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Review

Bioprocess challenges to the isolation and purification of bioactive peptides

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ABSTRACT

Food protein-derived bioactive peptides (BPs) have been reported to trigger certain physiological responses in the body, thereby influencing health positively. These peptides have attracted high research and consumer interests due to their huge potential of use in functional foods and other dietary interventions of disease control and health promotion. However, successful product development is limited by the fact that current manufacturing processes are either difficult to scale up, high in cost, or have the potential to affect the structure-activity properties of these peptides. To overcome these challenges, we have proposed in this review, the use of an integrated '-omics' approach comprising *in silico* analysis and '-omics' techniques (such as peptidomics) to respectively forecast and validate the biological and physico-chemical properties of the peptides. This information is then used for the rational design of suitable purification steps for peptides of interest. Downstream purification could also be undertaken by liquid chromatography using monolithic adsorbents physico-chemically engineered (using results of *in silico* analysis) for rapid isolation of peptides. By coupling the high throughput and predictive capability of '-omics' to the enhanced convective hydrodynamics of monolithic columns, it becomes feasible, even at preparative scale, to produce BPs that meet the requirements of high purity, potency, and cost-effectiveness.

Contents

Abbreviations: ACE, angiotensin-I-converting enzyme; DH, degree of hydrolysis; EMF, electromembrane filtration; MC, membrane chromatography; QSAR, quantitative structure–activity relationship; QSPR, quantitative structure–property relationship; HILIC, hydrophilic interaction liquid chromatography.

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1. Introduction

Over the years, it has become a known fact that health and nutrition are intricately linked. Not only do food nutrients supply the necessary biomolecules for various metabolic activities, but, in some cases, food nutrients are able to trigger certain desirable physiological responses in the body. Food proteins and hydrolysates thereof are amongst the most well studied bioactive molecules (Danquah and Agyei, 2012). Bioactive peptides have been defined as protein hydrolysates which, upon entry and absorption into the body, have the ability to induce certain desirable and physiologically measurable 'hormone-like' activities (Korhonen and Pihlanto, 2006). Some biological functions induced by these peptides include immunomodulatory, cytomodulatory, opiate, antihypertensive, antimicrobial, antithrombotic and metal-chelation activities (Möller et al., 2008). As natural products of food origin, bioactive peptides have a huge potential in health-promoting functional foods and therapeutic products (Korhonen, 2009). However, this potential is not being realized as a result of certain bioprocess challenges. The lack of commercially-viable processes for large-scale production of bioactive peptide has been a major hindrance to the percolation of bioactive peptides in marketable products (Agyei and Danquah, 2011). Further, basic research is lacking on the optimization of purification techniques tailored specifically for producing bioactive peptides at industrial scale (Korhonen and Marnila, 2013). The goal of peptide purification is not merely to achieve products of high purity and high recovery levels, but also, the purification technique must be economically viable (Desai et al., 2000). These challenges need to be overcome *via* the development of innovative, high throughput, and cost-effective platforms for the bioprocessing bioactive peptide. This review gives an overview on bioactive peptides, their production and functionalities; and also discusses the challenges encountered during the downstream purification of these peptides. A cascade of processing techniques that could offer some advantages over existing ones has also been proposed. Specifically, we propose (a) the use of '-omics' techniques (*e.g.* foodomics) and high throughput *in silico* simulation models to predict the properties of peptides that can be generated from a protein of interest. This prior knowledge aids the determination of suitable purification techniques that can be employed for specific peptides of interest. In addition, we propose (b) liquid chromatography using monolithic adsorbents specially designed for small sized and charged molecules (such as bioactive peptides). Some challenges encountered with the use of these proposed techniques and possible ways of overcoming them have also been presented.

2. Bioactive peptides: an overview

2.1. *Production of bioactive peptide*

Bioactive peptides are often inactive in the parent protein molecules and are released by processes such as (a) microbial fermentation of proteins by proteolytic microbes, (b) proteolysis by enzymes from plants or microorganisms, and (c) proteolysis by gastrointestinal enzymes. These production routes have been captured in Fig. 1. Each of these production alternatives has its own merits and demerits. For example, processes (a) and (b) are the only ones which can be pursued for the purposes of industrial scale production. Putatively, the production of bioactive peptide *via* (c) is to be expected, however, the amounts produced through such uncontrolled means may not be sufficient to induce the needed biological effect, especially in adult humans (Gauthier et al., 2006). Pathway (a) has been widely used in the past decades, especially for releasing bioactive peptides from dairy products (Korhonen and Pihlanto, 2006). In fact, there are several commercial products of the market that rely on this production method (Korhonen and Marnila, 2013). However, because fermented products usually contain other biomolecules such as live and dead bacteria cells, exopolysaccharides, and/or bacteriocins – all of which could exert some form of biological functions – it is inconclusive to assume that observed bioactivities is due to peptides released during fermentation (Martínez-Augustin et al., 2014).

