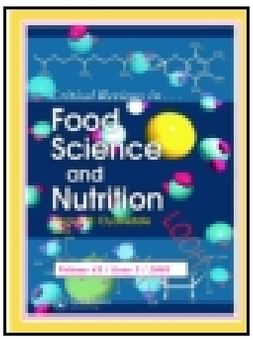

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Current Trends and Perspectives of Bioactive Peptides

Eric Banan-Mwine Daliri[a], Byong H. Lee[a,b] and Deog H. Oh[a,*]

[a]Department of Food Science and Biotechnology, Kangwon National University,Chuncheon, South Korea 200-701

[b]Department of Microbiology/Immunology, McGill University, Montreal, QC, Canada H3A 2B4

Deog H. Oh

Corresponding author Email: deoghwa@kangwon.ac.kr

ABSTRACT

The remarkable growth of therapeutic peptide development in the past decade has led to a large number of market approvals and the market value is expected to hit $25 billion by 2018. This significant market increase is driven by the increasing incidences of metabolic and cardiovascular diseases and technological advancements in peptide synthesis. For this reason, the search for bioactive peptides has also increased exponentially. Many bioactive peptides from food and nonfood sources have shown positive health effects yet, obstacles such as the need to implement efficient and cost-effective strategies for industrial scale production, good manufacturing practices as well as well-designed clinical trials to provide robust evidence for supporting health claims continue to exist. Several other factors such as the possibility of allergenicity, toxicity and the stability of biological functions of the peptides during gastrointestinal digestion would need to be addressed.

Keywords

Therapeutic peptides, *In vivo* tests, Antimicrobial peptides, Functional foods

INTRODUCTION

The past decade has seen an exponential increase in bioactive peptide research and the therapeutic peptide market value is projected to reach $25 billion by 2018. The significant issues with toxicity, poor response rate, and the emergence of resistance among many patients have made the administration of synthetic drugs less attractive. More so, an unhealthy diet is one of the four main lifestyle risk factors for non-communicable diseases (NCDs) and a global strategy that supports a healthy diet has become an integral part the World Health Organization's action plan for the prevention and control of NCD's (Mendis et al., 2015). Therefore, development of functional foods for specific health effects is on the increase. Bioactive peptides have proven to be particularly useful tools for inhibiting protein-protein interactions due to their small size, ease of production, exquisite potency and selectivity. Over the years, important bioactive peptides have been identified in both dietary and non-dietary sources. In foods, the search for bioactive peptides has mostly been carried out using empirical and bioinformatic approaches or an integrated approach to obtain *in vitro* data. The peptides remain inactive as long as they remain in their parent protein structure, but exhibit activity when cleaved intact by enzymes or by microbial fermentation (Figure 1) (Manzanares et al., 2015). Another means of biopeptide discovery is by screening large phage display libraries to find novel bioactive peptides that bind to targets of interest with desired biological properties. Peptides obtained by these methods become the basis for therapeutic molecule development. In this review, we discuss the discovery, production, functionalities of bioactive peptides including antihypertensive, antimicrobial, anti-oxidation, stimulatory, and immunomodulatory abilities. Challenges in development of therapeutic peptides as well as methods of improving their yields have also been included.

KEY MOTIVATIONS OF BIOACTIVE PEPTIDE RESEARCH

The increasing health care costs have prompted governments, researchers, health professionals, and the food industry to find solutions as to how the problem can be managed efficiently. Development of bioactive peptides from food proteins is propelled by the readily available and inexpensive protein raw materials. The increasing consumer awareness of functional food and their health promotion also motivates researchers to pursue food derived bioactive peptides as therapeutics for human disease treatments and prevention (Daliri and Lee, 2015). More so, the high selectivity, efficacy, stability, safety, bioavailability and tolerability of bioactive peptides make them better alternatives compared to therapeutic classes. Peptides are easily synthesized, optimized and evaluated as potential therapeutics for many diseases. These essential properties of peptides have attracted the attention of governments, companies and researchers towards developing peptide based therapeutics in recent years. In line with this, there is remarkable increase in the number of approved therapeutic peptides on the market as researchers continue to develop new peptides using novel strategies.

DISCOVERING BIOACTIVE PEPTIDES

A variety of biologically active peptides are present in almost all living organisms and hence, nature remains the most promising source of bioactive peptides. An alternative source of bioactive peptide is from genetic or recombinant libraries or from chemical libraries (Uhlig et al., 2014). According to current literature, the major approaches in bioactive peptide discovery in nature can be classified as empirical approach, bioinformatic approach and the integrated

approach (Figure 2). Other studies have also used peptide display technology for discovering important bioactive peptides (Wu et al., 2015; Hoogenboom, 2005).

The empirical approach

This is the most widely used method for the discovery of bioactive peptides from food proteins, and involves selection of protein sources of particular interest. The proteins are hydrolyzed with food-grade proteolytic enzymes of particular specificity to release numerous peptide fragments in the hydrolysate (Bougatef et al., 2008; J. Chen et al., 2012; Silvestre et al., 2012; Darewicz et al., 2014). Alternatively, the proteins could be fermented with specific microorganisms to enhance peptide release by microbial proteases (García-Tejedor et al., 2013; K. Nakamura et al., 2013; Ha et al., 2015). The fermentates or hydrolysates are then fractionized and purified based on their structural features. The fractions are then tested *in vitro* for their potential health benefit. The fractions of the highest *in vitro* ability are further fractionated to separate the peptides in the fraction. Usually, the peptides in the active fractions are identified using liquid chromatography coupled with mass spectrometry (Ahn et al., 2009; Garcia-Tejedor et al., 2014; Koyama et al., 2014). Using this approach, Bidasolo et al. (2012) digested donkey's milk casein with pepsin and Corolase PP® to recover a large number of peptides with various functional properties. In a similar way, we fermented whey with different lactobacillus species and obtained many different peptides some of which had antihypertensive activity (Ahn et al., 2009). Though this approach enhances the discovery of new bioactive peptides in various protein samples, it usually overlooks the activity of less concentrated peptide fragments which may also be potent. It is also expensive and very time consuming (if not impossible) to chemically synthesize all the peptides that may

be present in a fraction of digest for screening purposes. Therefore, bioinformatic approaches have been developed to help in identifying bioactive peptides.

