Bioactive peptides and protein hydrolysates: research trends and challenges for application as nutraceuticals and functional food ingredients

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Highlights

- Systematic design of experiments to optimize bioactive peptide production
- Empirical & bioinformatics complementary approaches to bioactive peptide discovery
- Complexity of methodology for quality assurance of bioactive peptide products
- Research needs for evidence of bioavailability and clinical efficacy
- Bitter taste and approaches for evaluating and debittering peptide products

Abstract

The past decade has seen a burgeoning of literature on food-derived peptides and protein hydrolysates with diverse biological activities, but to date, empirical and bioinformatics studies have provided primarily *in vitro* data and limited clinical evidence to justify the development of these bioactive peptides and hydrolysates as nutraceuticals and functional foods for promoting health. Several obstacles must be overcome on the road to commercialization of these products. In conjunction with the need to implement efficient and cost-effective strategies for industrial scale production, the successful transfer of the technology to market requires standardization of analytical methods for assurance of product quality, assessment of sensory properties for consumer acceptance, and most importantly, well-designed clinical trials to provide robust evidence for supporting health claims.

Introduction

The World Health Organization (WHO) reports that 36 million deaths result each year from non-communicable diseases (NCDs), including cardiovascular diseases, diabetes, cancers and chronic respiratory diseases [1] (Table 1). An unhealthy diet is one of the four main behavioural risk factors for NCDs, and strategies that advocate a healthy diet and physical activity in order to promote and protect health are an integral part of the WHO's "2008-2013 action plan of the global strategy for the prevention and control of noncommunicable diseases" [1].

At the same time, and over the last decade in particular, there has been an explosion of scientific research on the topic of bioactive protein hydrolysates and peptides derived from food, which display a broad scope of functions [2] (Table 1). While usually less potent in their effects than synthetic pharmaceutical drugs, these bioactive peptides are also less likely to accumulate in body tissues or to confer serious side effects because nature has provided the mechanism for their metabolism and utilization or excretion. Given the impressive array of functions that have been discovered for food protein-derived bioactive peptides, and the vast scope of available food commodities, processing by-products and under-utilized resources that can be used as sources to generate these value-added products, it may be surprising to know that few have reached the commercial market. What are the bottlenecks and what is needed to resolve them? The objective of this paper is to share some insights into the current status, trends and acute needs for further research in this field, which are necessary to capture the opportunities to develop these functional components for enhancing human health.

Discovery of bioactive peptides by an empirical approach

Bioactive peptides, or "cryptides" [3], are fragments that are nascent or encrypted in the primary sequences of proteins, and that confer functions beyond basic nutritional benefits. As illustrated in Figure 1, the classical, empirical approach to the discovery and production of bioactive protein hydrolysates and peptides involves first identifying a suitable protein source, and then releasing peptide fragments with bioactivity through hydrolysis of peptide bonds, usually by the proteolytic action of enzymes sourced endogenously (autolysis) or exogenously (commercial enzyme preparations), or via fermentation (by addition of starter cultures). The resulting crude protein hydrolysate may undergo fractionation processes to yield an enriched bioactive peptide preparation or additional purification steps to isolate single peptides. Following the identification of the sequence of the isolated peptides, bioactivity is validated by testing chemically synthesized pure peptides.

The plethora of literature abounding on bioactive peptides derived from proteins notwithstanding, most of these empirical studies have not recognized the importance of using a systematic approach for process development, to optimize the multiple factors that affect production and purification. Hanke and Ottens [4•] commented that trial-and-error and one-factor-at a time experimentation is largely obsolete, being replaced by systematic design of experiments (DOE) approaches incorporating the "science, process understanding and risk management to design the production process to consistently deliver the pre defined quality objectives". Knowledge based process development requires an understanding of the critical

process parameters (CPP) that affect critical quality attributes (CQA) [4]. Examples of CPPs for bioactive peptide production are characteristics of the starting source material (e.g. protein content, other major and minor constituents, pH, variability by season) and enzyme preparation (purity, substrate specificity, specific activity, single or multiple enzymatic activity, optimal pH and temperature conditions for activity and stability), as well as the process conditions (concentrations and relative ratio of enzyme to substrate, pH, temperature, time). Several CQAs may be identified for the protein hydrolysate or peptide fractions, and may require process optimization to obtain products with multiple functions, either within the same peptides (i.e. multifunctional peptides), or in different peptides each contributing to a specific function.

