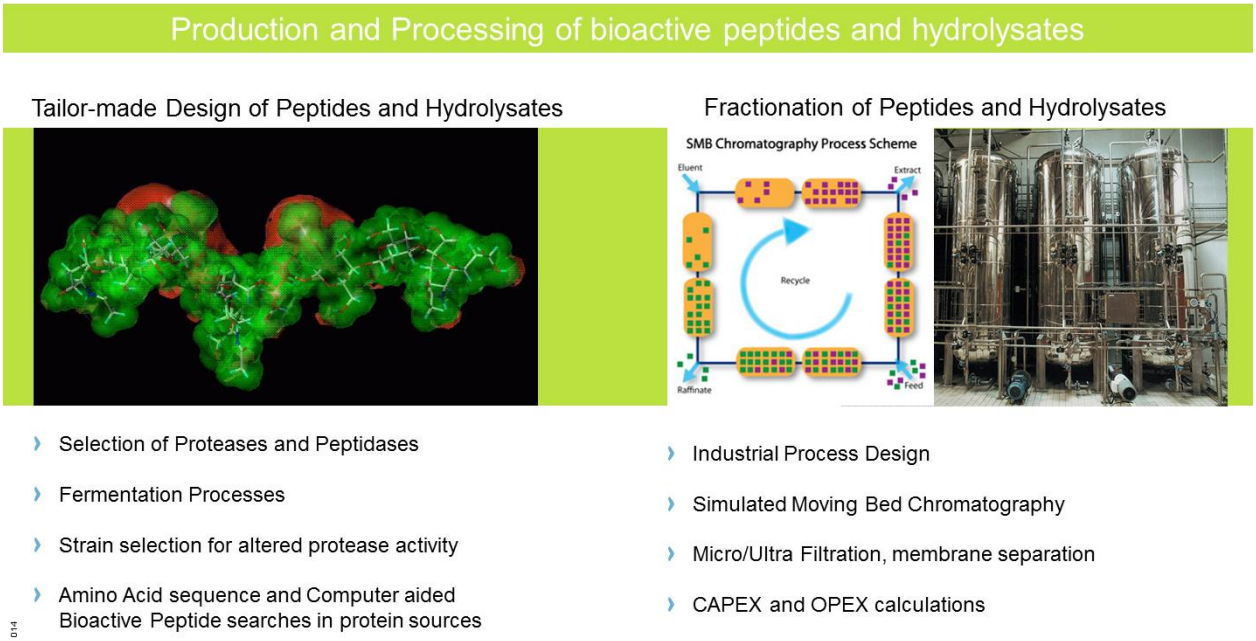
Bioactive Peptide Discovery Platform

Food-protein derived bioactive peptides are short amino acid chains that are inactive within the sequence of the parent protein, but can be released through hydrolysis by proteolytic enzymes. Several physiological effects have been attributed to these peptides, such as blood pressure reduction, scavenging of oxidative compounds, induction of satiety and reduction of cholesterol. Due to their ability to provide a positive impact on body functions or conditions, bioactive peptides and their application are of relevance for the food industry. TNO Triskelion and TNO jointly offer a technology platform that comprises advanced technologies and broad expertise to evaluate and optimize the bioactive potential of food proteins. An overview of this platform is presented in the figures below.



