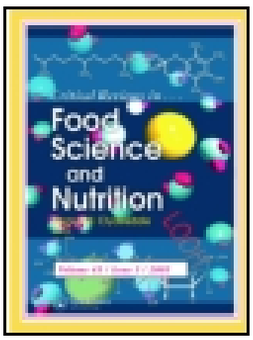

Critical Reviews in Food Science and Nutrition

ISSN: 1040-8398 (Print) 1549-7852 (Online) Journal homepage: https://www.tandfonline.com/loi/bfsn20

Food-derived antithrombotic peptides: Preparation, identification, and interactions with thrombin

Shuzheng Cheng, Maolin Tu, Hanxiong Liu, Guanghua Zhao & Ming Du

To cite this article: Shuzheng Cheng, Maolin Tu, Hanxiong Liu, Guanghua Zhao & Ming Du (2019): Food-derived antithrombotic peptides: Preparation, identification, and interactions with thrombin, Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2018.1524363

To link to this article: https://doi.org/10.1080/10408398.2018.1524363

Published online: 11 Feb 2019.

CRITICAL REVIEWS IN FOOD SCIENCE AND NUTRITION
https://doi.org/10.1080/10408398.2018.1524363

Taylor & Francis
Taylor & Francis Group

REVIEW

Food-derived antithrombotic peptides: Preparation, identification, and interactions with thrombin

Shuzheng Cheng[a,b,*], Maolin Tu[c,*], Hanxiong Liu[a], Guanghua Zhao[b], and Ming Du[a]

[a]School of Food Science and Technology, National Engineering Research Center of Seafood, Dalian Polytechnic University, Dalian, Liaoning, China; [b]Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, China; [c]Department of Food Science and Engineering, Harbin Institute of Technology, Harbin, Heilongjiang, China

ABSTRACT

Thromboembolism and its sequelae have been the leading causes of morbidity and mortality throughout the world. Food-derived antithrombotic peptides, as potential ingredients in health-promoting functional foods targeting thrombus, have attracted increasing attention because of their high biological activities, low toxicity, and ease of metabolism in the human body. This review presents the conventional workflow of preparation, isolation and identification of antithrombotic peptides from various kinds of food materials. More importantly, to analyze the antithrombotic effects and mechanism of antithrombotic peptides, methods for interaction of anticoagulant peptides and thrombin, the main participant in thrombosis, were analyzed from biochemistry, solution chemistry and crystal chemistry. The present study is intended to highlight the recent advances in research of food-derived antithrombotic peptide as a novel vehicle in the field of food science and nutrition. Future outlooks are highlighted with the aim to suggest a research line to be followed in further studies with the introduced research approach.

KEYWORDS

Thrombosis; anticoagulant peptides; thrombin; interaction analysis; inhibitory activity

Introduction

Thrombus and thrombosis

Cardiovascular diseases (CVDs), mainly caused by an embolism, are a group of disorders of the heart and blood vessels, including stroke, coronary heart disease, myocardial infarction and unstable angina (Yang et al. 2017; Rafiq et al. 2017). It is a major threat to human health both in developing and developed countries (Sabbione et al. 2016a; Ye et al. 2015; Herrera et al. 2016a). An estimated 17.7 million people died from CVDs in 2015, representing 31% of all global deaths, and of these deaths, an estimated 7.4 million were due to coronary heart disease and 6.7 million were due to stroke by the World Health Organization (WHO) (www.who.int/mediacentre/factsheets/fs317/en/). CVDs annually occupy the first place: more people die from CVDs than from any other cause. The mortality of stroke is rapidly increasing from 5.41 million in 2010 to 6.24 million in 2015 throughout the world. The ischemic heart disease caused by thrombus was at the top the list of top 10 causes of death globally in both 2000 and 2015, the death increased from 6.88 million to 8.76 million (Fig. 1). Thus, thromboembolism and its sequelae have been the leading causes of morbidity and mortality throughout the world (Jasuja et al. 2012), the situation is still grave. As well known, the most important behavioral risk factors of CVDs are unhealthy diet, physical inactivity, tobacco use and harmful use of alcohol. Although the mechanisms of all CVDs have not been exactly clarified, thrombus also is one of the chief culprits of the diseases.

Thrombosis mainly involves two principal mechanisms, i.e., activation of platelets and coagulation factors (Davie, Fujikawa, and Kisiel 1991; Kastelowitz et al. 2017). The thrombin (IIa), a serine protease at the end of the coagulation system, is a potent platelet activating factor and activated platelets would strengthen the coagulation system to produce more thrombin. The two systems of thrombosis are tied together in the organism. Platelets play an important role in blood coagulation by recognizing the vascular injury site, releasing agonists, adhering to each other, and interacting with the coagulation cascade to form a platelet plug through different receptors (Fig. 2) (McNicol and Israels 2003; Clemetson and Clemetson 2001). The coagulation system, the second step of thrombosis, is triggered in response to many factors such as endothelium rupture, activated platelets, atherosclerotic plaque. It involves a cascade of enzymatic reactions involving a series of clotting factors (Fig. 3), many of which are serine proteases subjected to activation and inhibition (Davie and Ratnoff 1964;

CONTACT Ming Du duming@dlpu.edu.cn School of Food Science and Technology, National Engineering Research Center of Seafood, Dalian Polytechnic University, Dalian 116034.
Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/bfsn.
*Equivalent contribution author to this work.

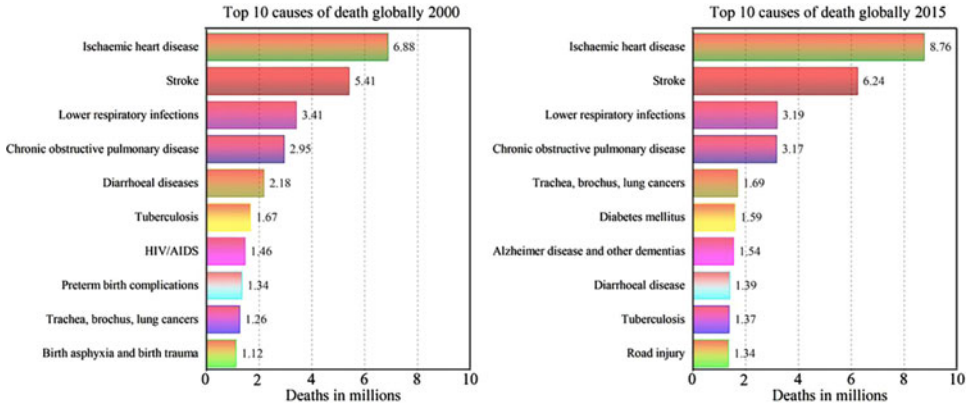

Figure 1. Top 10 causes of death globally 2000 and 2015.