Cheung and Li-Chan [5] used a Taguchi's L_{16} (4^5) fractional factorial design to investigate the influence of four CPPs, each tested at 4 levels, on three CQAs (the extent of hydrolysis, angiotensin-I converting enzyme (ACE)-inhibitory activity and bitterness) of protein hydrolysates produced from shrimp processing by-products. Using this DOE enabled the evaluation of hydrolysates produced under conditions associated with combinations of the four CPPs based on only 16 unique experiments, as opposed to either single-factor-at a time testing (holding three parameters constant while changing the fourth), or a full factorial design (requiring 256 unique experiments). Similarly, Marchetti et al. [6] applied DOE for "Quality by Design" to understand and design the CPPs for peptide separation and recovery by nanofiltration. While the benefits of Taguchi's and other DOE methods have been recognized in many other disciplinary sectors [7], its adoption by researchers in the field of bioactive peptides derived from food has been limited. In comparison, response surface methodology (RSM) has been more widely adopted [8]. It should however be noted that application of RSM for optimization of CPP conditions to attain the best product attributes assumes that the researcher has *a priori* knowledge of which CPPs are significant and should be investigated, as the number of experiments increases exponentially with the number of parameters to be optimized. Furthermore, the ability to fit the data to a statistically robust regression model for predicting the optimum depends on selection of an appropriate range of conditions for experimentation.

Effect of processing on the generation of bioactive peptides

Kopf-Bolanz et al. [9•] reported that processing can induce changes in protein degradation and peptide profiles generated within complex food matrices such as commercially available dairy products, and commented that many studies have investigated single proteins or peptides in isolation, without considering the influence of processing and/or other components on susceptibility to hydrolysis and rate of uptake. Lacroix and Li-Chan [10] compared the extent of hydrolysis and dipeptidyl peptidase IV (DPP-IV) inhibitory activity of dairy protein products (whey protein isolate (WPI), milk protein concentrate, skim milk powder and sodium caseinate) subjected to hydrolysis by various enzymes, including simulated gastrointestinal (GI) digestion with pepsin and pancreatin. The highest DPP-IV inhibitory activity was obtained in the 1-h peptic hydrolysate of WPI [10]. When hydrolysates were prepared from the individual whey protein constituents, higher bioactivity was obtained in the hydrolysate of α-lactalbumin than any of the other whey proteins including β-lactoglobulin, lactoferrin and bovine serum albumin [11]. However, the subsequent fractionation, isolation and identification of peptides in WPI

hydrolysate revealed unexpectedly that the most potent DPP-IV inhibitory peptides were in fact not from α-lactalbumin, but from β-lactoglobulin [12]. The co-existence of multiple protein substrates in WPI, possible conformational changes induced during commercial production and their resultant effects on accessibility and susceptibility of peptide bonds to peptic digestion, may have been responsible for the different profiles and DPP-IV inhibitory activity of peptides generated by peptic digestion of β-lactoglobulin in commercial food grade WPI, compared to research grade β-lactoglobulin isolated by milder processes. These results underscore the importance of using commercially relevant starting materials during the research and development stages for bioactive peptide discovery.

Trends for industrial scale production of bioactive peptides

Pilot scale production processes for bioactive peptides typically utilize membrane and liquid chromatographic processes sequentially for fractionation and isolation of bioactive components from the crude hydrolysates. The principle for designing processes for separation of the peptides may be based on molecular properties such as size, charge, and polarity or hydrophobicity, and may be informed by mechanistic modelling of the quantitative structure-activity relationship (QSAR). New strategies including coupling or integration of complementary processes are necessary to establish economical and efficient industrial scale processes not only for fractionation, but also for simultaneous and continuous production of peptides with different bioactive properties. For example, Wu et al. [13] reported that an enzymatic ultrafiltration (UF) membrane reactor in conjunction with chromatography could be used to achieve continuous hydrolysis and isolation of multi-functional peptides more effectively than the traditional mode using batch reactors. The selection of membranes with appropriate molecular weight cut-off followed by either size-exclusion chromatography or cation exchange chromatography enabled simultaneous production and isolation of peptides with ACE-inhibitory, calcium-binding and antimicrobial properties [13].

In recent years, a process coined “EDUF” for “electrodialysis with UF membranes” has been explored to separate molecules by electric charge and molecular mass [14,15•]. In EDUF, the driving force through the membranes is via an electric field (anode/cathode) rather than by pressure as is the case with conventional UF, thus mitigating the limitations of both chromatography (high cost) and pressure-driven membrane (fouling) processes. EDUF can be used to achieve simultaneous production and fractionation in a single step, and the higher resolution achieved by stacking differently sized UF membranes can result in purified peptide fractions with higher functionality and bioactivity [15•].