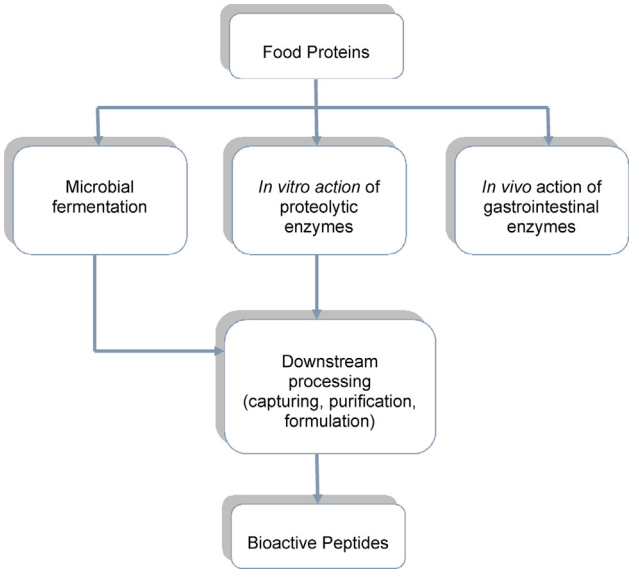

Fig. 1 – Production of bioactive peptides.
***Source*: Adapted from Danquah and Agyei (2012).**

2.2. *Bioactivities and applications*

As a result of the physiological activities they trigger, bioactive peptides have huge potential applications in the major

Table 1 – Some application of bioactive peptides in the major consumer industries.

Industry	Functional or bioactive property	Possible area of application
Food	Antimicrobial	As natural food preservatives for maintaining food safety and quality
	Antioxidant	
	Emulsifying	As food emulsifiers
	Antihypertensive	Marketing of food based on health clams or function
	Immunomodulatory	
Functional foods and nutraceuticals	Antihypertensive	Food and drug fortification
	Immunostimulatory and immunomodulatory	Immunonutrition
Pharmaceutics and health	Antithrombotic; opiate	Manufacture of peptide based therapeutic products
	Antihypertensive; cancer cell toxicity	Manufacture of peptide based therapeutic products
	Anxiolytic	As antipanic or antianxiety products
	Antimicrobial	As natural preservatives in pharmaceutical products
	Anorexic	For use in weight and satiety control products
	Antidiabetic	For improved glucose transport
Cosmetics	Antimicrobial	As natural preservatives in cosmetic products
	Emulsifying	Ingredient in cosmetic products
	Various biological activities for improved skin health and functions	Ingredient to combat wrinkles and skin aging; anti-inflammatory ingredients

consumer industries (Table 1). The food industry is one of the key sectors where proteolytic starters and/or gastrointestinal enzymes are used to break whole proteins to bioactive peptides. Several dairy products have begun to be marketed on the grounds of the health or functions claim based on their bioactive peptide composition (Korhonen and Pihlanto, 2006). Bioactive peptides may also be used in the fortification of products marketed as functional food and/or nutraceuticals.

Bioactive peptides have several traits which makes them suitable for use as therapeutic agents. These traits include (a) high biospecificty to targets; (b) high activity and wide spectrum of action, including some individual peptides with multifunctional properties; (c) low toxicity and reduced incidence of accumulation in body tissues, as compared to small molecules; (d) high structural diversity; (e) small size (when compared with antibodies) which is helpful for delivery, and also means these peptides have a low likelihood of triggering undesirable immune responses (Hancock and Sahl, 2006; Marx, 2005; Mason, 2010).

Certain peptides are able to trigger physiological activities that promote skin health, and are therefore used in non-food products such as cosmetics and dermatological products (Lintner and Peschard, 2000; Lupo and Cole, 2007). Some of these functions include modulation of cell and tissue inflammation, stimulation of collagen synthesis, control of angiogenesis and melanogenesis, and modulation of cell proliferation and cell migration (Fields et al., 2009; Lintner and Peschard, 2000). In one clinical study, a synthetic hexapeptide has been identified as a topical alternative to botulinum neurotoxins for controlling skin wrinkles. At 10% concentrations, this hexapeptide was able to reduce wrinkle depths by 30% after 30 days of topical application (Blanes-Mira et al., 2002). Argireline is now marketed as an anti-aging cosmetic product (Ruiz et al., 2007). Another topical peptide-based product is palmitoyl pentapeptide-4 (marketed under the name Matrixyl) which has demonstrated anti-wrinkle efficacy in clinical studies (Robinson et al., 2005). A number of patented dermopharmaceutical products that incorporates peptide ingredients are also already on the market (Lintner, 2000; Zhang et al., 2011).

3. Characteristics of bioactive peptides

As partially hydrolysed protein products, peptides still retain certain physico-chemical properties of proteins, some of which influence the behavior of bioactive properties, as reviewed below.

3.1. *Physico-chemical properties*

Proteins are complex ampholytic molecules, and when hydrolysed they usually results in peptides with completely different properties. Enzyme hydrolysis affects physico-chemical properties such as molecular weight, charge distribution, isoelectric point, acid/base group ionization, and hydrophilicity/hydrophobicity indices of resulting peptides. These primary and secondary structural changes are responsible for the biological properties observed in the resulting peptides. In many cases, the biological properties are attributed to the presence of certain key amino acids with peculiar properties. For example, most short, hydrophobic and cationic peptides are able to exhibit antimicrobial properties (Hancock and Sahl, 2006). Also, the amino acids proline and valine play a significant role in most antihypertensive peptides (Korhonen and Pihlanto, 2006). Hydrophobic properties and sulfhydryl groups imparts antioxidant properties (Park et al., 2010; Ren et al., 2015), whereas glycine and histidine promote immunomodulatory and antioxidant properties respectively in peptides (Pihlanto-Leppälä, 2002). For example, in a recent study, the action of cell envelope proteinase (CEP) from *Lactobacillus acidophilus* JQ-1 on β-casein resulted in the production of hydrolysates which had higher thermal stability (observed from differential scanning calorimetry) and an entirely different chemical composition when compared with hydrolysed proteins. The hydrolysates were rich in free sulfhydryl groups and possessed antioxidant and angiotensin-I-converting enzyme (ACE) inhibitory activities (Ren et al., 2015).