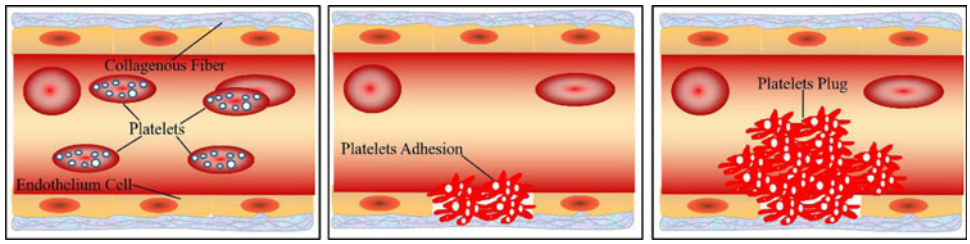

Figure 2. The simplified scheme of platelets adhesion and aggregation.

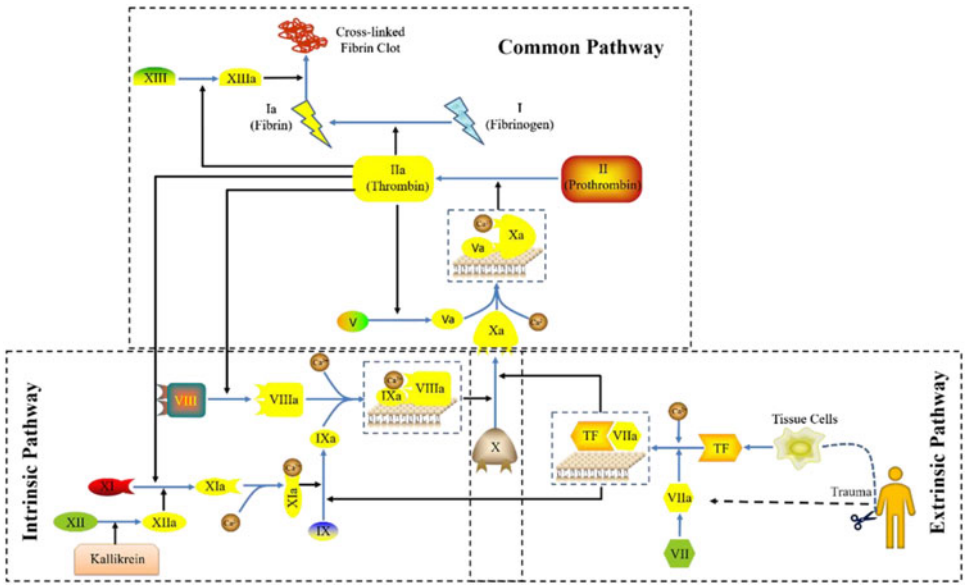

Figure 3. The simplified scheme of the coagulation cascade.

Macfarlane 1964). The coagulation cascade consists of an intrinsic way, so named originally, because the presumed initiative components were present in blood, and an extrinsic pathway, which requires sub-endothelial tissue factor (TF) for its activation (Jung and Kim 2009). The two pathways converge at the formation of factor Xa by intrinsic tenase complex (IXa/VIIIa/Ca^{2+}/PL) in an intrinsic pathway and extrinsic tenase complex (VIIa/TF/Ca^{2+}/PL) complex in an extrinsic pathway (Nasri et al. 2012). Factor Xa binds to factor Va on PL_3 to form prothrombinase (Xa/Va/Ca^{2+}/PL), and then proceeds to catalyze the conversion of prothrombin (II) to thrombin (IIa) in the common pathway (Weitz and Bates 2005). The thrombin (IIa) is responsible for converting fibrinogen to fibrin (Li et al. 2011) and activates factor XIII to XIIIa, which cross-links and stabilizes the fibrin to produce a tough fibrin meshwork, which reinforces the friable platelet plug (Jung et al. 2007).

In broad terms, platelet plug and coagulation cascade mainly involve the arterial thrombosis mainly and the venous thrombosis, respectively (Mackman 2008). Thus, the importance of anticoagulants and antiplatelets as treatments for the prevention of venous thrombosis and arterial thrombosis in patients with cardiovascular diseases is becoming increasingly recognized (Rajapakse et al. 2005). Remarkable progress has occurred over the last decade in the development of antithrombotic drugs.

Table 1. The action mechanism, administration route and side effects of main antithrombotic agents in clinical application.

Antithrombotic agents	Action mechanism	Administration route	Side effects
Anticoagulants			
Heparin	Enhance antithrombin activity, inactivate IIa, Xa	Intravenous injection	Bleeding, thrombocytopenia, allergy, osteoporosis
Rivaroxaban	Inhibit Xa	Oral administration	Bleeding
Hirudin	Inhibit IIa	Intravenous injection	Bleeding
Bivalirudin	Inhibit IIa	Intravenous injection	Bleeding, nausea, headache, hypotension
Argatroban	Inhibit IIa	Intravenous injection	Bleeding
Dabigatran	Inhibit IIa	Oral administration	Liver dysfunction
Antiplatelets			
Aspirin	Inhibit cyclooxygenase-1	Oral administration	Bleeding, gastrointestinal discomfort, allergy, asthma
Clopidogrel	Inhibit P_2Y_{12} receptor	Oral administration	Bleeding, thrombocytopenia, gastrointestinal discomfort, allergy, headache
Prasugrel	Inhibit P_2Y_{12} receptor	Oral administration	Bleeding
Tirofiban	Block fibrinogen receptors GP IIb/IIIa	Intravenous injection	Hypotension, thrombocytopenia, gastrointestinal discomfort, constipation, phlebitis
Iloprost	Activate adenosine cyclase, raise the platelet cAMP, decline the Ca^{2+}, activate plasminogen	Intravenous injection	Fever, headache, nausea, emesis, hypotension, diarrhea, stomachache
Abciximab	Block fibrinogen receptors GP II_b/III_a, inhibit the platelet activation	Intravenous injection	Bleeding, thrombocytopenia, immune response, hypotension, nausea, emesis
Thrombolytics			
Streptokinase	Activate plasminogen	Intravenous injection	Bleeding, antigenicity, allergy
Urokinase	Activate plasminogen	Intravenous injection	Bleeding, allergy
t-PA	Activate plasminogen	Intravenous injection	Bleeding
Monteplase	Activate plasminogen	Intravenous injection	Bleeding
Reteplase	Activate plasminogen	Intravenous injection	Less negative effect
Staphylokinase	Activate plasminogen	Intravenous injection	Bleeding, antigenicity, allergic reaction, hypotension

Disadvantages of antithrombotic agents

Based on the pathobiology of thrombotic and vascular disorders, antithrombotic drugs can be classified into 3 major categories, i.e., anticoagulants, antiplatelets and thrombolytics. Currently, the available therapeutic drug of thrombosis and thrombus includes heparin, low-molecular-weight heparin (LMWH), fondaparinux, aspirin and so on (Hirsh, O'Donnell, and Weitz 2005; Weitz and Bates 2005; Eikelboom and Weitz 2010). At the same time, several new parenteral (AVE5026, RB006) and oral anticoagulants (AZD0837, dabigatran etexilate) are in advanced stages of development (Viskov et al. 2009; Rusconi et al. 2002; Eriksson, Quinlan, and Weitz 2009; Wolowacz et al. 2009). Moreover, some new peptides derived from the salivary gland of the mosquito (Choi, Kim, and Kim 2016), the venom of snake (Ye et al. 2015; Liu et al. 2015a), the hydrolysates of centipedes (Kong et al. 2014) have been shown to be antithrombotic activity. The name, action mechanism, administration route and side effects of main antithrombotic agents in clinical application were summarized in Table 1.