Discovery of bioactive peptides by a bioinformatics driven approach

Bioinformatics, also known as *in silico* prediction and analysis, refers to computational methods applied to manage, curate and interpret information on biological systems, in this case, the bioactive peptides derived from food. Based on knowledge about structure and activity of peptides reported in the literature and deposited in pertinent databases, computational approaches may be applied to elucidate structure-function relationships, predict peptide sequences likely to exhibit specific activities, locate peptides encrypted in particular protein sources, envisage

release of those fragments by specific enzymatic cleavage, and propose the putative mechanism of action through molecular docking of binding sites [16].

Although there is a growing bank of databases pertinent to bioactive peptides and the proteolytic enzymes that may be used to release them from food proteins [17], the majority describe bioactive peptides found endogenously, i.e. of physiological relevance, rather than being derived from food. Moreover, the available databases on proteolytic enzymes describe properties of isolated biochemically well-characterized enzymes [18], which is in contrast to the less stringent substrate specificity and lower purity typical of most commercially available proteolytic enzyme preparations used for food applications.

The BIOPEP database developed at University of Warmia and Mazury in Poland is unique in that it focusses primarily on peptides of food origin [17]. It offers the user the ability to generate profiles of potential biological activity of the protein of interest as well as the frequency of occurrence of bioactive fragments in the protein. For example, *in silico* analysis was applied to assess the potential of different food commodities to serve as sources of peptides with inhibitory activity against the enzyme DPP-IV, which acts on incretin hormones that play a role in blood glucose regulation [19]. One limitation is that the DPP-IV inhibitors reported in the literature at the time of that study consisted primarily of di-and tri-peptides, in contrast to the much longer physiological substrates of the DPP-IV enzyme, GLP-1 and GIP.

Higher frequency of occurrence of bioactive sequences in a protein molecule does not necessarily correlate with the potential of that protein to serve as a good source of bioactive peptides unless the potency of each bioactive fragment and any overlaps of bioactive sequences are taken into account. To address these limitations, Nongonierma & FitzGerald [20] developed an *in silico* approach incorporating protein coverage and potency indices, and applied a peptide alignment strategy to investigate the relationship between sequence and activity. Potency is represented in the BIOPEP database by EC_{50} values, i.e. the concentration of the bioactive fragment corresponding to its half-maximal activity. Unfortunately, EC_{50} values are not always reported in the literature and moreover, may vary for identical sequences if assayed under different conditions. For example the concentration of a peptide required to inhibit an enzyme to its half-maximal activity (referred to as the IC_{50} value), can be influenced by assay conditions including enzyme and substrate concentrations. Thus unless the inhibitory activity is reported as the inhibitor affinity constant (K_i), potency of different peptides reported by different researchers may not always be comparable.

Molecular docking simulations have also been applied to elucidate which peptide sequences, either experimentally identified or predicted from bioinformatics investigation, may actually be able to interact with the proteins that are the target of the biological activity [21]. Acharya et al [22] noted that the dynamic conformational changes induced in both the bioactive peptide and the receptor target protein upon binding impose limitations on computational docking studies, and advocated for a 4D structural database documenting these changes. Nongonierma et al. [23] also found no direct correlation between the Vina scores (predicted affinity) obtained by molecular docking of tri-peptides to the active site of DPP-IV and their *in vitro* DPP-IV inhibitory properties, and recommended structure-activity relationship modeling rather than

docking to select peptides for experimental testing. These results may reflect the fact that binding of a peptide to a protein (or enzyme) molecule may arise from non-specific interactions or else occur at a site that is associated with an activity other than the one of interest, and these scenarios cannot be easily be ascertained by molecular simulations alone.

Predictive models can be generated by QSAR analysis of physicochemical characteristics (size, charge, polarity, secondary structure, sequence) reported for specific activities of peptides. Zhou et al [24•] used QSAR analysis in conjunction with quantum mechanics/molecular mechanics analysis of the structural basis and energetic profile involved in complexes of peptides with the ACE enzyme, to model ACE inhibitory activity and bitterness on peptide structural property and the interaction profiles between ACE receptor and peptide ligands. The correlation between ACE-inhibition and bitterness was strongest for di-peptides, and decreased markedly through tri- and tetra-peptides, which the authors explained as being due to the exponential increase in structural diversity with each additional amino acid in the peptide length. Moreover, structural and energetic analysis of ACE-peptide complexes indicated that while ACE-inhibitory potency suggested by binding energy increased from di- to tri- and tetra-peptides, insignificant changes were observed for longer peptides, presumably as the terminal residues reside out of the active pocket of the enzyme and thus have minor influence on the binding. Using a similar approach, Wang et al. [25] reported a positive significant relationship between ACE-inhibitory potency and antioxidative activity of tri-peptides, but only a modest correlation with bitterness, suggesting the potential to develop non-bitter functional peptide products with multiple bioactivities.