3.2. *Biological properties*

The biological properties of bioactive peptides are largely influenced by the type, number, sequence and properties of amino acids present in that peptide (Danquah and Agyei, 2012;

Kitts and Weiler, 2003). Whereas the primary sequence affects the structure and biological function (Korhonen and Pihlanto, 2006), the actual systemic binding characteristics of bioactive peptides have been attributed largely to the secondary structure (Kaur et al., 2007). In certain instances, some peptides, classified as multifunctional peptides, are able to induce more than one physiological activity under two key conditions, (a) if they have a unique primary structure able to resist proteolysis, and/or (b) if they consist of one or more overlapping sequences each of which trigger different biological responses (Meisel, 1998; Rutherfurd-Markwick and Moughan, 2005). Examples of food derived multifunctional proteins and peptides include lactoferrin, lactoferricin (Brock, 2002; Théolier et al., 2014); casein phosphopeptides (Arunachalam et al., 2012; Fan et al., 2013); hydrolysates of phosvitin from egg yolk (Xu et al., 2007); rapakinin from rapeseed proteins (Yamada et al., 2011); rubiscolin-6, rubimetide (Zhao et al., 2008); and soymorphins-5, -6, and -7 (Yamada et al., 2012). These constitute a new class of potent biologically active materials which can be exploited for food, pharmaceutical and cosmetic applications.

4. Protein processing methods that affect resulting peptide bioactivities

Considering their potential roles in human nutrition and health, it is imperative that production techniques for bioactive peptides must not compromise the biological activities. Research has however shown that certain processing methods pursued on proteins affect the potency of bioactive peptides released from those proteins after enzymatic hydrolysis.

4.1. *Degree of hydrolysis*

The kinetics of protein hydrolysis by enzymes is a complex process since there is always the possibility of sequential hydrolysis of peptides obtained from the parent protein molecule (Levison, 2003). Peptide products become reactant for further hydrolysis and the reaction can proceed until conditions such as (a) the production of peptides whose primary sequence differs from the biospecificity of the enzymes, (b) the production of species with resistant peptide bonds, and (c) loss of enzyme activity. These multiple sequential reactions make it difficult to model protein hydrolysis *via* simple kinetic models. The concept of 'degree of hydrolysis' (DH) is therefore used to characterize protein hydrolysis and it is defined as the proportion of cleaved peptide bonds in a protein hydrolysate (Levison, 2003; Rutherfurd, 2010). The end use of the proteolytic process will determine the allowable DH. For example, during protein waste treatment, higher levels of proteolysis (*i.e.* higher DH values) is desirable. However, in the food industry, the functional and bioactive properties of protein hydrolysates have been shown to be affected by high DH (Bandilla and Skinner, 2003; Vlakh and Tennikova, 2009). Limited protein hydrolysis (DH < 10%) often give hydrolysates that differ in functional properties such as foaming and emulsion capacity; whereas extensive hydrolysis is needed to obtain bioactive peptides for medical and dietary applications (Agyei et al., 2014). Over-hydrolysis however, may cause enzymatic degradation of the bioactive peptides resulting in loss of the bioactive properties. This has been observed in a number of studies where the biological activities of protein hydrolysates were lost when DH exceeded an optimum (Klompong et al., 2007; Wu et al., 2008).

4.2. *Processing method*

Processing methods have been shown to affect activity of certain proteins with biological properties (Korhonen et al., 1998). For example, the spectrum of antibacterial action of lysozyme and α-lactalbumin were seen to have improved after the two proteins were denatured by heat (Expósitoa and Recio, 2006; Ibrahim et al., 1996).

As observed in proteins, certain physical processing methods also affect the biological activities of peptides. For example, recently, Montoya-Rodríguez et al. (2014) reported that the physical process of extrusion improved the bioactive properties of amaranthus hydrolysates. In their study, extruded and unprocessed amaranth flour were subjected to *in vitro* simulated gastrointestinal digestion by pepsin and pancreatin. The resulting hydrolysates were screened for anti-inflammatory properties on lipopolysaccharide induced inflammations in human acute monocytic leukemia cell lines and mouse macrophage cell lines. The hydrolysates inhibited inflammation in both cell lines but the extruded hydrolysates had better anti-inflammatory properties.

Further, hydrostatic pressure has been used to enhance the hydrolysis of ovalbumin by a cocktail of enzymes (chymotrypsin, trypsin and pepsin) and the subsequent release of antihypertensive peptides (Quirós et al., 2007). Thermal treatment has also been shown to increase antioxidant (Fe^{2+}-chelating) capacity of lyophilized chickpea crude protein extracts, whereas antioxidant capacity was reduced in white bean crude protein extracts subjected to the same treatment (Yang et al., 2005). Majumder and Wu (2010) have identified very potent ACE inhibitory peptides from the action of thermolysin and pepsin on ovotransferrin, an egg protein. They also found that pre-treatment of the egg proteins by sonication improves ACE inhibitory activity by over 20 times. These results allude to the point that processing of certain proteins affects the biological activity of peptides released from those proteins *via* enzyme hydrolysis. These processing methods may have caused certain structural and conformational changes that better expose important peptide bonds to enzyme attack resulting in the release of potent bioactive sequences.