Those antithrombotic drugs have exhibited favorable pharmacokinetic characteristics. However, most antithrombotic agents can bring to a series of side effects, such as bleeding, thrombocytopenia, hypotension, allergy, etc. (Indumathi and Mehta 2016; Atanassov and Tchorbanov 2009). Furthermore, a narrow therapeutic window and a highly variable dose-response relation also limited their application. Aspirin produces its antithrombotic effect by blocking the synthesis of thromboxane A2. The effectiveness of aspirin is therefore limited because it fails to block platelet activation by other important agonists such as shear stress, thrombin, collagen, and ADP (Weitz and Hirsh 1999). Melagatran, a potent active-site inhibitor of thrombin and trypsin, has only low bioavailability (5.8%) after oral administration due to poor adsorption (Gustafsson et al. 2001).

Food-derived peptides with anticoagulation activity

Considering the limits of available antithrombotic agents, to find a new generation of safe and effective antithrombotic alternatives with larger therapeutic windows (that is, a larger difference between the dose that prevents thrombosis and the dose that induces bleeding), bioactive peptide with anticoagulation property, derived from daily diets, is needed to discuss for developing of new approaches for antithrombotic therapy.

In recent years, functional food with many biological properties and therapeutic effects in several health disorders, including cardiovascular disease, has captured the attention of many consumers and researchers. Proteins are a potential source of health-promoting biomolecules with medical, nutraceutical, and food applications. Bioactive peptides are usually encrypted and kept inactive within the primary structure of food protein and considered to promote diverse beneficial effects (Marcone, Belton, and Fitzgerald 2017), and have an array of attractive advantages (e.g., low toxicity and immunogenicity, good solubility property, distinct tissue

Table 2. Anti-thrombotic peptides derived from daily diets.

Source	Peptide Sequence	Functionality	References
Yellowfin Sole	TDGSEDYGILEIDSR	Coagulation factor (XIIa)	Rajapakse et al. (2005)
Blue Mussel	EADIDGDGQVNYEEFVAMMTSK	Coagulation factor (Xa)	Jung and Kim (2009)
Goby Mussel	LCR, HCP, CLCR, LCRR	TT, APTT	Nasri et al. (2012)
Mackerel skin	FGN	Platelet aggregation	Khiari et al. (2014)
Echiuroid worm	GELTPESGPDLFVHFLDGNPSYSLYADAVPR	Coagulation factor (IXa)	Jo, Jung, and Kim (2008)
Granulated ark	HTHLQRAPHPNALGYHGK	Coagulation factor (Xa)	Jung et al. (2007)
Casein	YQEPVLGPVRGPFPIIV	Thrombin (IIa)	Rojas-Ronquillo et al. (2012)
Peanut	WAQL, GNHEAGE, CFNEYE	Thrombin (IIa)	Zhang (2016b)
Oat, Highland barley, Buckwheat	ALPIDVLANAYR, EFLLAGNNKR, GEEFGAFTPK, QLAQIPR, LQAFEPLR, ALPVDVLANAYR, GEEFDAFTPK, QKEFLLAGNNK, TNPNSMVSHIAGK	Platelet aggregation	Yu et al. (2016)
Edible seaweed	NMEKGSSSVVSSRMKQ	APTT	Indumathi and Mehta (2016)
Amaranth	TEVWDSNEQE, NDQGQSVFDEELS	Thrombin (IIa)	Sabbione et al. (2016a)
Blue Mussel	ELEDSLDSER	Thrombin (IIa)	Qiao et al. (2018)

distribution pattern, favorable pharmacokinetic profile), which make them present an excellent starting point for the design of novel therapeutics (Fosgerau and Hoffmann 2015; Zvereva, Dudko, and Dikunets 2018). Thus, they have a wide range of applications in medicine for treatment of cancer (Nurdiani et al. 2017; Díazgómez et al. 2017; Tornesello et al. 2017), cardiovascular (Yu et al. 2016; Skrzypczak et al. 2017; Rafiq et al. 2017), inflammation diseases (Martínez-Sánchez et al. 2017; Ho et al. 2017; New et al. 2017), and in the food industry to produce nutraceuticals and functional food ingredients due to their health benefits (Caliceti et al. 2017). The past decade has seen an exponential increase in bioactive peptide research and the therapeutic peptide market value is expected to reach $25 billion in 2018 (Daliri, Oh, and Lee 2017a).

Diets with antithrombotic components offer a convenient and effective way of preventing and mitigating CVD incidence. Thrombosis is a complicated process in which a cascade of responses operates with several coagulation factors and platelets are involved; because of this, there are various mechanisms to inhibit thrombus formation (Rojas-Ronquillo et al. 2012). Over the last few decades, bioactive peptides with anticoagulant activity have been reported for certain food proteins from plants, animals and microorganisms.