As evident from the preceding discussion, a bioinformatics-driven approach can lead to the discovery of novel peptides. Holton et al [16] remarked that the tremendous strides in bioinformatics tools made in various disciplines including biotechnology, drug discovery, comparative genomics, molecular medicine and microbial genomics, have not been paralleled in food and nutrition science research, and the use of bioinformatics in food is "still in its infancy". They proposed establishment of a Food-Wiki database (FoodWikiDB) for sharing and managing the vast content of data being continuously generated. However, even though bioinformatics can provide insight at the molecular level of specific peptide sequences that would be of interest for further investigation, its limitations must be acknowledged. For example, *in silico* approaches cannot easily predict the bioactivity of combinations of peptides that are present in protein hydrolysates or fractions. Furthermore, the reliability and utility of bioinformatics is heavily dependent on the data repository used for *in silico* analysis. There is a paucity of knowledge of the structure-activity relationships of peptides longer than four amino acids, and these investigations are often limited by the high costs for chemical synthesis of longer peptides. Peptide array technology, also referred to as scanning peptide array or microarray technology, may offer a relatively cost-effective approach to generate an array of longer peptide sequences that can be probed on the array support, and used to investigate interactions of the peptides with physiologically relevant proteins or other molecules, e.g. peptide-protein interactions involved in allergenic epitope analysis, enzyme-substrate, enzyme-inhibitor investigations [26,27]. Peptide array technology may thus offer a high throughput approach as a complement to classical and bioinformatics-driven approaches to select peptide sequences for further investigation (Figure 1).

Challenges to commercialization: Complications in methodology for quality assurance

In the end, both the traditional (empirical) and newer (bioinformatics driven) approaches converge at a common point (Figure 1), namely the need to test the activity of specific peptide sequences that have either been identified by the experimental data or suggested by *in silico* analysis, and then to verify that these sequences are actually released and exist in the end-products, whether the latter be unfractionated protein hydrolysates containing bioactive properties, or else partially purified fractions with enriched concentrations of the bioactive sequences. Compared to synthetic small-molecule drugs, which are single identifiable entities, in most cases, the target end product for bioactive peptides derived from food is not usually a single peptide with 99% purity – not only due to the unacceptable high cost and low yield that would be involved, but also because products containing only single peptide entities would ignore any additive, synergistic or antagonistic effects among peptides. Moreover, peptides possessing bioactivity are often hydrophobic in nature and exhibit poor aqueous solubility at high concentrations. Formulating products with several peptides each at lower concentration can ameliorate the solubility problem while conferring the same level of bioactivity. Thus, the minimum level of information for quality assurance should include not only verification of specific peptide sequences in the complex matrix that are associated with the activity but also the bioactivity of peptide mixtures under standard conditions.

Mass spectrometry, or more specifically liquid chromatography tandem mass spectrometry (LC-MS/MS) is recognized as the primary tool for sequencing peptides and identifying proteins, but requires particular paradigms for the analysis of bioactive peptides derived from food. Databases used for "BLAST" (Basic Local Alignment Search Tool) searches in life sciences research are commonly based on tryptic digests of proteins, while enzymes typically used to produce food protein hydrolysates have diverse and often less stringent substrate specificity, and combinations of enzymes may also be employed either concurrently or in tandem. Furthermore, instead of a single purified protein as the precursor for generating peptides, food protein sources typically are composed of multiple constituents, for example α_{s1}-, α_{s2}-, β-, and κ-caseins are all present in sodium caseinate. Thus, the number of unique peptide sequences generated in these protein hydrolysates is usually massive.