4.3. *Method of purification*

The complexity of biological materials and differences in properties of reaction mixture components poses challenges for the bioprocess industry in downstream processes (Saxena et al., 2010). This is because the downstream purification and fractionation steps impact directly on the potency of biological activities as well as on economics of production. The exploitation of alternative purification routes and selection of the most optimal is therefore of utmost importance in the bioprocessing of bioactive peptides (Agyei et al., 2013). Although the mainstream approaches used for the purification of proteins are generally applicable for peptide purification, due to the severing of bonds, the resulting peptide fractions often have their own peculiar properties. To develop an efficient purification process it is therefore necessary to explore the effect of the isolation or purification method on the physical, chemical and biological properties of bioactive peptides.

Table 2 – Classical isolation and purification methods for peptides – pros and cons.

Method	Advantages	Disadvantages
Selective precipitation	Can be used at all stages of purification from bulk recovery to selective isolation	Difficult for hydrophobic peptides with low solubility in aqueous and organic solvents
	Easy to scale up, especially for $(NH4)_2SO_4$ precipitation	$(NH4)_2SO_4$ precipitation is not effective for selective precipitation of some proteins and peptides
	Has high throughput and possibility of continuous operation	Strong precipitation agents can compromise properties of the peptides *via* undesirable reactions, *e.g.* sulfhydryl oxidation
	Usually simple, rapid, and inexpensive	Often requires a subsequent 'cleaning' step to remove precipitation agent from the peptide of interest
	Achieves some degree of product concentration during separation	
Membrane filtration	Has high throughput; is scalable and allows continuous operation	Often results in low mass transfer rates for highly concentrated substances
	Maintains protein and peptide biological activity and integrity	Results in increased viscosity of the retentate
	Uses an insoluble separating agent	Membrane fouling
	Allows selective transport and good separation	Reduced selectivity when separating similar sized proteins or peptides
	Does not require additives and can be performed isothermally at and at a fixed pH	
	Easy to upscale and downscale; can be combined with other processes	
	Achieves some degree of product concentration during separation	
Chromatographic methods	Highly selective, high resolution separation often in a relatively short time	Highly expensive, especially for scale-up applications
	Uses an insoluble separating agent	Slow binding and reduced capacity due to mass transfer resistance from diffusional limitations and steric hindrance
	Can concentrate dilute starting materials, and stabilize target molecules	Difficulty in treating viscous materials (as can be observed with protein hydrolysates) which can cause plugging and reduced flow rates
		Scale up limitations due to bead deformation with higher pressure drops
		Solvent (acetonitrile) waste from reversed-phase chromatography poses environmental concern regarding toxicity if not handled properly
		Often results in product dilutions (especially for size exclusion chromatography)

Sources: Bargeman et al. (2002a), Feng et al. (2009), Li and Chung (2008), Lovrien and Matulis (2001), Niederauer and Glatz (1992), Orr et al. (2013), Saxena et al. (2009), Shi et al. (2011), Strancar et al. (2002), van Reis and Zydney (2007) and Yang et al. (2005).

5. Bioprocess challenges to peptide purification

To establish an efficient downstream processing strategy for bioactive peptides largely depends on the production techniques, as well as properties and intended use of the peptide. This poses a number of challenges. For example, in order to conform to acceptable standards, production methodology must be strictly monitored to ensure there are no deviations from the established protocols. Further, although some methods extensively used in protein purification can be applied for peptides, these methods have to be fine-tuned to obtain more specific operation steps that are peculiar to the properties of the desired peptides. Purification of bioactive peptides from protein hydrolysates is usually achieved by selective precipitation techniques, membrane filtration systems, and chromatographic methods (Korhonen and Marnila, 2013). Each of these methods has its own merits and demerits (Table 2) and must therefore be considered before the selection of purification method(s) for peptides.

It must be mentioned that a number of research advances have been made that combines two of the aforementioned

methods and thus achieves better separation efficiency. Two of such combined methods include electro-membrane filtration (EMF) and membrane chromatography (MC). EMF achieves separation of charged molecules by combining the principles of membrane filtration and electrophoresis (Bargeman et al., 2002b). Due to the fact that membrane pressure and electrical fields are the dominant factors to achieve separation, EMF is able to improve protein solutions permeation flux, thereby controlling concentration polarization and membrane fouling (Oussedik et al., 2000; Saxena et al., 2009). EMF has been used in a number of studies for the isolation of various highly charged and neutral bioactive peptides (Bargeman et al., 2002a; Doyen et al., 2011a, b; Lapointe et al., 2006). In a similar way, membrane chromatography (MC) is achieved by grafting ligands onto membrane surface to act as 'traps' for protein/peptide mixture during convective flow through the membrane pores (Etzel, 2003; Ghosh, 2002; Saxena et al., 2009). This technology overcomes the challenge of pore diffusion observed in conventional chromatography. Also, specific ligands can be selected to work on ion exchange or affinity adsorption principles, demonstrating the use of MC for protein purification. However, studies aimed at developing ligands for peptide purification based on hydrophobic interaction and reversed-phase principles are still lacking (Saxena et al., 2009). Further research is therefore needed to understand the effect of various operational parameters in order to exploit the purification potential of these new technologies for bioactive peptides.

During peptide purification, a sequence of more than one method is often employed in a 'trial and error' fashion to attain peptides of desired purity and activity. Such 'trial and error' approach is cumbersome, time consuming, and often costly. In fact, it is estimated that around 70% of process cost is accounted for by separation and purification processes alone (Brady et al., 2008). Every bioprocess operations should aim at a reduced number of unit operations and the attainment of cheap, simple isolation procedures (Robins and Gordon, 2011). However, without prior knowledge of the properties of bioactive peptides it becomes difficult to rationally design suitable purification steps, especially when one is interested in the identification of novel peptides whose properties have never been explored.