For instance, quercetin-3-rutinoside blocks thrombus formation at concentrations that are generally ingested as nutritional supplements (Flaumenhaft 2013). Nasri et al. (2012) isolated four novel bioactive peptides with high anticoagulant activity from goby muscle protein hydrolysates and identified the peptide sequences (LCR, HCP, CLCR, LCRR). Shimizu et al. (2009) found that antithrombotic activity of the purified peptide fraction from protein hydrolysates of pork meat via papain was equivalent to that of aspirin at 50 mg/kg mice body weight. Moreover, Jung and Kim (2009) reported a potent anticoagulant oligopeptide with an approximately 2.5 kDa molecular mass, which could potently prolong both the thrombin time and the activated partial thromboplastin time, and specifically interact with blood coagulation factors: IX, X, and II. Khiari et al. (2014) reported that bioactive gelatin peptides from mackerel (*Scomber scombrus*) skin hydrolysates were able to significantly inhibit platelet aggregation by about 30%, corresponding to moderate antithrombotic activity. Jo, Jung, and Kim (2008) isolated a novel inhibitory peptide against blood coagulation factor IXa from the marine echiuroid worm, which potently prolonged the activated partial thromboplastin time (APTT). A novel inhibitory protein against blood coagulation factor Va was purified from muscle protein of granulated ark, which could potently prolong thrombin time (TT), analogous to inhibition of thrombin (IIa) formation (Jung et al. 2007). The plancinin, a new anticoagulant peptide isolated from the starfish *Acanthaster planci*, significantly inhibited prothrombin activation by the prothrombinase complex (Xa/Va/Ca^{2+}/PL) factor X activation by both the intrinsic (IXa/VIIIa/Ca^{2+}/PL) and extrinsic (VIIa/TF/Ca^{2+}/PL) tenase complexes (Koyama et al. 1998). Herrera et al. (2016b) hydrolyzed the pods of *Mucuna pruriens* with the sequential pepsin pancreatin system to obtain peptide fractions, which exhibited the inhibition of human platelet aggregation ranged from 1.59% to 11.11%. Sabbione, Scilingo, and Añón (2015) reported that amaranth seed protein was first hydrolyzed with alcalase and then with trypsin, the hydrolysates prolonged thrombin time from 19.5 ± 0.7 s (control group) to 81.0 ± 8.5 s (sample group). Martínez-Sánchez et al. (2017) studied the effects of consuming Spanish dry-cured ham on platelet function, which decreased after ADP stimulation. Thus, this research strongly suggests the existence of a mechanism that links dietary biopeptides and beneficial health effects. The antithrombotic peptides derived from daily diets are shown on the Table 2.

In this review, we summarize the common approaches for preparing and identifying anticoagulant peptides. Moreover, methods for interaction of anticoagulant peptides and thrombin, the main participant in thrombosis, were analyzed from biochemistry, solution chemistry and crystal chemistry. The detailed contents will be discussed in the following sections.

Preparation and identification of food-derived antithrombotic peptides

Preparation of antithrombotic peptides

In recent years, generation of food-derived antithrombotic peptides mainly involves classical methods used by most researchers and a bioinformatic approach as a new method to identify bioactive peptides. The classical approach may be

associated with food-grade enzymes (e.g., pepsin, trypsin, chymotrypsin, alcalase) hydrolysate, microbial fermentation, food processing, or artificial synthesis using genetic method. On the other hand, with the development of the computational biology technology, bioinformatic (*in silico*) approach depends on the information available in a database, promoting the further development of classical approaches.

From literature, the most widely used method to produce antithrombotic peptides is by enzyme of specificity hydrolysis of food proteins. Specific food-grade enzymes that can cleave the identified segments from the parent protein are chosen to hydrolyze the proteins obtaining bioactive peptides with antithrombotic activity (Daliri, Lee, and Oh 2017b; Yu et al. 2016; Zhang et al. 2016a). In this process, the protein material is subjected to enzyme hydrolysis at a given optimal temperature and pH to produce the hydrolysate containing short peptide sequences (He et al. 2016; Huang et al. 2016a; Silva et al. 2017). Several of factors including enzyme strains, enzymolysis time, and a ratio of enzyme to substrate would determine the bioactivity and production of peptides in hydrolysates. Although no certain proteolytic enzymes are known to produce hydrolysates containing specific bioactive peptides with different amino acids sequences, characterization with various functional properties in foods, subtilisin hydrolysis tends to yield low molecular weight peptides with higher biological activity than samples hydrolysis by papain and trypsin (Huang et al. 2017; Zhang, Yokoyama, and Zhang 2012). Also, peptide sequences and their biological activities in enzyme hydrolysates may differ depending on the enzymolysis time and the enzyme to substrate ratio (Rodríguez-Díaz et al. 2011; Song et al. 2012). After enzymatic hydrolysis, the supernatant of hydrolysates isolated by centrifugation which contains low molecular weight peptides is recovered by freeze-drying, desalting, cross-flow membrane filtration and membrane ultrafiltration or column chromatography (Nimalaratne, Bandara, and Wu 2015; Ferri et al. 2017; Zhang et al. 2016a; Zhang, Yokoyama, and Zhang 2012).

This approach often produces the milk-derived bioactive peptides with some bacteria or yeast on milk protein substrates to hydrolyze the proteins with their enzymes as they grow (Korhonen and Pihlanto 2006; Wada and Lönnerdal 2014). The growing bacteria secret their proteolytic enzymes into the protein material so that continuous peptides can be produced from the parent proteins. In addition, microbial enzymes have different cleavage sites in comparison with isolated enzymes, and so the peptides generated by these enzymes may differ from those generated during enzymatic hydrolysis (Marcone, Belton, and Fitzgerald 2017). Usually, the bacterium of choice is grown to its exponential phase in a broth at a temperature suitable for the bacterial growth. The cells are then harvested, washed and suspended in sterile distilled water and used as a starter to inoculate a sterilized protein substrate (Aguilar-Toalá et al. 2017; Rizzello et al. 2017). The extent of hydrolysis would depend on the strain used, the type of protein and the fermentation time. Then the fermented broth is treated as the almost invariable procedure with hydrolysates.

The current generation regimens of targeted bioactive peptides, mainly enzymatic hydrolysis and microbial fermentation, are complex and could be affected by many factors (pH, temperature, time, et al) (Zhou 2014). To analyze the bioactivity of targeted peptides, the purified bioactive peptides need to be synthesized by other methods. With the development of technologies and methodologies for structural elucidation, organic synthesis is increasingly applied to the production of bioactive peptides (Wang et al. 2017). Organic synthesis usually chooses a solid-phase synthesis method using a series of solvents and synthesis methods to obtain the target peptides, and the coarse product is identified by mass spectrometry to test whether it is consistent with the theoretical molecular weight. Its further biological activity would also be verified. Organic synthesis would realize high-volume production of the target peptides. As the identified antithrombotic peptides by enzymatic hydrolysis and microbial fermentation are very difficult, so the simple method to find antithrombotic peptides is needed. The bioinformatic 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 (Daliri, Lee, and Oh 2017b), and thus this approach enables identification of known peptides from unknown protein sources. Once the primary structure and amino acid sequences are identified, the bioactive peptides are synthesized to research their bioactive future. Several researchers (Pripp et al. 2004; Wu, Aluko, and Nakai 2006; Wu and Aluko 2007) have used this approach to detect and study potent bioactive peptides.