According to Panchaud et al [28] and Lahrichi et al. [29••], proteomics (for biomarker discovery) and peptidomics (for bioactive peptide discovery) have in common the necessity for identification and validation on the peptide level. However, the majority of peptides generated by specific enzymes such as trypsin in biomarker proteomics analyses fall in the range of 7-25 amino acids in length; in contrast the typical length of peptides occurring in protein hydrolysates produced by enzymes for food applications may range from 2-100 amino acids, and will vary in properties including charge state and hydrophobicity. Different technological challenges must be considered in the analysis of small (< 7 amino acids), medium (7-25 amino acids) and large (>25 amino acids) peptides. Size exclusion chromatography on columns capable of separation in the ~100-10,000 Da range was suggested as a fractionation step prior to mass spectrometry [28], and the application of LC-MS/MS with multiple reaction monitoring (MRM) was reported to address challenges of analysis for even very complex peptide sets with large isobaric clusters

[29••]. Promising results were obtained in the analysis of a set of 117 peptides composed of di-, tri- and tetra-peptides of the three branched chain amino acids (V, L, I) in a model system as well as in a complex matrix (whey protein hydrolysate), by optimizing chromatographic separation followed by LC-MS/MS analysis with MRM scan mode and using a combination of retention time, diagnostic ion as well as ratios of key diagnostic ions [29••]. Further research is crucial for expansion of this approach to the analysis of other peptide sizes likely to be found in food protein hydrolysates.

Challenges to commercialization: Sparse data on bioavailability and metabolic fate

Picariello et al. [30] commented that "pharmacokinetics" and "pharmacodynamics", which are integral to understanding drug metabolism, are "still elusive for dietary peptides", with most studies on food-derived bioactive sequences paying little attention to the susceptibility of the peptides to degradation by gastric, pancreatic and small intestinal brush border membrane enzymes, and the likelihood that only nano- or even pico-molar concentrations of the original peptide may pass into the systemic circulation. Although some peptides may exhibit their bioactivity locally in the GI tract, for example by inhibition of the DPP-IV enzyme that acts on the incretin hormones [31] or by preservation of the intestinal mucosa integrity against oxidative stress induced conditions such as inflammatory bowel diseases and colon cancer [32], the vast majority of therapeutic peptides exert their bioactivity via the systemic circulation. Therefore, information on *in vivo* stability, availability and accessibility of identified bioactive peptide sequences as well as their absorption, distribution, metabolism and excretion is critical [33].

Boutrou et al [34•] detected and sequenced 356 and 146 peptides in jejuna effluents of healthy adults after consumption of 30 grams of milk casein and whey proteins, respectively, and suggested that these levels of peptides were sufficiently high so as to confer bioactivity, including for example opioid and antihypertensive action associated with the identified peptide sequences originating from β-casein. However, the absorption and bioavailability of these oligopeptides was not determined. On the other hand, Dia et al [35] did detect lunasin (a 43-amino acid peptide from soybean and several other plant sources, and reported to possess anti-inflammatory and anti-cancer properties) in plasma samples of healthy male subjects who consumed soy protein daily for five days after a preliminary washout period. The daily dose of 50 g soy protein represented a total daily intake of 155.5 mg lunasin, of which 97% was estimated to be destroyed by GI digestion, resulting in only about 4.7 mg lunasin that might be available for absorption in the intestine [35]. The levels of 71.0 ng lunasin per mL of plasma measured in the human subjects was calculated to represent an average of 4.5% absorption of the lunasin [35]. Given these numbers, and the doses associated with chemopreventive effects in cell culture model systems, the authors concluded that large amounts of soy protein would have to be consumed to achieve bioactive levels of lunasin in humans, albeit consumption over a prolonged period of time could reduce the required amount. Indeed, further investigation demonstrated the ability of lunasin to inhibit human colon cancer cell metastasis in a mouse model when administered intraperitoneally at a dose of 8 mg/kg bw, whereas the same dose by oral gavage was not effective [36••]. Higher oral doses were suggested for further study.

The uptake of lunasin by macrophages particularly under inflammatory conditions was investigated by Cam et al. [37], who concluded that the internalization of this peptide is primarily facilitated by endocytic mechanisms involving clathrin-coated structures and macropinosomes. There is a fine balance between ability of peptides to enter cells (desirable for intracellular activity) and potential hemolytic and toxic properties associated with cell-penetrating peptides. More research in this area is crucial, and may require tapping into databases for peptide sequences and predicting structural features that may be requisite to membranolytic activity such as hemolysis [38] or to cell penetration [39], in conjunction with those associated with the mechanism of action for bioactivity.

Challenges to commercialization: Inadequate clinical evidence of bioefficacy

To date, ACE-inhibitory and/or antihypertensive peptides are probably the most intensively studied class of bioactive peptides derived from food [40]. Despite this vast body of literature, the European Food Safety Authority (EFSA) has rejected health claims proposed for bonito protein peptide [41], the C12-peptide (FFVAPFPDVFGK) [42], as well as the milk tri-peptides IPP and VPP [43], citing inadequate human studies and/or "major methodological limitations" in the reported studies, and a lack of convincing evidence for the mechanism responsible for the claimed effect at the proposed dose.