The bioinformatic-driven (*in silico*) approach

The *in silico* approach involves the use of information accrued in databases to determine the frequency of occurrence of encrypted bioactive peptides in the primary structure of proteins. These data bases contain many protein sequence information which make it possible to identify specific amino acid sequences in the parent protein which have been reported to be bioactive. Since the occurrence of the peptides does not necessarily indicate liberation of the encrypted peptides, certain bioinformatic software are also able to simulate proteolytic specificities of enzymes in order to generate profiles of peptides *in silico*. This approach enables identification of known peptides from unknown protein sources. Such proteases can then be used to hydrolyze the food protein and the potential health effects tested to establish their potency. The software may also predict which peptides in the simulated digest could be bioactive. New peptides identified from the *in silico* simulations could also be synthesized and their bioactivities established through *in vitro* and *in vivo* experiments. Several researchers (Pripp et al., 2004; Wu et al., 2006a, 2006b; Wu and Aluko, 2007) have used this approach to identify potent bioactive peptides. The use of *in silico* platforms alone for predicting the bioactivity of peptides may be simple, but it may not be very dependable since not all the predicted peptides from the simulated enzymatic digestion may be active or exhibit the expected potentials when tested in vitro or in vivo. Many researchers therefore compare their data obtained from HPLC-MS with several

house data bases to enable identification of less concentrated but potent peptides (Sagardia et al., 2013).

The integrated approach

Due to the limitations associated with the aforementioned approaches, an integrated bioinformatic process could be used in the discovery of bioactive peptides. The strengths of both empirical and bioinformatic approaches can be combined as and when needed to enhance the discovery and use of peptides in functional food and health applications. Bioactive peptides identified in food proteins through the *in silico* approach could be chemically synthesized. This approach can lead to the discovery of new peptides from new sources (Udenigwe and Aluko, 2011). However, many important bioactive peptides of low concentrations may still be lost if they have not been captured in the database already. Hence, other technologies such as peptide display technologies are currently employed in the search for bioactive peptides.

Peptide display technologies

In view of the challenges associated with bioactive peptide screening, recombinant peptide libraries linked to their coding sequence (peptide display technologies) could be applied as powerful tools in identifying potent bioactive peptides. Peptide display technologies have been used as potent research tools for high-throughput screening of protein interactions (Sidhu, 2000; Hoogenboom, 2005). Of all available molecular display techniques such as yeast and bacterial display, ribosome display, mRNA display, CIS and covalent antibody display, phage display has been widely applied (Ullman et al., 2011; Wu et al., 2016). Phage display is a selection technique which involves the fusion of a peptide or protein with a bacteriophage protein coat displayed on

the surface of a virus. The phage-displayed random peptide libraries allow functional access to biopeptides and separates binding peptides from nonbinding peptides by affinity purification. Phage-displayed random peptide library screening is a powerful means to identifying peptides that can bind and regulate the functions of target molecules. This technique has been applied in selecting bioactive peptides bound to receptors (Houimel et al., 2012; Hanes et al., 2015), disease-specific antigen mimics (Liu and Higgins 2013;Sarkar et al., 2016), cell-specific peptides (Wu et al., 2015), peptides bound to non-protein targets (Bose et al., 2015), or organ-specific peptides (Nemudraya et al., 2016), and development of peptide-mediated drug delivery systems (Wu et al., 2016). Screening for bioactive peptides using phage display technology is therefore a useful technique that could be applied in screening for food-derived bioactive peptides. Bioactive peptides obtained this way can be cloned and overexpressed to increase their quantities. They may also be synthesized using hybrid technology combining solid and liquid syntheses.

THE ROLE OF BIOACTIVE PEPTIDES IN HUMAN HEALTH

Antihypertensive peptides

Synthetic antihypertensive drugs are noted for side effects such as dysgeusia, dizziness, headache, angioedema, and cough (Daliri et al., 2016) and so the search for antihypertensive biopeptides has increased over the years (Table 1). These biopeptides may be safe because they are food derived (Chakrabarti et al., 2014) and are known to have higher tissue affinities than synthetic drugs (Fujita and Yoshikawa, 1999). For these reasons, we fermented whey with several Lactobacillus species and found that *Lactobacillus helvelticus* fermentates had angiotensin 1-converting enzyme (ACE) inhibitory peptides AQSAP, IPAVF, APLRV and

AHKAL which, at least in part, accounted for the inhibitory effect of the fermentate. The presence of AEKTK in *Lactobacillus brevis* fermented whey also contributed to its ACE inhibitory effect (Ahn et al., 2009). Casein derived peptides such as Val-Pro- Pro (VPP) and Ile-Pro-Pro (IPP) have shown positive results in reducing blood pressure in animal models and humans (Fekete et al., 2015). These ACE inhibitory tripeptides were first reported by Nakamura et al. (1995) after they fermented β-casein with *Lactobacillus helveticus* CP790 and *Saccharomyces cerevisiae.* Several other antihypertensive peptides from food have been effective in reducing blood pressure in spontaneous hypertensive rats (SHR). *Kluyveromyces marxianus* fermented lactoferrin contained several antihypertensive peptides including DPYKLRP which reduced systolic blood pressure (SBP) by 27mmHg in SHRs (Garcia-Tejedor et al., 2014) while Asp-Tyr in aqueous extracts (boiled water, filled liquid, and squeezed juice) of bamboo shoots reduced SBP by 18 mmHg (Liu et al., 2013). Also Fitzgerald et al. (2014) hydrolyzed *Palmaria palmata* with papain to obtain IRLIIVLMPILMA which also reduced SBP by 33mmHg. Several mechanisms by which these peptides may reduce blood pressure have been proposed. The peptides may be (1) inhibitors of ACE, a metalloprotease that converts angiotensin-I into vasoactive angiotensin II which binds with receptors on the vascular wall to cause blood vessel contractions (Garcia-Tejedor et al., 2014), (2) inhibitors of renin, an enzyme that hydrolyses the Leu10-Val11 peptide bond of angiotensinogen to produce angiotensin I (Malomo et al., 2015), (3) enhancers of nitric oxide production (Yuan et al., 2012; Majumder et al., 2013) and (4) blockers of angiotensin II receptors (Aluko, 2015). Many separation methods usually based on peptide size, charge, and/or hydrophobicity are applied in purifying the peptides from the hydrolysates. Active fractions can also be separated by chromatography and their amino