Identification of antithrombotic peptides

Peptides with antithrombotic bioactivity usually contain 3-20 amino acids residues (Wang et al. 2017). To future investigate the antithrombotic mechanism, the accurate compositions and the sequences of bioactive peptides need to be addressed, so it is important to identify the peptide structure (Zheng et al. 2017; Zanfardino et al. 2017; Ji, Zhang, and Ji 2017; Neves et al. 2017). The composition of many hydrolysates and fermentation liquor containing bioactive peptides must be complex, thus it is hard to elucidate which peptide in a complex mixture exerts a specific bioactivity (Nongonierma and Fitzgerald 2017; Capriotti et al. 2016). Therefore, the isolation techniques must be employed to reduce the compositional complexity of hydrolysates and fermentation liquor for identification of the targeted peptides. During the fractionation and/or peptide enrichment process, bioactivity determination is used as a mean to rank different hydrolysates in terms of their biological potency. The hydrolysates and fermentation broth may be future fractionated according to the peptides physicochemical properties such as molecular mass, hydrophobicity, and isoelectric point by using different the separation and purification technologies including membrane filtration, ion-exchange chromatography, gel or size filtration chromatography, high-performance liquid chromatography or combined methods (Mechmeche et al. 2017; Singh and Vij 2017;

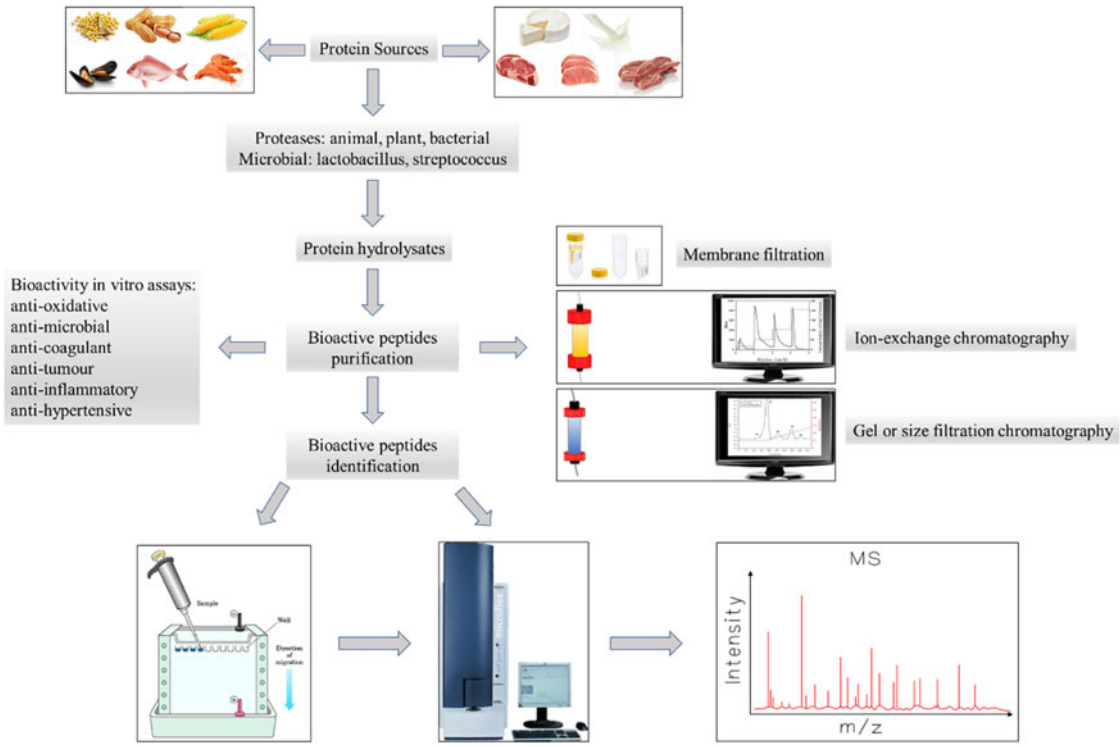

Figure 4. Schematic diagram for preparation, purification and identification of biological peptides.

Lemes et al. 2016; Dallas et al. 2015). When a single and highly bioactive fraction has been obtained, it can be further characterized by identification of the peptide sequences therein with front end separative techniques (e.g., Matrix assisted laser desorption ionization flight time mass spectrometry, MADI-TOF-MS, Ultra Performance Liquid Chromatography time-of-flight mass spectrometer, UPLC-TOF-MS) (Lahrichi et al. 2013; Koehbach et al. 2016; Liu et al. 2015b; Mejri et al. 2017; Català-Clariana et al. 2013).

The schematic diagram for preparation, purification and identification of biological peptides was illustrated in Fig. 4. The identification of bioactive peptides is crucial for research involving dietary peptides as these sequences are liable to be relevant to human health. Also, the bioactive peptides which are assessed in vitro for their bioactive properties would be synthesized based on the identification results and then researched future.

Interaction of antithrombotic peptides and thrombin

Active sites of thrombin

Blood coagulation cascade, a physiological response to vascular injury, is activated by sequential proteolysis of circulating zymogens of serine proteinases resulting in fibrin clot formation (Brahma et al. 2017). Imbalances in blood coagulation may give rise to either loss of clotting activity, leading to hemorrhagic disorders, or unwanted clot formation, resulting in thrombosis. As the main actor of the blood coagulation cascade, thrombin can convert soluble fibrinogen into fibrin filaments and activate the factor V, VII and XI by the negative feedback regulation, then accelerate the formation of thrombus, which makes it a promising target for the treatment of thrombotic diseases.

The crystal structure of thrombin (IIa) was first determined in 1989 (Bode et al. 1989). IIa is activated from a larger precursor protein (prothrombin), and its active form (α-thrombin) consists of a 36-aa light chain (L chain) that is covalently linked by a disulfide bridge to the 259-aa peptidase domain (H chain) (Davie, Fujikawa, and Kisiel 1991). Alpha-thrombin has three main structural domains: a catalytic site and two exosites (I and II) (Fig. 5a).

The active sites of IIa are structured for specific catalytic cleavage of physiological substrates. IIa cleaves, among others, sequences GGGVR-GP and PPSAR-GH in fibrinopeptides A and B, respectively (Herbert 2012). Thrombin strongly prefers arginine as the residue preceding the scissile peptide bond (P_1 site). Substrate/inhibitor residues are denoted P_n, …, P_1, P_1', …, P_m', from N- to C-terminal end, where P_1-P_1' is the scissile peptide bond; the corresponding proteinase subsites that accommodate these residues are termed S_n, …, S_1, S_1', …, S_m' according to the nomenclature (Schechter and Berger 1967). Compared to other blood coagulation serine proteinases, thrombin has a prominent active site cleft, which is deep and narrow, includes four specific pockets: S_1, S_2, S_3 and S_4 (Fig. 5b). The active site contains the classical catalytic triad-His57, Asp102 and Ser195. Two insertion loops (called the 60-loop with residues Leu59-Asn62 and the autolysis loop (149-loop), residues Leu144-Gly150) form the wall of the cleft (Koh et al. 2011). As a predominantly hydrophobic specificity pocket, S_1 contains an aspartic acid residue at its bottom end (Asp-189) that serves as the recognition site for the basic side chain. The S_2 sites of IIa, an insertion loop around residue Trp-60D, occlude a hydrophobic pocket that

can accept larger aliphatic residues such as valine and proline. The S_3 sites are flat and exposed to the solvent. The S_4 sites are again hydrophobic in character, including a conserved Trp-215 and two aliphatic amino acid Ile-174 and Leu-99 (Herbert 2012). Other than the active site of thrombin, the anion binding exosites I and II always play an essential role in the function of IIa (Fig. 5a). Exosite I, a special structure domain formed by 30S and 70S loop, locates on the other side of catalytic regions as specific binding site of fibrinogen and thrombomodulin, interacts specifically with thrombin substrates (e.g., factor V, VII, VIII, protein C and the protease-activated receptors) (Handley et al. 2015; Gandhi, Chen, and Cera 2010; Aisiku et al. 2015). Exosite II, the heparin-binding exosite, extends from "top" to "back" of the thrombin in classical orientation, plays a vital role in interaction with platelet membrane glycoprotein and substrates recognition (Richardson et al. 2000; Bode et al. 1989, Bode and Huber 1992).