The results of clinical studies have been inconsistent. Pooled effects of 5.23 and 2.42 mm Hg reduction of systolic blood pressure (SBP) and diastolic blood pressure (DBP), respectively were observed in a meta-analysis of placebo-controlled clinical trials on food protein-derived peptides and their effect on blood pressure [44]. On the other hand, Qin et al. [45] concluded from their recent meta-analysis of randomized controlled clinical trials that the blood pressure lowering effect of the milk tri-peptides VPP and IPP, while statistically significant, is small in magnitude, with pooled mean effects of only 1.66 and 0.76 mm Hg reduction in SBP and DBP, respectively. Reductions of 1.30 and 0.57 mm Hg were observed for 24-h ambulatory blood pressure response to the intervention. Interestingly, these values for mean blood pressure reduction were less pronounced than those reported by the same authors from a previous meta analysis reported in 2008, as most of the more recent studies did not show reduction. Qin et al. [45] expressed a need for well-designed and larger scale clinical investigations, particularly randomized double blind trials with ambulatory blood pressure monitoring, in order to conclusively determine efficacy of the milk tri-peptides.

Challenges to commercialization: The bitter taste of peptides

According to Temussi [46], "the taste of peptides is seldom one of the most relevant issues when one considers the many important biological functions of this class of peptides". Unfortunately, protein hydrolysates and peptides are notorious in exhibiting bitterness [47,48], necessitating suitable formulation of the bitter peptides with other ingredients such as cocoa powder and aspartame [49], or fructose, pectin, natural and artificial flavours and colors [50].

Bitter taste is recognized by the T2R family of Ca^{2+}-bound G protein coupled receptors (GPCRs), with 25 human T2R bitter taste receptors being identified to date. Although the receptor hTAS2R1 was initially reported to be more specific and sensitive to bitter peptides than other types of bitter compounds including caffeine, more recent research by Kohl et al. [51••]

other types of bitter compounds including caffeine, more recent research by Kohl et al. [51••] has revealed that in fact at least five or six members of the human T2R bitter taste receptor family are activated by amino acids and peptides. The advances in knowledge of bitter taste receptors and ligands notwithstanding, many questions remain to be answered in order to understand the complexity of taste sensations, including the subtle relationship between sweet and bitter tastes, potential involvement of the T1R1-T1R3 umami receptor in sensing bitter molecules, as well as the possible contribution of sourness and saltiness in modulating sweet, bitter and umami modalities for acceptance of foods [52]. New approaches are therefore needed to elucidate the structural features associated with bitterness of amino acids and peptides, and to devise strategies to reduce the bitter sensation, including spray-drying encapsulation with maltodextrin and cyclodextrin as carriers [53], addition of bitter masking or inhibiting ingredients [54•], or enzymatic exopeptidase treatment [55].

Sensory-guided fractionation, involving multi-step separations followed typically by mass spectrometry and "sensomics mapping", is a recognized approach to identify the peptide sequences responsible for undesirable bitter taste of protein hydrolysates and their fractions [47], but requires evaluation by humans to verify the taste of individual peptides in the isolated fractions or chemically synthesized peptides. Not only is this a time-consuming and expensive process, there are technological challenges related to the small quantities of peptides typically available, as well as safety concerns for taste evaluation considering the non-food grade solvents and chemical reagents used in peptide synthesis, fractionation and purification. Panelist fatigue, the limited number of samples that can be evaluated at a time, and difficulty with standardization particularly over long periods of time are also important considerations. QSAR may be useful to complement human sensory evaluations by providing clues to elucidate the bitterness of food-derived bioactive peptides [24•,25,56-58], but, as previously mentioned, the validity and usefulness of the QSAR approach hinges on the information available for building the prediction model.

Although human sensory evaluation will always be the "gold standard" for assessing taste attributes and acceptability, in view of the aforementioned limitations, there has been increasing interest to develop instrumental taste sensing systems and electronic tongues for screening of large numbers of fractions and samples, thus lightening the burden of human taste panel evaluation [59,60]. Instrumental sensors have been applied to analyze taste of amino acids, peptides and protein hydrolysates [61-63,64••], and to assist in screening for compounds to mask their bitterness [65]. Cell based assays also show promise as an alternative to human panelists for screening of peptides for bitter taste. Using engineered cell lines expressing the TAS2R and the chimeric G protein α-subunit (Gα16gust44), positive interaction of peptides with the TAS2R receptor is detected fluorometrically by an influx of extracellular calcium indicator that is taken to represent activation by bitter peptides [51•]. This approach has been applied for discovery of synthetic and natural bitter taste receptor antagonists [66] and may provide structure-function information on the contribution of different peptide sequences to the bitter taste modality, as well as guide the discovery of natural bitter taste receptor agonists which can mitigate the problem of bitterness in bioactive protein hydrolysates. This avenue of research is still in its infancy, and research is needed to resolve problems of the current assay, including interferences from other

compounds in the complex sample matrix which may induce a non taste-receptor mediated response by the cells [67].