We propose in this report an isolation/purification pathway which can replace the 'trial and error' approach. This involves a cascade of techniques based on the use of (a) '-omics' techniques (such as foodomics) and high throughput simulation models that can be used to predict beforehand the properties of the peptides and thereby predetermine the isolation and purification steps that can be used for the peptides of interest; (b) perfusion chromatography by employing monolithic columns specially designed for peptides (*i.e.* charged molecules that are also small in molecular weight). This approach has been explored further in subsequent sections of this review.

6. Novel bioprocessing approaches for bioactive peptides

6.1. *Integrated '-omics' techniques and in silico analysis*

Recent advances in '-omics' techniques and *in silico* prediction and analysis offer tremendous advantage in terms of throughput and the possibility of predicting the types and properties of peptides obtainable from a particular protein.

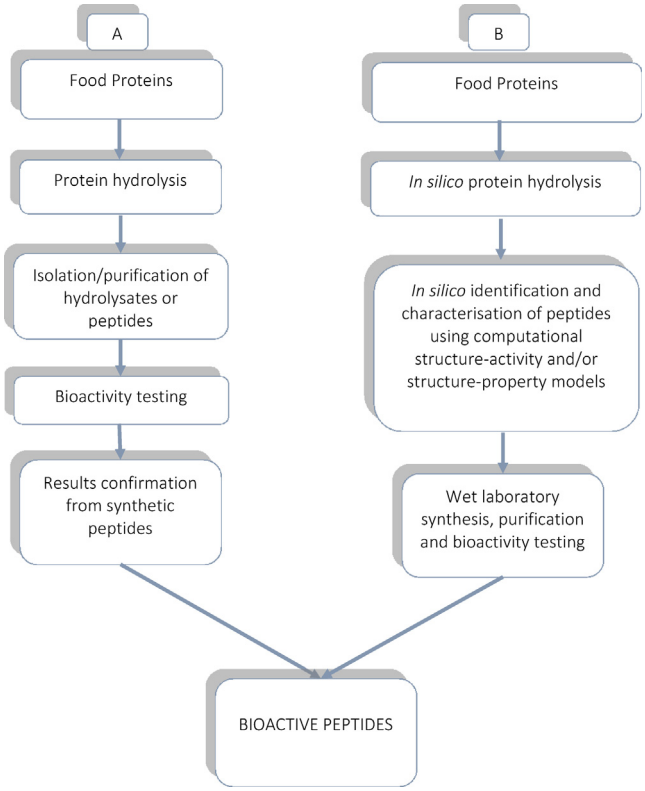

Fig. 2 – Classical (A) and integrated '-omics' (B) approaches to the study and production of bioactive peptides. *Source*: Adapted from Agyei et al. (2015).

Foodomics is a term that has been coined to describe the composition of food and how it is impacted by biotechnological processes, as well as the effect of food consumption on health (Picariello et al., 2012). It can be viewed as a toolbox of other '-omics' techniques such as proteomics, epigenomics, genomics, metabolomics and transcriptomics and the role they play in ensuring the bioactivity, safety, quality and traceability of food, thereby meeting consumer expectations of health and well-being (Cifuentes, 2013). Specifically, nutrigenomics, food proteomics, and food peptidomics have a huge impact on the study and production of food bioactive peptides. This is because they serve as analytical tools for studying and in validating biological (*e.g.* bioactivity, allergenicity, toxicity), chemical (*e.g.* authenticity), and physico-chemical (*e.g.* functional, organoleptic) properties of bioactive peptides that have been predicted from *in silico* studies (Han and Wang, 2008; Minkiewicz et al., 2008a; Picariello et al., 2012; Soloviev and Finch, 2006).

The classical approach to identifying and processing bioactive peptides is captured in Fig. 2A. This approach involves identification of a protein followed by *in vitro* protein digestion and chromatographic purification of hydrolysates. After testing for biological activities, the actual peptide sequence(s) of the active fraction(s) are then identified. This is usually followed by a confirmation of bioactivity by chemically synthesizing the peptide sequence(s) and conducting bioactivity tests on them. This approach has been used to identify bioactive properties in a host of food proteins and peptides (Fernández-Musoles et al., 2013; Ishiguro et al., 2012; Li et al., 2007; Marczak et al., 2003; Minkiewicz et al., 2008a; Saito et al., 1994). The major drawback with the classical approach is that it gives low yields, and there is a limit to the number of peptide species that can be studied at a time (Udenigwe, 2014). Moreover, this

approach does not give any prior indication of the types of peptides that can be expected from a particular food protein. As such, during purification, one has to rely on a more or less 'trial and error' approach which is often expensive, laborious, time consuming and could compromise the bioactive properties of some peptides.

The '-omics' integrated approach (Fig. 2B) on the other hand relies on high throughput *in silico* protein digestion and *in silico* peptide prediction techniques that provide biological and chemometric information on peptide sequences of interest (Mena and Albar, 2013; Saavedra et al., 2013; Udenigwe and Aluko, 2012). By this approach, important information on bioactive peptides can actually be predicted from food proteins quickly and effectively prior to wet-laboratory synthesis. This is an integrated approach because it relies on the predictive powers of *in silico* digestion and computational analysis, coupled to validation using '-omic' techniques such as foodomics and peptidomics. The key steps in this approach are as follows. First, protein databases are consulted to select desired proteins with known amino acid sequences. Next, the proteins are digested *in silico* using appropriate proteolytic enzymes for the selected protein. The *in silico* generated peptides are then characterized to identify structural properties, and potential biological activities, including toxicity and allergenicity. Wet laboratory synthesis, followed by validation with mass spectrometry and '-omic' techniques are then pursued after the successful identification of peptides with the desired physico-chemical and biological properties. The '-omics' integrated approach allows for the concurrent discovery of bioactive peptides from several proteins and proteolytic enzymes (Udenigwe, 2014) and eliminates guess work at several stages of the bioprocess chain, making the entire process quicker, relatively cheaper and less laborious (Carrasco-Castilla et al., 2012; Minkiewicz et al., 2008a).