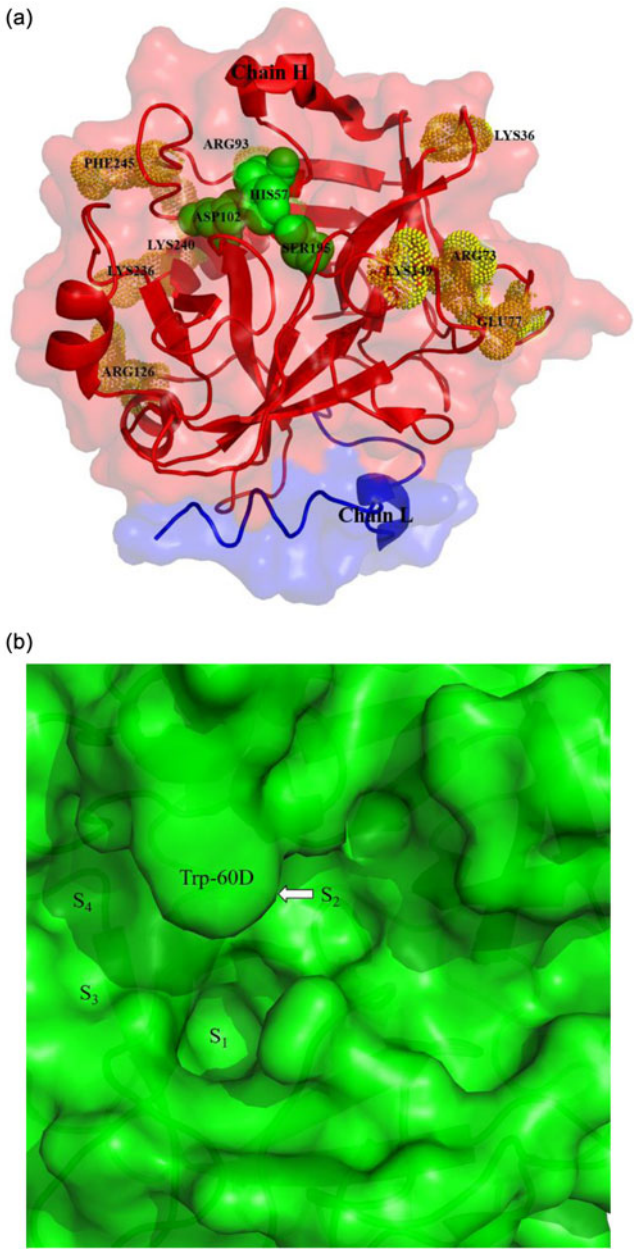

Figure 5. Molecular structures of the IIa (PDB: 2BVR). (a) Ribbon representation of the light chain (blue) and the heavy chain (red). Spheres representation of the catalytic site (His57, Asp109 and Ser195, green). Dots representation of the exosite I (Arg75, Arg77A, Lys149E, yellow) and exosite II (Arg93, Arg126, Lys236, Lys240, Phe245, yellow). (b) Surface representations a magnification of the active site cleft of IIa. The most prominent feature is the deep S_1 pocket. The 60-loop insertion of IIa with the prominent Trp-60D occludes the hydrophobic S_2 pocket. The S_4 pocket floor is hydrophobic in nature, formed by a conserved Trp-215 residue. In IIa, Ile-174 and Leu-99 form a rather shallow pocket that prefers to bind aromatic moieties.

Interaction analysis by solution chemistry

To investigate the interaction mechanism of thrombin and inhibitors in solution, various research methods are utilized according to their own principle (e.g., polyacrylamide gel electrophoresis, biofilm interferometry, isothermal titration calorimeter, surface plasmon resonance, et al.). In contrast to free thrombin, the inhibitory peptides coupled with thrombin to form higher molecule weight complex, which would migrate slower on non-denaturing polyacrylamide gel electrophoresis. Human α-thrombin and madanin (an inhibitory peptide) was mixed in equimolar amounts formation of a single species with a slightly different migration profile from thrombin alone was observed by electrophoretic mobility shift assay, suggesting the formation of a 1:1 thrombin-madanin complex (Figueiredo, De, and Pereira 2013). Richardson and Macedo-Ribeiro also studied the binding behavior of human α-thrombin-haemadin and α-thrombin-boophilin in solution by band shift (Richardson et al. 2000; Macedo-Ribeiro et al. 2008). Based on the similar principle, the size exclusion chromatography also is a practical approach to assess whether the thrombin could interact with the inhibitory peptides (Figueiredo, De, and Pereira 2013).

The thrombin inhibitory peptides binding to immobilized human α-thrombin measured by surface plasmon resonance (SPR). The anophelin[Aa,] an anticoagulant from the malaria vector, binds with high affinity to human α-thrombin to form a stable complex ($K_D = 3.65$ nM) (Figueiredo et al. 2012). Jung applied the surface plasmon resonance to examine the binding affinity between coagulation factors (IX, X, II) and bioactive peptide, isolated from blue mussel, *Mytilus edulis*, suggesting that the inhibitory peptide has lower binding affinity for thrombin (Jung and Kim 2009).