There is currently a dearth of information on the taste attributes of bioactive protein hydrolysates or peptides. Research applying sensomics mapping, instrumental taste sensing or cell-based systems to the study of bioactive peptides could accelerate the acquisition of important knowledge in this field.

Conclusions

Bioactive peptides and protein hydrolysates hold great promise as valuable functional ingredients in healthy diets to fight the global epidemic of non-communicable diseases. However, in order to realize this potential, several challenges must be addressed (Table 2). The high cost and multi-step nature of existing processes for bioactive peptide production implores the need to apply a systematic approach for identifying the best conditions to release "cryptides" with target bioactivity from the parent protein source, and for developing innovative production and purification strategies to obtain peptide fractions with high potency and yield. Bioinformatics tools may be useful to guide the empirical approach and may also provide a better understanding at the molecular level of the peptide structure-activity relationship. Standardized methodology for analysis and robust clinical trials to evaluate efficacy and metabolic fate of the established products are of critical importance for quality assurance and justification of health claims. Finally, research must be conducted on the taste and other sensory quality attributes of bioactive peptides to ensure their successful adoption as functional food ingredients that can lead to better health.

Acknowledgements

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Table 1. Non-communicable diseases (NCD) of global health concern, behavioural risk factors and other underlying metabolic/physiological causes of NCD, and examples of biological properties for peptides and proteins derived from food that may play a role in mitigating the NCD epidemic.

Non-Communicable Diseases (NCD) of global health concern[1]	**Risk factors and other underlying causes for NCD**[1]	**Examples of biological properties reported for peptides and proteins derived from food**[2]
➢ Alzheimer's disease ➢ Cancer ➢ Cardiovascular disease ➢ Chronic respiratory diseases ➢ Diabetes	• Tobacco use • Insufficient physical activity • Harmful use of alcohol • Unhealthy diet o High blood pressure o Overweight and obesity o Raised cholesterol o Obesity o Cancer-associated infections	❖ Anti-aging ❖ Anti-cancer ❖ Anti-cariogenic ❖ Anti-diabetic ❖ Anti-hypertensive ❖ Anti-microbial ❖ Anti-oxidative stress ❖ Anti-inflammatory ❖ Cholesterol lowering ❖ Growth enhancing ❖ Immunomodulatory ❖ Mineral binding ❖ Radical scavenging ❖ Regulation of glucose & insulin homeostasis ❖ Satiety regulating ….

[1] WHO 2011 [1]