acid sequences determined by mass spectrometry. Since a large database of antihypertensive peptides is available at AHTPDB (Kumar et al., 2015), ACEpepDB (http://www.cftri.com/pepdb/) and other platforms, researchers can make informed decisions about the right proteases required for hydrolyzing and releasing antihypertensive peptides when the amino acid sequence of their substrate is known. The integrated approach for antihypertensive peptide search could alleviate the expensive and time consuming empirical approach (Capriotti et al., 2016). Though a large number of antihypertensive peptides have been identified and confirmed to be active in animal studies, more human intervention studies are required to confirm their health effects in humans.

Opioid peptides

Opioid peptides function as neuro-hormones and neurotransmitters involved in stress reactions, nociception control, sedation, breathing tone, depression, hypotension, appetite, gastrointestinal digestion and other physiological actions. Many opioid peptides are formed during digestion of food proteins in the gut and the peptides modulate several physiological functions. In view of this, Fukudome et al. (1992) isolated four opiod peptides: Gly-Tyr-Tyr-Pro-Thr (exorphins A5), Gly-Tyr-Tyr-Pro (exorphins A4), Tyr-Gly-Gly-Trp-Leu (exorphins B5), Tyr-Gly-Gly-Trp (exorphins B4) and Tyr-Pro-Ile-Ser-Leu (exorphins C) after pepsin, trypsin and chymotrypsin digestion. Both administration of Gly-Tyr-Tyr-Pro-Thr and Tyr-Gly-Gly-Trp-Leu were found to modulate pancreatic endocrine functions (Fukudome and Yoshikawa, 1993; Fukudome et al., 1995). Other peptides such as β-casomorphins from milk interact with opiate receptors to influence food absorption, electrolyte balance and insulin secretion (Jahan-Mihan et al., 2011).

Enkephalins, endorphins, dynorphins, and endomorphins are endogenous opioid peptides found in peripheral tissues and in the central nervous system and they are involved in producing analgesia and euphoria (Bodnar, 2014). Leu-enkephalins preferably bind to δ_1, and δ_2 subtypes of δ-opioid receptors while β-endorphin preferably binds to the μ_1, μ_2 and μ_3 subtypes of μ-opioid receptors (Ninkovic and Roy, 2013). Dynorphins A and B as well as Neoendorphins bind to κ_1, κ_2 and κ_3 subtypes of K-opioid receptors (Oka et al., 1982; O'Connor et al., 2015). Though opioid peptides have shown promise in many studies, their possibility to cause dependence and addiction need to be investigated. More studies are also needed to establish the potential safety concerns that may prevent the use of opioids for pain management.

Antioxidative peptide

The ability of antioxidant peptides to mitigate pathological effects caused by free radical-mediated lipid oxidation and oxidative stress has been studied. Free radicals may attack membrane proteins, lipids and DNA to result in diseases such as cancer, inflammatory diseases, diabetes mellitus and neurodegenerative diseases (Lobo et al., 2010). The potential risk associated with synthetic antioxidants such as their potential carcinogenic effects in the kidneys, liver and lungs (Taghvaei and Jafari, 2015) has intensified the search for bioactive antioxidative peptides. Several antioxidant peptides from food have been identified. Recently, Agrawal et al. (2016) isolated a new antioxidative peptide Ser-Asp-Arg-Asp-Leu-Leu-Gly-Pro-Asp-Glu-Glu-Gln-Tyr-Leu-Pro-Lys after hydrolyzing *Pennisetum glaucum* proteins with digestive enzymes. In another study, hydrolysis of hoki frame protein yielded Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn which has a strong antioxidant activity (Kim et al., 2007). Liu

et al. (2015) also isolated Asp-His-Thr-Lys-Glu, Phe-Phe-Glu-Phe-His and Met-Pro-Asp-Ala-His-Leu which have strong oxygen radical-scavenging activity. Bioactive peptides Ala-Glu-Glu-Arg-Tyr-Pro and Asp-Glu-Asp-Thr-Gln-Ala-Met-Pro isolated from hydrolyzed chicken egg white showed strong oxygen radical absorbance capacities (Nimalaratne et al., 2015) while Asp-Cys-Gly-Tyr and Asn-Tyr-Asp-Glu-Tyr showed strong hydroxyl radical scavenging activities (Fan et al., 2012). Meanwhile, *Hylarana guentheri* protein hydrolysates yielded two dipeptides, Leu/Ile-Lys and Phe-Lys, with strong oxygen radical absorbance capacities (Gu et al., 2014). Subtilisin hydrolyzed *Crassostrea talienwhanensis* also released two peptides Pro-Val-Met-Gly-Asp and Gln-His-Gly-Val with strong antioxidant activities (Wang et al., 2014). Though antioxidative peptides have shown free radical scavenging activities in many *in vitro* studies, further research on the undesirable side reactions, safety and allergenicity is needed to establish their therapeutic use. Also, *in vitro* studies are required to establish how these antioxidants may have therapeutic effects in humans.