BIOPEP is one of the well-used application databases for *in silico* study and identification of food bioactive peptides. It is designed to interlink three databases of protein sequences, bioactive peptides, and proteolytic enzymes. BIOPEP also has inbuilt programs that aids the prediction of allergenic and toxic peptides (Dziuba and Dziuba, 2010; Minkiewicz et al., 2008b). Captured in Table 3 are some databases with deposited protein/peptide sequences, as well as databases and servers for *in silico* digestion and prediction of biological properties.

Characterization of *in silico* peptides is attained by the use of computational tools which are used to develop and validate relationships between peptide structure and its activity/property. Models such as the quantitative structure–activity relationships (QSAR), and quantitative structure–property relationships (QSPR) are often utilized, and these work on the principle that physico-chemical or structural properties such as electronic charge, hydrophobicity and steric properties can be used to predict the activity of a molecule (Carrasco-Castilla et al., 2012; Norris and FitzGerald, 2013; Pripp et al., 2005).

Choosing the most appropriate structural descriptors is very important for QSAR studies, and over the years a number of amino acid descriptors based on 2D and 3D spatial conformations have been developed (Li and Li, 2013). Amino acids descriptors play a key role in structural variation of the peptide and this guides the modeling and prediction of biological activity as a function of molecular structure. These descriptors are used to score amino acid principal properties such as hydrophilicity/hydrophobicity, molecular size/bulkiness, and electronic properties/charge (Kim and Li-Chan, 2006). From this data matrix, the properties of the entire peptide can be forecasted. Examples of 2D amino acid descriptors include the amino acid hydropathic scale (Kyte and Doolittle, 1982); amino acid z-scales for hydrophilicity/hydrophobicity (z1), molecular size/bulkiness (z2), and electronic properties/charge (z3) (Hellberg et al., 1987); molecular electronegativity edge vector (MEEV) scales (Li et al., 2001); the divided physicochemical property scores (DPPS) descriptor (Tian et al., 2009); the vectors of hydrophobic, steric, and electronic properties (VHSE) scales (Mei et al., 2005). Some 3D amino acid descriptors on the other hand include the side-chain descriptors that uses isotropic surface area and electronic charge index (ISA-ECI) (Collantes and Dunn, 1995); and molecular surface-weighted holistic invariant molecular (MS-WHIM) scales (Todeschini et al., 1994; Zaliani and Gancia, 1999).

There are practical limitations to some of the aforementioned amino acid descriptors. For example, descriptors such as z-scores, MEEV scales, and MS-WHIM are derived from the principal component analysis of a data matrix of the amino acids. As such, properties of different categories are combined in each principal component and are usually of little physicochemical meaning (Mei et al., 2005). Descriptors such as VHSE on the other is based on principal component analysis of 50 physico-chemical properties grouped according to 18 hydrophobic properties, 17 steric properties and 15 electronic properties. Principal component analysis is applied separately to each of these three categories of properties followed by selection of appropriate principal component scores that can be used as vectors (Mei et al., 2005). VHSE therefore overcome the limitations of the previously mentioned descriptors because it is obtained from a wide spectrum of physicochemical properties and has better predictive powers and ease of interpretability (Mei et al., 2005; Xie et al., 2013).

It is worth mentioning however that, as a relatively recent technique, there are some setbacks with the '-omics' approach to bioactive peptides discovery. It has been argued that the generation on *in silico* peptides is no guarantee that they can be reproduced experimentally. Also, there is the possibility of missing novel peptides with hitherto unknown bioactive properties since the currently used techniques only takes into consideration the sequences that exist in peptide databases (Udenigwe, 2014). Thus, research studies are needed to unravel effective solutions to these challenges. Developments of sophisticated amino acid/peptide descriptors as well as improvements in mathematical expressions and algorithms for QSAR modeling hold promise in overcoming these challenges. All the same, '-omics' techniques and QSAR have been applied to successfully identify and study a range of bioactive active peptides from several food proteins (Norris et al., 2012; Wu et al., 2006). For example, in a number of studies, *in silico* digestion and QSAR model prediction were used to identify ACE-inhibitory peptides from egg proteins (Majumder and Wu, 2010), cheese (Sagardia et al., 2013), soybean proteins (Gu and Wu, 2013), as well as forecasting ACE-inhibitory peptides from several common food products such as livestock meat (pork, beef and chicken), milk, egg, soybean, canola, salmon and cereals (oat and barley) (Gu et al., 2011). Not only is the '-omics' integrated useful for the discovery of new protein precursors that contain bioactive sequences, it also provides vital structural and physico-chemical properties which can be used in designing suitable isolation/purification techniques for the peptides. This makes it a suitable option for overcoming some of the challenges encountered during the industrial scale bioprocessing of bioactive peptides.

Table 3 – Some databases for protein/peptide sequences, *in silico* digestion, and biological property prediction.