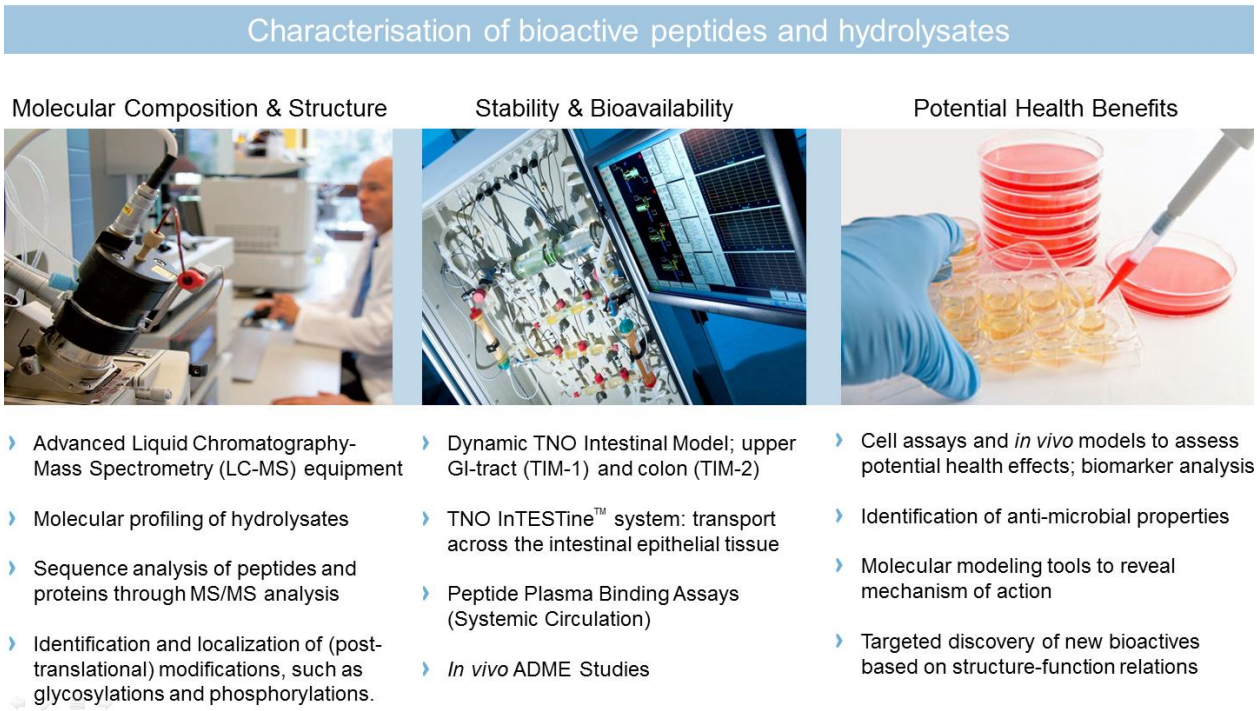
Peptide processing & fractionation

a. <u>Tailor-made design of peptides and hydrolysates</u>

The choice of proteolytic enzymes or micro-organisms as well as processing conditions used in industrial production of hydrolysates has a profound impact on the composition of the peptides released. Production of tailor-made protein hydrolysates with a fixed composition and/or the release of specific peptide sequences therefore requires a combination of robust analytic tools and hands-on experiences utilizing industrial processes. The *suicide substrate biotech* platform, a proprietary TNO technology, allows the direct selection of micro-organisms with modified or novel protease activity. This results in reduced or increased degradation due to the released proteases during the late “auto-lytic” stages of fermentation and DSP. These opportunities allow the development of new, tailor-made hydrolysates of food proteins.

b. <u>Fractionation of peptides and hydrolysates</u>

Industrial fractionation of hydrolysates is TNO core technology. Examples of process designs for peptide separations include units operations such as ultrafiltration for pre-separation and ion-exclusion *Simulated Moving Bed* chromatography using pH gradients for selective fractionation. Based on the latter technology, processes with low-cost affinity adsorbents for the selective separation of small peptides, such as ACE-inhibiting peptides, have been developed. This selective separation technology has been implemented on industrial scale by food ingredient producers and the dairy industry.



Peptide characterisation

a. Molecular Composition & Structure

TNO Triskelion is well-experienced in the design and execution of analytical studies of proteins and hydrolysates, using Liquid Chromatography-Mass Spectrometry (LC-MS). TNO Triskelion developed and optimized in-house protocols for digestion and IgG purification. After sample preparation, chromatographic separation is performed using UPLC (-nanoTile™). Eluting peptides or proteins are detected with state-of-the-art triple-quad Xevo or Orbitrap systems, the latter of which provides very accurate m/z determinations. Experience includes:

- Design of protein purification protocols and dedicated sample preparation procedures, including enzymatic treatments, to answer the research question at hand.
- Molecular profiling of (complex) hydrolysates
- Sequence analysis of peptides and proteins through MS/MS analysis and database submission.
- Identification and localization of (posttranslational) modifications, such as glycosylations and phosphorylations.
- Highly sensitive and selective quantification of proteins in biological matrices using signature peptides. The sensitivity for peptides in final extracts typically is 10-100 ng/l.
- Intact protein analysis for determination of intact protein mass and glycosylation profiles.
- *In silico* signature peptide prediction.

b. Functional characterization: Intestinal Stability & Bioavailability

The health-promoting effects of bioactive peptides depend on their stability during gastrointestinal passage and their bioavailability. The stability and availability for absorption can be investigated using the TNO gastro-intestinal model (TIM). This dynamic *in vitro* model realistically simulates gastrointestinal conditions of the stomach and intestine. Besides different age groups, such as adults and infants, the TIM system can be set to mimic healthy and diseased conditions or animals. Samples can be taken at several sites to measure the intact luminal peptides and the fraction that is available for absorption. Exposing TIM samples to intestinal segments, using the InTESTine system, allows to study the passive and active transport and interactions with mucus and the brush border enzymes. Data from those physiologically relevant GI models can subsequently be combined with mathematical models to predict the rate of absorption and bioavailability of bioactive peptides in humans.

c. Functional characterization: Health Effects & Mechanism of Action

Bioactive peptides are known to trigger antioxidative, antimicrobial, antihypertensive, cytomodulatory and immunomodulatory effects in the human body. Potential health effects can be demonstrated through in vitro cell assays and in vivo models covering relevant health issues such as body weight disorders and metabolic syndrome. The *in vitro* assays include a diverse selection of cell lines, enabling the study of receptors, transporters, induction of digestion and satiety hormones, metabolism and biotransformation enzymes and a range of immunological and inflammatory markers. Expertise on biomarker analysis comprise

immunoassays such as ELISAs, radio immunoassays (RIA), multiplex assays (Luminex, Mesoscale), and a wide range of gas/liquid-chromatography based biomarker platforms.

Bioactive peptides also feature antimicrobial activity. TNO has broad expertise in analyzing antimicrobial activities against pure cultures of bacteria, yeasts and fungi as well as against naturally occurring populations of micro-organisms such as the intestinal microbiota. Analyses can be directed at identifying growth inhibition of single or multiple micro-organisms but also at analyzing specific stress responses evoked by these peptides. Medium throughput systems mimicking the natural situation as much as possible allow for rapid and cost-efficient screening and only need small amounts of peptide molecules.

Design of peptides based on relevant structure-function parameters can lead to the discovery of new, potent bioactive peptides. The *in silico* targeted discovery approach is well-known and frequently used for drug discovery in pharmaceutical industry. Cheminformatics, bioinformatics and molecular modeling tools provide methodologies to discover and identify bioactive peptide sequences present in the parent protein, to describe their 3D-structure and to elucidate their mechanism of action and related targets of interest. Computational chemistry at TNO is based on a broad palette of experience in pharmaceutical drug discovery, and application of this knowledge towards food industry may lead to new insights through elucidation of mechanistic knowledge on agents such as bioactive peptides.

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