Antimicrobial peptides (AMP)

Antimicrobial peptides are oligopeptides with a broad spectrum of targeted microorganisms. AMPs may be membrane-active or intracellular active. Membrane-active AMPs are thought to attach to the membrane lipids of target organisms to form trans-membrane pores. On the other hand, intracellular active AMPs interact with intracellular targets such as DNA, RNA and proteins leading to cell death (Xiao et al., 2015). Several eukaryotic cells including lymph, phagocytes, gastrointestinal and genitourinary epithelial cells have the ability to produce antimicrobial proteins such as defensin (Bahar and Ren, 2013). Antimicrobial peptides may have

different target organisms. For example, neutrophil antibiotic peptide NP-1 isolated from rabbit neutrophils inhibits the migration of herpes simplex virus type 2 protein VP16 which plays a role in cell-to-cell spread of viral particles (Sinha et al., 2003). Magainin 2 isolated from frog skin is effective against many Gram negative and Gram positive bacteria (Strandberg et al., 2013). Dermaseptin S4 isolated from amphibian skin also strongly inhibits many bacteria, yeast, filamentous fungi, herpes simplex virus type 1, and HIV-1 infections (Lorin et al., 2005). Hydrolyzing casein with chymosin released caseicidin peptide which inhibited *Streptococcus pyogenes, Sarcina* spp., *Bacillus subtilis* and *Staphylococcus* spp. (Lahov and Regelson, 1996). Lactoferrampin from lactoferrin hydrolysis also strongly inhibits *E. coli, Streptococcus mutans, Pseudomonas aeruginosa* and *B. subtilis* (van der Kraan et al., 2005). Caseinomacropeptide obtained from casein hydrolysates effectively inhibits *S. mutans* and *E. coli* while Isfracidin and lactoferricin B inhibit *Candida albicans* (Mohanty et al., 2015). Other antifungal biopeptides recovered from the gut, epididymis and lungs include SMAP-29, BMAP-27, BMAP-28, protegrin-1 and indolicidin which strongly permeate and destroy *Candida albicans* and *Cryptococcus neoformans* cell membranes resulting in microbial cell death (Benincasa et al., 2006). Antimicrobial activity of two peptides casecidin 15 and 17, found naturally in bovine colostrum has also been reported (Birkemo et al., 2009). Also, soy peptides PGTAVFK and IKAFKEATKVDKVVVLWTA effectively inhibit the growth of *Listeria monocytogenes* (Dhayakaran et al., 2016). Though AMPs have shown good potentials in many studies, their pharmacokinetics are still not well understood (Xiao et al., 2015). This calls for more studies into the fate of AMPs *in vivo*.

Immunomodulatory peptides

Many immunomodulatory peptides have been identified in milk, soybeans, honey, etc. Recently, Mesaik et al. (2015) isolated honey glycopeptides that suppressed the production of TNF-α in human monocytic cell lines. Several peptides and protein hydrolysates in milk have been identified to be immunomodulatory. Short peptides (<5 kDa) isolated from whey enhanced *in vitro* proliferation of lymphocytes isolated from murine spleen (Jang et al., 2008). Trypsin and pepsin hydrolyzed αs1--CN peptides suppressed the proliferation of lymphocytes while β- and κ-CN derived peptides increased the proliferation of lymphocytes in human blood (Sütas et al., 1996). Hou et al. (2012) obtained the peptides Asn-Gly-Met-Thr-Tyr, Asn-Gly-Leu-Ala-Pro and Trp-Thr after hydrolyzing *Alaskan pollock* (a fish). The peptides stimulated lymphocyte proliferation in mice spleen cells. Val-Glu-Pro-Ile-Pro-Tyr from human β-casein was found to stimulate the phagocytosis of opsonized sheep red blood cell by murine peritoneal macrophages. Intravenous administration of the peptide to adult mice enhanced resistance to *Klebsiella pneumoniae* infection (Parker et al., 1984). Immunomodulatory peptides in fermented milk have been found to inhibit the proliferation of human breast cancer cells (Chen et al., 2007) and a similar reason may account for the ability of regular milk consumption to lower the risk of colorectal cancer (Buckland et al., 2010; Makino et al., 2010; Pala et al., 2011). Also, gastrointestinal-resistant peptides (<10kD) from rice bran hydrolysates have been shown to strongly inhibit the proliferation of Caco-2 and HepG2 cells relative to control treatments (Kannan et al., 2008). Recently, Rayaprolu et al. (2017) isolated an 18 kDa (158 amino acid residues) peptide after hydrolyzing soybean protein with Alcalase®, pepsin and pancreatin. The peptide showed a strong inhibition against human colon cancer cell proliferation when the cells were treated with 700µg/ml of the peptide.

A challenge in the development of immunomodulatory peptides as therapeutics has been the mechanism by which they stimulate the immune system. Meanwhile, some bioactive peptides have been proposed to stimulate P2X (7) receptors in embryonic kidney cells to transactivate epidermal growth factor receptor in epithelial cells. The peptides may also interact with formyl peptide receptor-like 1 in many cell types and enhance TLR3 signaling in response to viral dsRNA (Haney and Hancock, 2013). More studies are however required to understand the mechanism of action of bioactive immunomodulatory peptides. Other important bioactive peptides are shown in table 1.

LARGE-SCALE BIOACTIVE PEPTIDE PRODUCTION

Purification of bioactive peptides from enzymatic digestion or microbial fermentation is always laborious and time consuming. Also, both fermentation and enzymatic digestion methods do not guarantee high quantities of specific bioactive peptides and are hardly reproducible. Therefore, several studies have focused on producing bioactive peptides by cloning them in microbial hosts or by modern chemical synthesis. The most suitable technology to be used largely depends on the size of the desired peptide (Uhlig et al., 2014).