Databases	Name	Webpage
Protein/peptide sequence databases		
	BIOPEP	http://www.uwm.edu.pl/biochemia/index.php/en/biopep
	PepBank	http://pepbank.mgh.harvard.edu/
	BioPD	http://biopd.bjmu.edu.cn/
	SwePep	http://www.swepep.org/
	EROP-Moscow	http://erop.inbi.ras.ru/
	MilkAMP	http://milkampdb.org/home.php
	UniProtKB	http://www.uniprot.org/
	NCBI Protein database	http://www.ncbi.nlm.nih.gov
	PeptideDB	http://www.peptides.be/
	AMPer	http://marray.cmdr.ubc.ca/cgi-bin/amp.pl
In silico digestion databases		
	BIOPEP	http://www.uwm.edu.pl/biochemia/index.php/en/biopep
	PeptideCutter	http://web.expasy.org/peptide_cutter/
	POPS	http://pops.csse.monash.edu.au/pops-cgi/index.php
	NeuroPred	http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py
Potential bioactivity prediction		
	PeptideRanker	http://bioware.ucd.ie/~compass/biowareweb/
	BIOPEP	http://www.uwm.edu.pl/biochemia/index.php/en/biopep
	AntiBP2	http://www.imtech.res.in/raghava/antibp2/
Toxicity/allergenicity prediction		
	ToxinPred	http://www.imtech.res.in/raghava/toxinpred/
	BIOPEP	http://www.uwm.edu.pl/biochemia/index.php/en/biopep
	AlgPred	http://www.imtech.res.in/raghava/algpred/
	Allerdictor	http://allerdictor.vbi.vt.edu/
	EPIMHC	http://bio.dfci.harvard.edu/epimhc/
	SORTALLER	http://sortaller.gzhmu.edu.cn/

6.2. *Perfusion chromatography with monolithic columns*

Monolithic chromatographic columns are highly porous continuous stationary support materials that are gaining significant attention for the separation of substances such as proteins, peptides, plasma DNA and viruses (Jungbauer and Hahn, 2004; Tennikova and Reusch, 2005). Due to their unique physical and chemical structures, monolithic supports have scalable feasibilities and better hydrodynamics, and have therefore been reported to have improved chromatographic performances compared to conventional particulate supports. The reason lies in the fact that, unlike bead-based resins, monolithic materials comprise a single structure with a highly interconnected network of channels that span macroporous (greater than 50 nm) and mesoporous (between 2 nm and 50 nm) ranges (see Fig. 3). The presence of interconnected pores in monolithic sorbents allows convective transport mechanism instead of diffusion which features as the transport mechanism for particulate supports. That monolith hydrodynamic property is predominated by convective transport mechanism is an important feature which overcomes diffusion limitations, allowing the chromatography of larger molecules that are unable to penetrate into the internal structure of particulate supports (Jungbauer and Hahn, 2008; Kłodzińska et al., 2006). Monolithic support also features a lower pressure drop property that varies with different pore structure orientations (Miheličˇ et al., 2005; Yang et al., 2005). Such features allow for higher mobile phase flow rates to be applied which could enhance the separation efficiency (Bandilla and Skinner, 2003; Levison, 2003). Also, the intended productivity/yield can be improved by increasing the flow rate without compromising separation efficiency. Monolithic structures exhibit good mechanical strength (Vlakh and Tennikova, 2009), and by their nature and properties, monolithic columns can be cast to a desired volume – from as high as 8 L volume (Yang et al., 2005) to as small as 50 μL (Rieux et al., 2006). Also, with monoliths, a greater capacity for separation is made possible by increased volume – a thing which is a limiting factor for particulate supports (Roberts et al., 2009). These attributes have allowed monolithic supports to be used in all types of liquid chromatographic methods including reversed-phase, normal-phase, hydrophilic interaction liquid chromatography (HILIC), ion-exchange applications, and in two-dimensional separation systems (Jandera, 2013; Pruim et al., 2008).

Monolithic supports can broadly be classified into inorganic (mostly silica) and organic polymer monoliths and both classes of adsorbents can be engineered to have the desired pore diameter that does not impedes convective mass transport. Polymeric monoliths are applied in the purification of macromolecules such as proteins and polynucleotides whereas silica-based monolithic columns on the other hand exhibit high bed porosity and as such a lower pressure drop (Jungbauer and Hahn, 2008). Silica-based monolithic columns are therefore well adapted for the purification of smaller molecules such as peptides (Ishizuka et al., 2002; Minakuchi et al., 1997; Siouffi, 2003).

Monolithic materials can be prepared *in situ* and based on the desired end use, optimization of the polymerization mixture or post-polymerization modifications can be used to alter the pore size and morphological properties of the resulting column. Functionalization of monolithic columns can also be achieved with functional groups (such as ion-exchangers and zwitterionic reagents) to attain monoliths with the desired chemical surface properties. By these techniques, the mass transfer, flow dynamics as well as surface chemistry can be fine-tuned as desired.