Like the SPR, biolayer interferometry (BLI) based on spectral interferometric principle can monitor the kinetic behavior of the protein adsorption onto the biomaterial surfaces *in situ*, in real-time and without labeling. Wartchow et al analyzed the interaction between three proteins (BCL-2, eIF4E, JNK1) associated with human disease and enzyme protein via BLI technology and concluded the results as the same with SPR and time resolved fluoroisnmunoassay (Wartchow et al. 2011). Melanie comparative analyzed the specialty of enzyme-linked immunosorbent assay, surface plasmon resonance and biolayer interferometry for screening of deoxynivalenol in wheat and wheat dust and determined the affinity among deoxynivalenol-ovalbumin and antibody of wheat/wheat dust (Melanie et al. 2016). Biolayer interferometry is widely used in the analysis of biomolecular

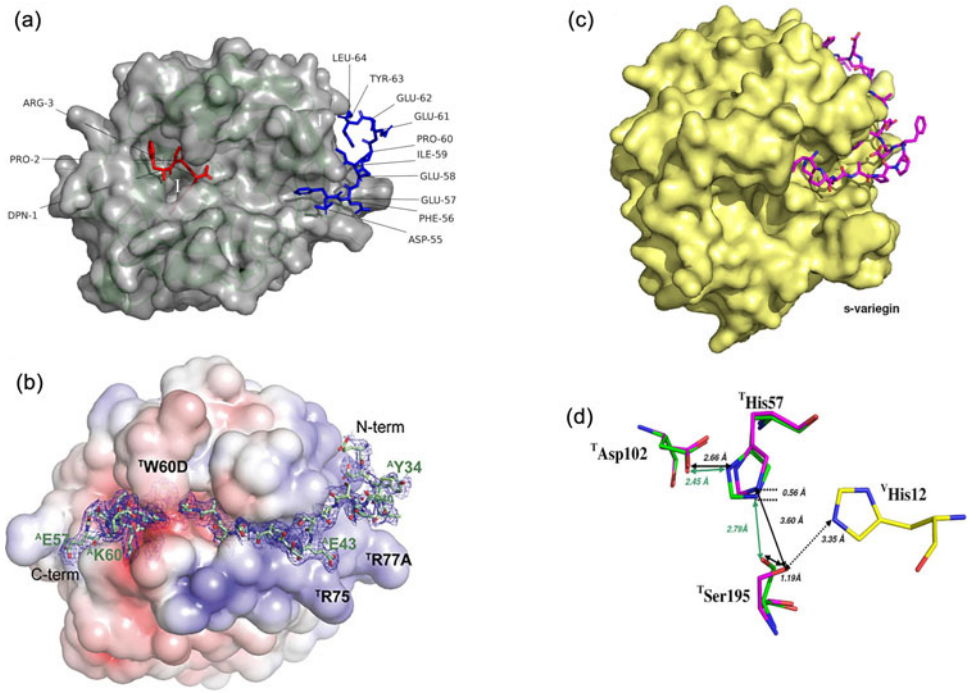

Figure 6. (a) Overall structure of the human α-thrombin–bivalirudin complex. The short fragment I is the dFPR-COOH fragment from bivalirudin, which binds to the active site of α-thrombin. Fragment J is the C-terminal fragment from bivalirudin bound to the exosite I of α-thrombin. (Yamada et al. 2013). (b) Anophelin inhibits α-thrombin in a unique reverse-binding mode. (A) The acidic E32-F45 segment of anophelin (stick model with nitrogen atoms in blue, oxygen in red, and carbon in green) binds to the exosite I of thrombin (positive surface electrostatic potential in blue and negative surface electrostatic potential in red), whereas the downstream D50-L55 segment occupies the active site cleft of the proteinase. The unbiased Fo-Fc electron density (1.5-σ cutoff) for anophelin is displayed as a blue mesh. The thrombin molecule is shown in the standard orientation for serine proteinases (i.e., substrates would run from left to right or exactly opposite to the path followed by the Anopheles inhibitor) (Figueiredo et al. 2012). (c) The structure of thrombin (yellow) in the classical orientation in complex with s-variegin (pink) together with its electron density map (2Fo – Fc) shown contoured at 0.9 s (Koh et al. 2011). (d) Thrombin catalytic triad ^{T}His57, ^{T}Asp102 and ^{T}Ser195 in thrombin-hirugen structure (green) and in thrombin-variegin structure (pink) are superimposed. The ^{T}Ser195 Oγ in thrombin -variegin structure is displaced by 1.19 Å compared to thrombin-hirugen structure. The displacement of ^{T}Ser195 Oγ in thrombin -variegin structure (pink) is due to interactions with ^{V}His12 of s-variegin through hydrogen bond (dotted arrow), rendering ^{T}Ser195 a weak nucleophile that is incapable of catalysis. The imidazole ring of ^{T}His57 also rotated, resulted in a displacement of its Nε by 0.56 Å. Overall, the distance between Nε of ^{T}His57 and Oγ of ^{T}Ser195 increases to 3.60 Å (black arrow) from 2.79 Å (green arrow), disrupts the catalytic charge relay system (Koh et al. 2011).

dynamics, protein-protein (Sun et al. 2013; Brochier and Ravault 2016; Huang et al. 2016b), protein-micro-molecule (Li et al. 2011), protein-nucleotide (Ciesielski, Hytönen, and Kaguni 2016; Grabherr et al. 2011), protein-lipid (Wallner et al. 2013), protein-carbohydrate (Digiandomenico et al. 2012).

The interaction between thrombin and inhibitory peptides in the solution could also be detected by other label-free analysis and detection methods, such as isothermal titration calorimetry (Velazquezcampoy, Leavitt, and Freire 2004), static light scattering (Kameyama and Minton 2006), dynamic light scattering (Gallagher and Woodward 1989), and so on. The information obtained by solution chemistry only determines whether the inhibitory peptides combined with thrombin, but it could not infer the binding sites and forces among them. So, the structural chemistry is needed to apply for investigating the precise inhibitory mechanism.

Interaction analysis by structural chemistry

Since the crystal structure of thrombin was determined in 1989, amounts of thrombin-inhibitor complex were established by X-ray diffraction analysis. Thus, a great deal of high-efficiency inhibitors derived from various species, especially the hematophagous animals (e.g., leeches, mosquitoes, ticks). Bivalirudin, a mimic of hirudin which is a well-known direct thrombin inhibitor, which is a 64-residue anticoagulant peptide originating from the saliva of medicinal leeches (Tanaka-Azevedo et al. 2010), was developed as an artificial peptide composing of 20 residues (Maraganore et al. 1990), had been applied in the medical as a novel anticoagulant for treatment of embolism. The entire sequence of bivalirudin is dFPRP-GGGG-^{53}NGDFEEIPEEYL^{64}, and it has an active site blocker, ($_{D}$-Phe)-Pro-Arg-Pro (dFPRP) at the N-terminus, a glycine linker, and a short peptide mimicking the C-terminal region of hirudin (fragment 53-64 of hirudin) (Yamada et al. 2013). Bivalirudin was hydrolyzed during the crystallization to form a dFPR fragment and a hirudin peptide. α-Thrombin-bivalirudin complex contains the dFPR fragment in the active site and hirudin peptide fragment in the exosite I through hydrogen bonds involving water molecules, and non-covalent interactions (Fig. 6a) (Yamada et al. 2013).