Chemical synthesis

The main chemical methods for peptide synthesis are solution phase synthesis (SPS) and solid phase peptide synthesis (SPPS). SPS is usually carried out by coupling single amino acids in solution. Synthesis of long peptides is possible by first synthesizing short fragments of the desired peptides and condensing them to yield long peptides (Chandrudu et al., 2013). This method of SPS is called the fragment condensation method. In SPS, intermediate products can be

deprotected and purified to obtain a high purity of the desired peptide (Nishiuchi et al., 1998; Carpino et al., 2003). Peptides including Aequoria green fluorescent protein, human pleiotrophin (Sakakibara, 1999), melanotan II (Ryakhovsky et al., 2008), oxytocin (Vigneaud et al., 1953), human insulin, adrenocorticotropic hormone, desmopressin, leuprolide, goserelin, and octreotide (Andersson et al., 2000) have been produced by SPS. SPS is inexpensive and can easily be scaled up however, the long reaction time remains a drawback.

On the other hand, the SPPS method involves the synthesis of a peptide using resin as a support for the growing peptide chain. The reactive side chain and the alpha amino group of an amino acid are first protected (mostly by using Fmoc or Boc) and the C-terminus of the amino acid is attached to the resin (Stawikowski and Fields, 2012). The protection group is removed usually by using trifluoroacetic acid or 20% piperidine in dimethylformamide after which the resin is washed before subsequent amino acids are added. After the required sequence is completed, the peptide is cleaved from the resin (Chandrudu et al., 2013). Currently, SPPS based on Fmoc chemistry is commonly used for therapeutic peptide synthesis due to its cheaper cost of production as well as advancements in chromatographic equipment (Chandrudu et al., 2013).

Long peptide or protein chains can also be synthesized using chemical ligation techniques. The native chemical ligation (NCL) is an optimized peptide ligation method. To ligate peptide fragments, an unprotected peptide segment possessing an N-terminal cysteine is reacted with another unprotected peptide-α-thioester to produce a thioester-linked intermediate which is later transformed into a peptide bond. This method makes it possible for the synthesis of high molecular weight peptides such as the multivalent peptide-based nonsymmetrical dendrimer

(Dirksen et al., 2006) and collagen-like polymers (Lovrinovic and Niemeyer, 2007). The high stability of the starting materials in NCL, the well-established chemical methods for producing peptide thioesters and the high chemoselective nature of the peptides are advantages of this method.

Genetic cloning of bioactive peptides

Renye and Somkuti (2008) were first to describe the cloning and expression of synthetic genes of milk derived bioactive proteins in lactic acid bacteria. They successfully engineered *S. thermophiles* to produce an antimicrobial peptide RRWQWRMKKLG and an ACE inhibitor FFVAPFPEVFGK. In a similar way, Yang et al. (2015) transformed probiotic *L. plantarum* NC8 with antihypertensive peptides YFP and TFP from *Limanda aspera*. The recombinant probiotics reduced and maintained a low blood pressure (relative to controls) in spontaneous hypertensive rats even after ten days of administration. The HNH motif from colicin E7 with known Zinc-binding ability has also been cloned and expressed in *E. coli* (Gyurcsik et al., 2013) to improve its yield. Several immunomodulatory peptides have been successfully cloned and expressed in various hosts including bacteria and insects. For instance, immunomodulatory protein p36 was cloned and expressed in both bacteria and insect cells and actively suppressed T-lymphocyte-mitogen-driven production in mice splenocytes (Alarcon-Chaidez et al., 2003). Lin et al. (2009) have also cloned and overexpressed immunomodulatory proteins from *Ganoderma lucidum* in *Pichia pastoris*. The recombinant proteins were found to induce human leukemia-NB4 apoptosis and also stimulated mouse spleen lymphocytes proliferation while enhancing the expression levels of interleukin-2 in mice splenocytes. Recently, Pushparajah et al. (2016) isolated and

characterized an immunomodulatory protein from *Lignosus rhinocerotis* (Tiger milk mushroom). The proteins were cloned and expressed in BL21 cells to yield recombinant proteins which effectively killed mcf-7, HeLa and A549 cancer cell lines. Human Cystatin C is known for its anti-inflammatory (Frendéus et al., 2009), anti-viral (Björck et al., 1990) and anti-bacterial (Blankenvoorde et al., 1998) functions however, low yields of the protein have been reported after expression and purification (Zhang et al., 2014). Chauhan et al. (2016) therefore applied a strategy in which different chaperones were co-expressed with cystatin C so that overexpression of the chaperons resulted in high expression of a fully functional protein in the soluble fraction. Cloning and expression in *E. coli* yielded 2mg/L of the fully functional recombinant proteins which inhibited H3 protease and cathepsin L enzyme activities. Several appetite regulatory peptides have also been cloned. Volkoff and Wyatt (2009) cloned apelin, an orexigenic factor from *Carassius auratus.* Intraperitoneal injection of the peptide caused a significant food intake in gold fish. However, Drougard et al. (2016) have recently reported that central apelin infusion could contribute to the development of type 2 diabetes in humans since it alters energy expenditure and thermogenesis. Nesfatin-1, (an orexigenic inhibitor) plays an important role in regulating energy homeostasis related with food and water intake. Oh et al. (2006) successfully cloned and expressed nesfatin-1 in *E coli* and the peptide significantly reduced food intake in rats. The peptide has been reported to enhance satiety and suppress weight gain in animal models (Shimizu et al., 2009; Oh et al., 2006) making nesfatin-1 a potential anti-obesity drug. In 2015 and 2016, the United States Food and Drugs board approved seven new recombinant therapeutic peptides to be sold on the market (Table 2).

CHALLENGES TO COMMERCIALIZATION

Therapeutic peptides are purified peptide-based drugs meant for pharmacological treatments while hydrolyzed food proteins (fermented and enzyme treated) can serve as functional foods for nonpharmacological treatments. In both cases, several challenges need to be addressed before the products can be commercialized.

