: β-Lactoglobulin
       - Part of amino acid chain (total 178 residues)
       - In vivo released peptides may result in blood pressure decrease
BIOPEP database

- Biopep database

→ Contains many bioactivity properties of peptides

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Miniaturized microtiter plate assays

- Assays available for the human enzymes:
  - ACE; cardiovascular system; blood pressure
  - DPP4; glucose metabolism; insulin action
  - α-Glucosidase; glucose absorption in intestine
  - CETP
  - Lp-PLA2
  - COX

- Readily introduced:
  - eNOS
  - NADH Oxidase
  - sPLA2

Robotic liquid handling system for automated enzyme / immunochemical assays

Microarray for printing proteins on substrates; volumes down to 100 pl
Miniaturized microtiter plate assays

- Antimicrobial compounds (peptides); various projects executed such as an EU project on alternative AMPs in food products
  - Alternatives to chemical antibiotics and inspired by increasing antibiotic resistance
  - Miniaturised microbial assays (bacteria, fungi, yeasts) and toxicity assays with eukaryotic cells (e.g. erythrocytes)

- Other antimicrobial compounds (such as glycosinolates and alkaloids) can be tested as well
  - AIR2-CT94-1356 (1994-1998) Agro-Industrial applications of antifungal proteins from plant seeds
  - EU FAIR CT97-3135 (1996-2000) Antimicrobial peptides - Studies Aimed at Application in Food and Food Products

- Other antimicrobial compounds (such as glycosinolates and alkaloids) can be tested as well

Miniaturized microtiter plate assays

- Antioxidant compounds in relation to 'low-grade inflammation'; various diseases linked to this status
  - Antioxidant in vitro assays available
ACE mechanism

- Angiotensin Converting Enzyme (ACE) converts angiotensin I to angiotensin II => blood pressure ↑

\[
\text{DRVYIHPFHL} \rightarrow \text{DRVYIHPF + HL}
\]

angiotensin I  \hspace{1cm} \text{ACE} \hspace{1cm} \text{angiotensin II (hypertensive)}

Inhibition of ACE: antihypertensive effect = blood pressure ↓

\[
\text{DRVYIHPFHL} \rightarrow \text{angiotensin I}
\]

DPP4 mechanism

- GLP-1 is inactivated by Dipeptidyl Peptidase-4 (DPP4)
- ↑GLP-1 → ↑insulin → faster uptake of glucose from the blood
Metabolic syndrome

- The Metabolic Syndrome constitutes significant risk for artherosclerosis
  
  - Atherosclerosis major contributor to cardiovascular disease (CVD)
  - CVD major cause of death (33%) in The Netherlands
Metabolic syndrome

- Also a risk factor for Type 2 Diabetes mellitus (T2DM)
  - Diabetes is a chronic metabolic disorder that affects about 8.5% of the population in Europe (in 2010 source: www.idf.org/diabetesatlas) and accounts for over $100 billion in medical costs
  - Type 2 diabetes, a metabolic disorder (± 90%; generally after the age of 40) involves progressive development of insulin resistance leading to hyperglycemia

Metabolic syndrome

- Type 2 Diabetes mellitus
  - Regulation of blood glucose levels
  - Major organ systems involved
### Obesity in Europe

- **Obese: BMI > 30**

  **BMI = Body Mass Index**

### Metabolic Syndrome

- **How to combat risk factors?**
  - Drugs
  - Physical activity
  - Reduced caloric intake
  - Consumption of mildly bioactive (preventive) functional foods/food ingredients

- **However, complex relationships exist between metabolic pathways involved in the regulation of the various risk factors of the MBS: Which enzymes/proteins to target?**
Combat the Metabolic Syndrome

- ACE is a regulator in blood pressure homeostasis
- ACE-inhibiting drugs also show beneficial effects on:
  - angiotensin-II ↓
  - bradykinin ↑
  - hypertension ↓
  - endothelial function ↑
  - insulin-resistance ↓
  - inflammatory responses ↓
  - individual health profile ↑

Example

Development of NWT-03 from lysozyme
Development of NWT-03

- Several sequential projects (started in 2003, still ongoing) in collaboration with:
  - Newtricious BV
  - Clinical Pharmacology (University of Groningen)
  - Maastricht University
  - Aroma Uden BV
  - Bouwhuis Enthoven BV

- Projects:
  - EU EggPressure
  - FND metabolic syndrome
  - FND NWT03
  - Characterization

Example: antihypertensive egg hydrolysate

- \textit{In silico} score with priority on shortest peptides
  - The lower the value, the better the theoretical activity