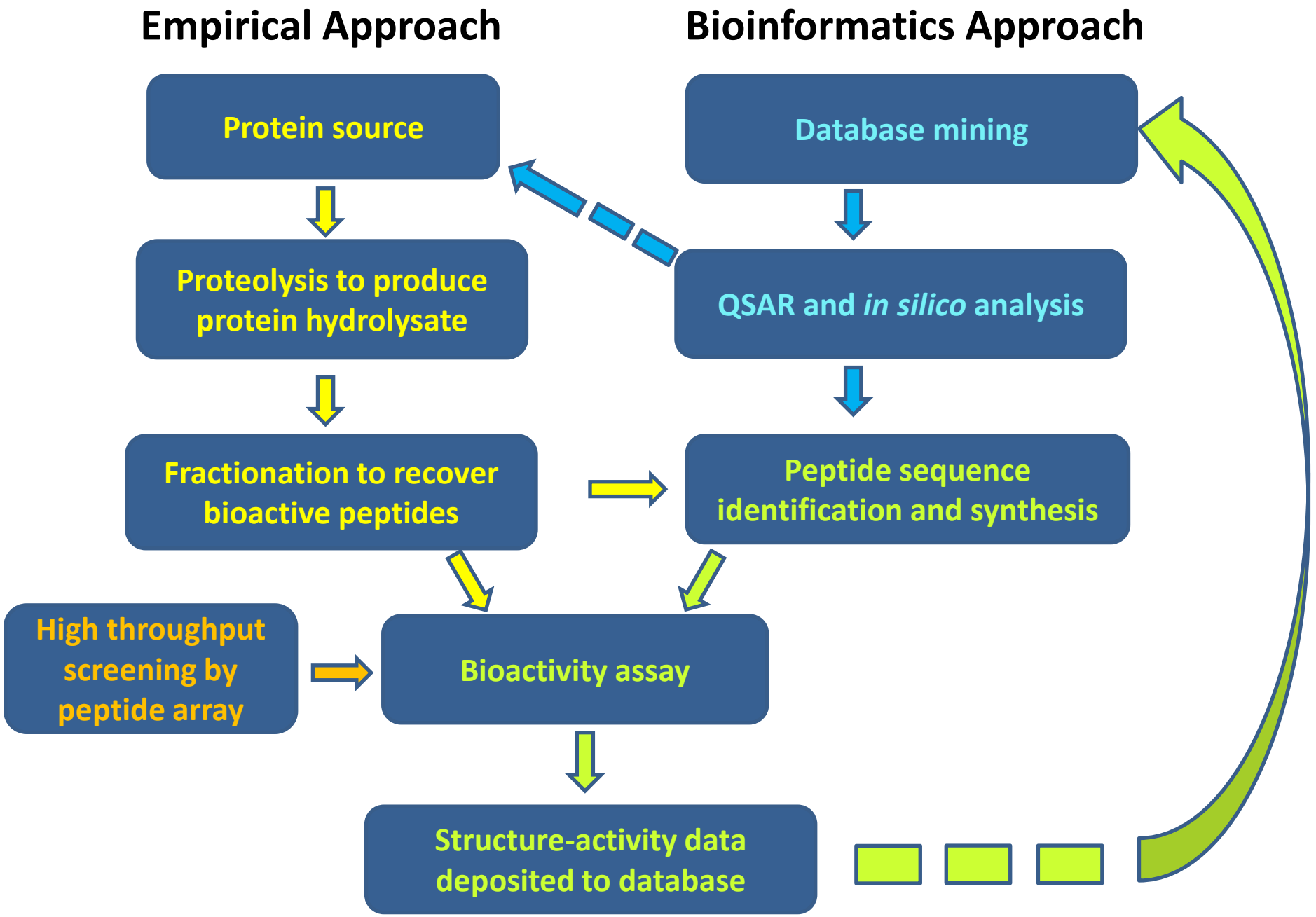
[2] Mine et al. 2010 [2]

Table 2. Challenges & bottlenecks versus strategies & research needs to achieve commercialization of food-derived bioactive peptides as nutraceuticals and functional foods.

Challenges & Bottlenecks	Strategies & Research Needs
Related to production:	
➢ High cost of production	❖ Use economical starting materials (byproducts, underutilized resources)
➢ Selection of "best" protein source as the precursor of peptides with target activity	❖ Bioinformatics tools and peptide array technology to guide protein precursor selection and discovery of novel peptides
➢ Selection of enzyme(s) and conditions for hydrolysis to achieve best activity and yield	❖ Systematic design of experiments approach to optimize process parameters for best quality attributes
➢ Multi-step isolation/purification procedures; industrial scale-up	❖ Innovation through coupling and integration of complementary processes
Related to quality:	
➢ Limited quantity and poor solubility of purified peptides	❖ Production of bioactive peptide mixtures that can act synergistically
➢ Analysis of bioactive peptides in product	❖ LC-MS/MS analysis of target sequences; assay of bioactivity of peptide mixtures
➢ Mechanism of action and safety	❖ Research on stability, bioavailability, absorption, distribution, metabolism, excretion
➢ Health claims	❖ Robust clinical trials for efficacy in target populations
➢ Consumer acceptance	❖ Debittering, encapsulation or masking. Human sensory evaluation. High-throughput screening using instrumental taste sensors and cell-based assays

Figure 1 Caption

Discovery of bioactive peptides by an empirical approach involves proteolysis of the selected protein source to produce the hydrolysate, followed by fractionation to recover bioactive peptides. Peptide sequences are identified by mass spectrometry, bioactivity is verified using chemical synthesized peptides, and structure-activity data deposited to the database. The bioinformatics driven approach applies computational methods to information in the database to guide selection of critical process parameters for experimental production of hydrolysates as well as to identify novel peptides for synthesis and bioactivity testing. Peptide array technology offers a high throughput approach for screening of a large number of sequences identified by empirical or bioinformatics approaches.


Empirical Approach
Bioinformatics Approach
Protein source
Database mining
Proteolysis to produce protein hydrolysate
QSAR and *in silico* analysis
Fractionation to recover bioactive peptides
Peptide sequence identification and synthesis
High throughput screening by peptide array
Bioactivity assay
Structure-activity data deposited to database