Functional foods

Though certain functional food processing factors and conditions may result in protein degradation, many studies do not consider the effects of processing on the anticipated peptide profiles nor do they consider the possibility of a change in the bioactivity of the peptides after hydrolysis. Processing conditions such as temperature and the duration of fermentation or substrate hydrolysis may result in the production of non-reproducible peptide profiles especially when the substrate contains a mixture of proteins (Lacroix and Li-Chan, 2012). However, the processing methods that may be detrimental to some peptides may improve the activity of other bioactive peptides. For instance, the antibacterial activity of α-lactalbumin (Agyei et al., 2016) and lysozyme (Takahashi et al., 2016) increased after they were denatured by heat. Therefore, the optimum conditions within which functional foods could be processed to provide maximum health effects must be investigated and applied so as to develop foods with specific health effects.

The extent of protein hydrolysis during the production of bioactive peptides in foods is critical in functional food production. However, kinetic modelling of protein hydrolysis using enzymes or by microbial fermentation is very challenging since peptide products serve as substrates for further hydrolysis. The multiple sequential hydrolysis may result in peptides with reduced or lost

activities due to degradation as has been observed in some studies (Naqash and Nazeer, 2013; Agyei et al., 2016).

Generally, food-derived bioactive peptides are not single peptides of high purity and this is partly due to the associated high cost and low yield that would be involved (Lemes et al., 2016). However, after purification, single peptide entities may lose their potential additive or synergistic effects with other food components such as polyphenols or other peptides and this may reduce their potency when used as nutraceuticals.

A potential risk of bioactive peptides is that, they could react with other food components such as carbohydrates or lipids to form toxic, allergenic or carcinogenic substances in the food mix. The peptides may even be involved in unwanted side reactions to cause unintended physiological effects when consumed. More *in vivo* studies on the potency and the safety of biopeptides are therefore required before they are commercialized since *in vitro* results do not always reflect *in vivo* results.

One other drawback in food-derived bioactive peptide research has been that, results from some human interventional studies on many bioactive peptides have been inconsistent. For instance, the antihypertensive ability of IPP and VPP has yielded controversial results in humans (Fekete et al., 2015) as the lacto-tripeptides were effective in reducing blood pressure in Japanese but not in Europeans. There is therefore the need to establish why some peptides may be active in one group (ethnic groups, gender, age, etc) but not in others. There is also the need for large scale clinical investigations for ascertaining the effects of bioactive peptides since small changes in health effects (such as blood pressure) may be not be significant in studies involving small

populations. Proteomics and other technologies will also be needed to study the impacts of bioactive peptides on gene expression as well as the health effects of these compounds.

The sensory properties of bioactive peptides in foods also affect their acceptance by consumers. For example, the bitter taste of some bioactive peptides makes some functional foods less acceptable by consumers. It is therefore important that sensomics mapping be included in bioactive peptide research to curb this problem.

Therapeutic peptides

It is challenging to test the bioavailability of therapeutic peptides in human blood after oral ingestion, due to their short half-life (generally less than 2 hours) and their low maximal plasma concentration (Iwai et al., 2005; Ichikawa et al., 2010). Therefore, preliminary pharmacokinetic studies are required before human interventional studies are conducted. Also, more novel strategies must be designed to enhance the stability, increase the bioavailability and enhance the effective delivery of bioactive peptides that have shown potent in *in-vitro* studies. Peptides can be protected against enzymatic cleavage by inserting a structure inducing probe tail (Kaspar and Reichert, 2013), lactam bridges (Houston et al., 1996), and by stapling or cliping peptide sequences (Timmerman et al., 2007), or by cyclization (Sim et al., 2012). The half-life of the peptides could be prolonged using strategies such as peptide acylation (Knudsen, 2010), insertion of albumin-binding peptide elements in the peptide backbone, or conjugation to albumin-binding antibody fragments (Bao et al., 2013).

The nature of the most desired peptide product remains puzzling. The preferred therapeutic form would probably be a capsule or tablet containing the active peptide that is stable at room

temperature at affordable price. While an oral formulation may not always be possible, other non-parenteral or alternative delivery platforms such as nasal, pulmonary, intra or transdermal, and topical administration as well as implantable devices may help to achieve a suitable drug form with high efficacy and satisfactory compliance. It may be challenging to reach that goal, yet an evaluation of the therapeutic peptide and its subsequent market at the start of its development can help achieve this.

CONCLUSIONS AND FUTURE PERSPECTIVES

Bioactive peptides hold great promise as valuable functional ingredients in healthy diets to fight the global epidemic of non-communicable diseases. More than 60 therapeutic peptides have been approved for the market while hundreds of novel bioactive peptides are in the clinical pipeline (Kaspar and Reichert, 2013; Fosgerau and Hoffmann, 2015). This high number of approved peptide therapeutics is likely to draw even much attention to the research and development of bioactive peptide (Kaspar and Reichert, 2013). The success can be attributed to the specificity, potency and yet safety of the peptides. Microbial fermentation will remain a promising strategy for generating a wide range of bioactive peptides in foods as microbial proteolytic systems (especially lactic acid bacteria) yield numerous peptides of diverse potentials during fermentation. This therefore calls for genomic and proteomic characterization of newly identified strains to predict their proteolytic profiles for their use in functional food development. However, bioinformatics coupled with appropriate high-throughput peptide display technologies will also remain important tools in the search for bioactive peptide. In the coming years, the application of medicinal peptide chemistry in a modular fashion coupled with cost-effective scalable

technologies would result in the construction of highly efficacious multifunctional therapeutic peptides with enhanced pharmacokinetic properties, improved targeted delivery and reduced cost.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Table 1. Biologically active peptides and their functions.