- Of all egg proteins lysozyme had the best score
Development of NWT-03

- Hydrolysis of lysozyme with approximately 20 different food grade proteases and protease mixtures
  - Percentage and hydrolysis time optimized
- Sources of enzymes: bacterial, fungal, plant, mammalian
- Characterization / Purification of hydrolysates by:
  - C18 reverse phase HPLC (analytical and preparative)
  - Size-Exclusion Chromatography (specific for peptide separation)
  - Cation-/Anion exchange chromatography
  - Membrane ultra- and nano-filtration
  - LC-Mass-Spectrometry analyses to identify peptide amino acid sequences

NWT-03: ACE inhibition assay

- IC50 ACE inhibition = 0.085 mg/mL

ACE inhibition assay

- [c] (mg/mL in sample)
- % ACE inhibition
Example: antihypertensive egg hydrolysate

- Acute effect of Lysozyme hydrolysate (NWT-03) on systolic blood pressure in anaesthetized Spontaneously Hypertensive Rats (SHR)

Part of this study has been executed in the framework of EU-CRAFT EGGPRESSURE, QLKT1-CT-2002-71943

Example: antihypertensive egg hydrolysate

- Acute effect of Lysozyme hydrolysate (NWT-03) on plasma ACE activity in anaesthetized Spontaneously Hypertensive Rats (SHR)

Part of this study has been executed in the framework of EU-CRAFT EGGPRESSURE, QLKT1-CT-2002-71943
Example: antihypertensive egg hydrolysate

- Long-term (2 months) effect of Lysozyme hydrolysate (A; NWT-03) on systolic blood pressure in Spontaneously Hypertensive Rats (SHR)

Part of this study has been executed in the framework of EU-CRAFT EGGPRESSURE, QLKT1-CT-2002-71943

Example: antihypertensive egg hydrolysate

Patent application:
- Antihypertensive functional food products
- Amerongen, A. van; Beelen, M.J.C.; Bent, A. van der; Buikema, J.H.; Gilst, W.H. van; Loonen, M.H.J.; Merck, K.B.; Nelissen, J.; Thielen, W.J.G.; Togtema, K.A.
- Patent nr. WO 2006/009448

Hen egg lysozyme
NWT-03 also inhibitor of DDP4

- Hydrolysate has inhibitory effect on both ACE and DDP4 activity

![Graph showing DDP4 inhibition](image)

Purification & characterization

- Further purification of active peptides is only possible if a cost-efficient process can be developed
  - Often not cost-efficient
  - Full hydrolysate used as functional food ingredient

- Purification and characterization of the hydrolysate can be used for:
  - IP purposes
  - Quality Assurance of the product
Example: lysozyme hydrolysate

- Purification of enzyme inhibiting peptides
  - Column fractions tested in miniaturized inhibition assays for Angiotensin Converting Enzyme (ACE) and DiPeptidylPeptidase-4 (DPP4)
Effect of egg protein hydrolysate (NWT-03) on renal function

Effect of NWT-03 on renal function

- Effect NWT-03 and drug vildagliptin on renal function in Zucker Diabetic Fatty (ZDF) rats

Effect of NWT-03 on renal function

Focal glomerulosclerosis (FGS)

- Beneficial effect of NWT-03 on focal glomerulosclerosis (FGS) as compared to untreated ZDF rats
Effect of NWT-03 on renal function

- Beneficial effect of NWT-03 on aorta endothelium-dependent relaxation (EDR) as compared to untreated and vildagliptin-treated ZDF rats

Egg lysozyme hydrolysate (NWT-03)

- Next human trial ongoing (Newtricious)

- Market introduction is expected by the end of 2017
NWT-05: Ovomucin mouth spray

- Project on NWT-05 (ovomucin): Mouth spray for dry-mouth-syndrome
- Sjögren syndrome (auto immune disease)
- Symptoms:
  - Hyposialy (less secretion of saliva)
  - Xerostomy (dry mouth feeling)
  - Dry eyes
- Prevalence: 1-1.5%
- Other important causes of less secretion of saliva and dry mouth symptoms:
  - Radiotherapy head-neck area
  - Side effects of (multiple) medication

Acknowledgements

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- Dr. Luc Sterkman,
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- Dr. Sanne van der Made
- Resy Smeets

Bouwhuis Enthoven BV
- Jan Zijderveld
- Tjesca Eilert

Aroma Uden BV
- Esther Keepe
Questions?

Thank you for your attention!