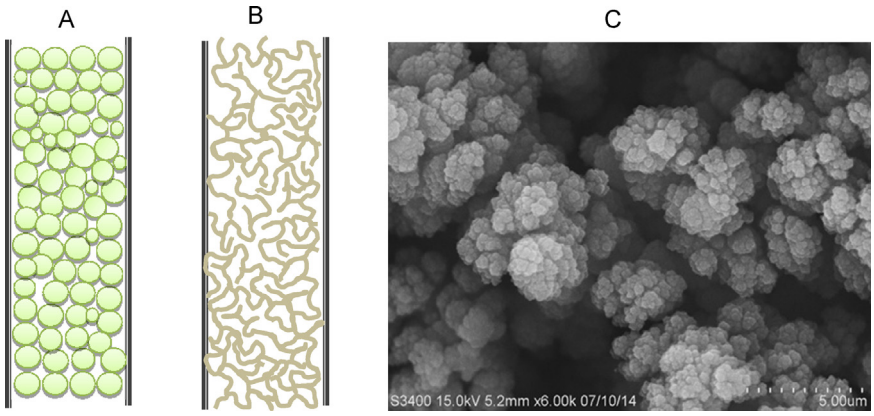

Fig. 3 – Cross-sectional schematic of particulate (A) and monolithic (B) stationary phases; (C) scanning electron microscopy image of polymethacrylate monolith showing the macroporous morphology.

We have described in the previous sections of this review that integrated '-omics' techniques can be used to forecast the properties of peptide species obtainable from combining a protein of known sequence and proteolytic enzyme of known catalytic specificity. Once the physico-chemical properties of the expected peptides have been forecasted, one can then rationally engineer monolithic columns that allow tailored isolation and/or purification of the peptides of interest in fewer steps. Generally, the properties of the monolith (*e.g.* pore size, binding capacity, and functional groups) can be specifically altered and optimized if the physico-chemical properties of the analyte is known. This strategy has been used to develop a model framework for the purification of plasmid DNA using monolith columns and ion exchange chromatography (Ongkudon and Danquah, 2010). Using predicted physico-chemical properties of peptides (*e.g.* pI value, molecular weight, hydrodynamic size) could greatly save time in developing monoliths that will ensure preferential adsorption of the peptides at optimum resolution. Monoliths have been used for the purification of several peptide-based biomolecules including peptides mixtures (*e.g.* tryptic digest of cytochrome c) (Levkin et al., 2008; Xiong et al., 2004); peptide growth factors and hormones (*e.g.* methionine enkephalin; tripeptide Tyr-Gly-Gly; luteinizing hormone releasing hormone; somatostatin) (Levkin et al., 2008); and synthetic phosphopeptides (Miyazaki et al., 2004).

Although monolithic chromatography have proven useful on a smaller scale (including chip-based separation processes), a number of challenges are encountered during scale up processes. Some of these bottlenecks include cracking of bed volume, wall channel formation, and the formation of heterogeneous pore sizes (Danquah et al., 2008; Ongkudon et al., 2014; Podgornik et al., 2000; Siouffi, 2003; Stachowiak et al., 2003; Strancar et al., 2002). Cracking of bed volume is caused by accumulated exothermic heat and pressures derived from the reaction and which far outweigh the mechanical strength of monolith. The resulting temperature gradient formed across the monolith adsorbent leads to the formation of heterogeneous pore size distribution (Danquah and Forde, 2008b; Danquah et al., 2008; Ongkudon et al., 2014; Podgornik et al., 2000; Stachowiak et al., 2003; Svec and Frechet, 1995). Indeed, controlling the process kinetics and getting temperature under control is one of the most important keys to achieving the desired macroporous structure (Danquah and Forde, 2008b). However, recent advances in research are providing practical solutions to overcome the aforementioned challenges. These advances provide strategies for limiting the formation of temperature gradients throughout the monolith (Danquah and Forde, 2008a; Danquah et al., 2008; Peters et al., 1997); ensuring homogeneity in monolith pore sizes (Feng et al., 2009); and controlling wall channel formation (Levkin et al., 2008; Ongkudon et al., 2014; Shi et al., 2011; Siouffi, 2003; Stachowiak et al., 2003).

7. Conclusion and future outlook

Due to the physiological responses they trigger and the spectrum of application, bioactive peptides have attracted huge interest from researchers, manufacturers and consumers alike. However, in order to meet future market potential, certain downstream bioprocess challenges have to be overcome with strategies that also meet the requirements of products purity and potency, at feasible economic costs and time. The integrated '-omics' approach described in this review combined the powers of *in silico* analysis and '-omics' techniques such as peptidomics to predict, analyze and validate bioactive peptides from various food proteins. This has paved the way for the rational design of peptides with desired biological and physico-chemical properties, while guiding the choice of subsequent purification techniques that can be employed for peptides of interest.

We have highlighted the prospects of liquid chromatography with monolithic columns as a suitable downstream isolation and purification technique for bioactive peptides. Polymeric monoliths are highly 'pliable' to obtain the desired morphology and surface chemistry. Unlike, bead-based resins, monolithic stationary phases can be specially designed and used in multi-mode separations involving ion-exchange, HILIC, zwitterionic, normal phase and reverse-phase mechanisms. As such, they are very attractive for the separation of peptides. Also, monolithic columns, with their advantages of scalability, speed, better transport mechanisms and hydrodynamics, and improved separation efficiency could well present a breakthrough in the bioprocessing of bioactive peptides at large scales.

Future developments in the production and application of bioactive peptide hinges on several other factors. For example, this '-omics' integrated techniques will rely heavily on the successful development of mathematical algorithms and statistical tools for the analysis of huge and bulky data that is often associated with peptide research. The elucidation of the mechanism of action of various bioactive peptides, and the regulation and legislation of bioactive peptides as therapeutic ingredients, also need to be looked into. However, in the

bioprocessing arena, we can expect the combination of integrated '-omics' techniques/*in silico* predictions and monolithic media to offer new possibilities in the discovery, characterization and bioprocessing of bioactive peptide at industrial scale for the food and therapeutics markets.

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