Peptide	Function	Reference
Anti-cancer		
DTP3 (a D-tetrapeptide)	Interacts with MKK7 to activate JNK signaling and induce cancer-cell--specific apoptosis in a dose dependent manner.	(Tornatore et al., 2014)
P6-LR(LSCQLYQR)	Allosterically stabilizes the inactive form of thymidylate synthase (TS) and inhibits ovarian cancer cell growth with stable TS and decreased dihydrofolate reductase expression.	(Cardinale et al., 2011)
WK_{18}LLL-ve and WK_{18}LLnV-ve	Inhibits the chymotrypsin-like activity of proteasomes	(Martin and Rice, 2007)
Cbz-Leu_4-SF, N_3Phe-Leu-Leu-Leu-ψ-[CH_2SO_2]-F, Ac-Ile^1-Ile^2-Thr-Leu-ψ[CH_2SO_2]-F, N_3CH_2C(O)-Ile-Ile-Thr-Leu-ψ-[CH_2SO_2]-F	Inhibits chymotrypsin-like activity of proteasomes and shows β5 subunit selectivity.	(Brouwer et al., 2012)
Anti-obesity		
DIVDKIEI	Reduces total adiponectin and	(Kim et al., 2015)

	high-molecular weight adiponectin levels.	
Galanin-like peptide	Suppresses energy intake and promotes energy expenditure.	(Kageyama et al., 2016)
Cholesterol-lowering		
PGPL	Interacts with receptors of blood or brain cells, and mediate blood cholesterol reduction through a series of reactions.	(Miasoedov et al., 2012)
Lupin peptides	Interfere with the HMGCoAR activity, up-regulate LDL and SREBP-2 proteins via the activation of PI3K/ Akt/ GSK3β pathways and increase LDL uptake.	(Lammi et al., 2014)
DE	Binds cholesterol in the gut and enhance bile acid uptake resulting in cholesterol reduction.	(Fatma and Wahyu, 2013)
5A-DWLKAFYDKVAEKLKEAFPDWAKAAYD-KAAEKAKEAA	Increases cholesterol efflux from macrophages and other peripheral tissues	(Amar et al., 2010)

A = alanine, R = arginine, N = asparagine, D = aspartic acid, C = cysteine, E = glutamic acid, Q = glutamine, G = glycine, H = histidine, I = isoleucine, L = leucine, K = lysine, M = methionine, F = phenylalanine, P = proline, S = serine, T = threonine, W = tryptophan, Y = tyrosine, V = valine.

JNK: c-Jun N-terminal kinase; MKK7: MAP kinase kinase 7; LDL: Low-density lipoprotein

Table 2. 2015 and 2016 FDA APPROVED RECOMBINANT THRAPEUTIC PEPTIDES (USFDA 2016 Biological License Application Approvals).

Trade name	Indication for Use	Manufacturer	Approval Date
AFSTYLA; Solchayn Antihemophilic Factor (Recombinant), Single Chain	Antihemophilic Factor, Single chain is indicated in children and adults with hemophilia A (congenital Factor VIII deficiency) for: (1) on-demand treatment and control of bleeding episodes, (2) routine prophylaxis to reduce the frequency of bleeding episodes, and (3) perioperative management of bleeding.	CSL Behring Recombinant Facility AG, Switzerland	05-25-2016
KOVALTRY Antihemophilic Factor (Recombinant), Full Length	Antihemophilic Factor is indicated for use in adults and children with hemophilia A (congenital Factor VIII deficiency) for: (1) on-demand treatment and control of	Bayer HealthCare LLC	3-16-2016

Trade name	Indication for Use	Manufacturer	Approval Date
	bleeding episodes; (2) perioperative management of bleeding; and (3) routine prophylaxis to reduce the frequency of bleeding episodes.		
IDELVION Coagulation Factor IX (Recombinant), Albumin Fusion Protein	Coagulation Factor IX, Albumin Fusion Protein is indicated for (1) control and prevention of bleeding in the perioperative setting, (2) control and prevention of bleeding episodes, and (3) routine prophylaxis to prevent or reduce the frequency of bleeding episodes.	CSL Behring Recombinant Facility AG, Switzerland	3-4-2016
VONVENDI von Willebrand factor (Recombinant)	von Willebrand factor is indicated for on-demand treatment and control of bleeding episodes in adults	Baxalta US Inc., USA	12-8-2015

Trade name	Indication for Use	Manufacturer	Approval Date
	diagnosed with von Willebrand disease (VWD).		
ADYNOVATE Antihemophilic Factor (Recombinant), PEGylated	Indicated for adolescent (12 to less than 18 years) and adult (≥18 years) patients with hemophilia A (congenital factor VIII deficiency) for: control and prevention of bleeding episodes and routine prophylaxis to prevent or reduce the frequency of bleeding episodes.	Baxalta US Inc., USA	11-13-2015
Nuwiq Antihemophilic Factor (Recombinant), rAHF	Indicated in adults and children with Hemophilia A for: • On-demand treatment and control of bleeding episodes • Perioperative management of bleeding	Octapharma Pharmazeutika Produktionsges.m.b.H.,Austria	9-4-2015

Trade name	Indication for Use	Manufacturer	Approval Date
	• Routine prophylaxis to prevent or reduce the frequency of bleeding episodes.		
IXINITY Coagulation Factor IX (Recombinant)	Control and prevention of bleeding episodes and peri-operative management in patients with hemophilia B.	Cangene Corporation, Canada.	4-29-2015

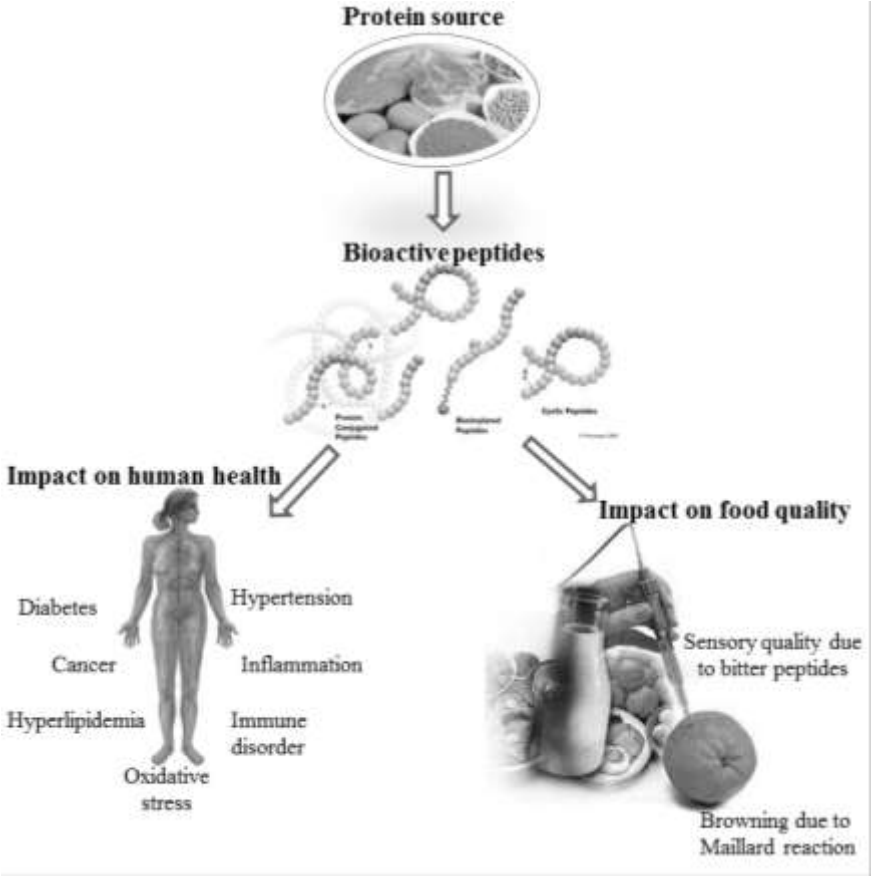

Figure 1. Bioactive peptides from food sources and their health effects.

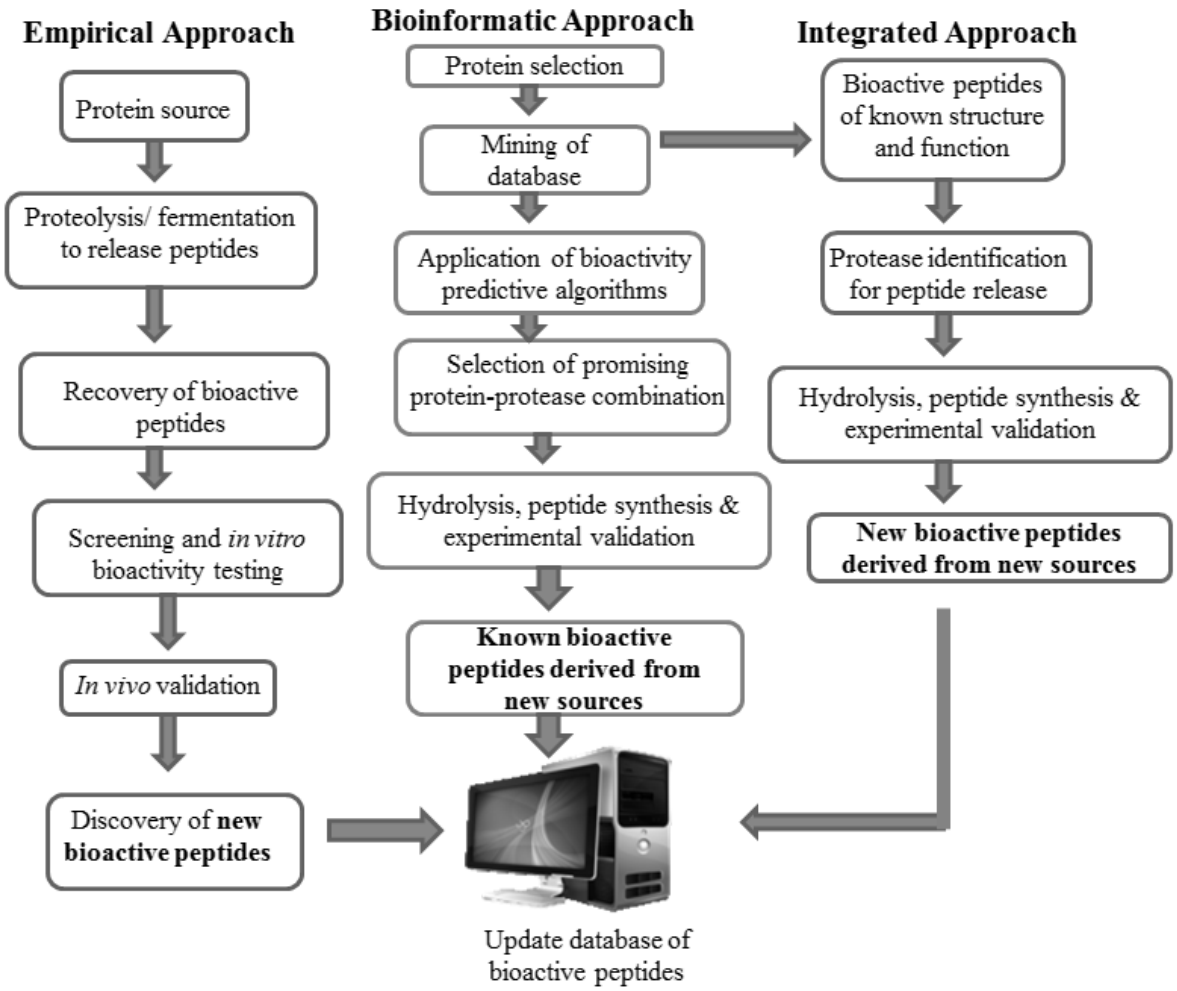

Figure 2. Methods for discovering bioactive peptides