The only mosquito-derived thrombin inhibitor described to date is anophelin (family I77 in the MEROPS classification; http://merops.sanger.ac.uk) (Rawlings, Barrett, and Bateman 2012), first isolated from *A. albimanus* salivary gland extracts (Valenzuela, Francischetti, and Ribeiro 1999; Francischetti, Valenzuela, and Ribeiro 1999). Figueiredo reported the unique molecular mechanism of thrombin

Table 3. Thrombin inhibitors from different animals.

Name	Origin	Target	Molecular Weight	Inhibition constant (K_i values)	References
Hirudin	*Hirudo medicinalis*	Thrombin catalytic site and exosite-1	7,000 Da	20 fM	Markwardt (1970)
Hirullin P18	*Hirudinaria manillensis*	Thrombin exosite-1	7,000 Da	7.8 pM	Steiner et al. (1992)
Haemadin	*Haemadipsia sylvestris*	Thrombin catalytic site and exosite-2	5,000 Da	100 fM	Scharf, Engels, and Tripier (1989)
Triabin	*Triatoma pallidipenis*	Thrombin exosite-1	18,000 Da	3 pM	Salzet et al. (2000)
Rhodniin	*Rhodinius prolixus*	Thrombin catalytic site	11,000 Da	20 pM	Noeske-Jungblut et al. (1995)
Savignin	*Ornithodoros savignyi*	Thrombin catalytic site and exosite-1	12,430 Da	4.89 pM	Van et al. (1996)
Boophilin	*Rhipicephalus (Boophilus) microplus*	Thrombin catalytic site and exosite-1	13,900 Da	1.8 nM	Lai et al. (2004)

inhibition by anophelin (Fig. 6b). In contrast to previously characterized natural bivalent inhibitors that contact one of the exosites through their C-terminal regions (PAR-1, hirudin), anophelin displays an unexpected reverse binding mode to thrombin (Figueiredo et al. 2012), namely, this inhibitor adopts a mostly extended conformation and binds to the proteinase in a reverse orientation (i.e., opposite to substrates) (Fig. 6b). Anophelin interacts with both the active site region (through its C terminus, ^{50}DPGRRL^{55}) and the exosite I (through its N terminus, ^{34}YAAIEAS^{40}) of the thrombin.

Koh identified a novel fast and tight binding thrombin inhibitor from the tropical bont tick and investigated the molecular interactions between thrombin and variegin by the crystal structure of the thrombin-variegin complex at 2.4 Å resolution (Fig. 6c) (Koh et al. 2007; Koh et al. 2011). Like hirudin/hirulog, variegin is a competitive inhibitor of thrombin which binds to thrombin active site through residues 8-14; residues 15-32 binds to thrombin exosite-I; and residues 1-7 (specifically, residues 5-7) are needed for its fast binding. However, unlike hirudin/hirulog, the cleavage product of variegin potently and noncompetitively inhibits thrombin whereas cleaved hirulog 1/bivalirudin, paradoxically, activates the function of the thrombin active site (Koh et al. 2009). Compared to the three catalytic residues of thrombin, the most striking differences are with the Oγ atom of ^{T}Ser195 and the orientation of the imidazole ring of ^{T}His57. The ability of thrombin to cleave its substrates is dependent on the attack of the hydroxyl oxygen of the catalytic ^{T}Ser195 to the carbonyl carbon of the substrate P_1 residue (Figueiredo, De, and Pereira 2013). The change distance between Nε of ^{T}His57 and Oγ of ^{T}Ser195 breaks the crucial strong hydrogen bond needed to form the catalytic charge relay system, which helps attacking the backbone C of the substrate (Fig. 6d). This accounts for this observed classical noncompetitive inhibition for the cleavage product. Another thrombin inhibitor, boophilin, isolated from the salivary glands of *R. microplus*, inhibited thrombin by a different mode of action, namely, it inhibited thrombin in a noncanonical manner (Macedo-Ribeiro et al. 2008). Modeling experiments showed that the C terminal domain of boophilin interacts with exosite-I of thrombin, while the N-terminal domain interacts with thrombin active site.

Currently, a variety of bioactive peptides with anticoagulant function derived from food sources were identified and studied for its interaction mechanism with the thrombin, however, few researches would explore the precise inhibitory mechanism by using the X-ray diffraction analysis for its complex operation. Recently, with the development of computational biology, bioinformatics has been used in research on bioactive peptides and is considered as an effective and practical method to identify novel peptides (Minkiewicz and Dziuba 2008a; Minkiewicz et al. 2008b; Minkiewicz, Dziuba, and Michalska 2011), and predict and reveal the behavior of bioactive peptides from food-derived proteins *in silico* (Cheung et al. 2009; Udenigwe 2014; Vercruysse et al. 2009). Docking algorithms of the computer has become a new approach to investigate thoroughly the interaction between enzymes and inhibitors. Our recent studies have identified 39 bioactive peptides from the *Mytilus edulis* hydrolysate by UPLC-Q-TOF-MS/MS and predicted the anticoagulant function of 26 peptides through structure-activity relationship and affinity activity to thrombin by molecular docking used the unligands thrombin (PDB: 2BVR) (Feng et al. 2018). The interaction of anticoagulant peptide (KNAENELGEVTVR) has similar and strong affinity with an "-CDOKER-_ Energy" of 190.077 kcal/mol as compared with the control (hirudin) of 181.530 kcal/mol. The interactional sites included ^{T}Lys110, ^{T}Met84, ^{T}Pro111, ^{T}Ser83, ^{T}Leu65, ^{T}Ile82, ^{T}Gln38, ^{T}Arg67, ^{T}Thr74 and ^{T}Arg73 which were also involved in the interaction site on the S_1 pocket of thrombin by non-bounded interaction (e.g., hydrogen bound; electrostatic and other hydrophobic categories) (Biela et al. 2012; Feng et al. 2018). As the same with Feng et al (2018), we selected the unliganded thrombin (PDB: 2BVR) as the receptor molecule to study the interaction between thrombin and bioactive peptides from casein screened by bioinformatics based on sequences predicted from *in silico* enzymatic digestion with trypsin (Tu et al. 2017). In contrast with hirudin, FQSEEQQQTEDELQDK, identified from hydrolysate of casein *in silico* simulation, had similar target domain (^{T}Lys36-^{T}Gln38-^{T}Arg73-^{T}Thr74-^{T}Lys81-^{T}Ile82-^{T}Lys110), the partial structure of exosites I and II of thrombin (Fig. 5a), and this result may explain why it showed similar inhibitory activity on thrombin when compared with hirudin (Tu et al., 2017). Structural chemistry, either the X-ray crystalline diffraction or molecular docking, is an important method to present the structural information of thrombin-inhibitors complexes and explains the interaction mechanism on

atomic-level. We can see the active site between the thrombin and inhibitors, which are more convincing than the solution chemistry.

Interaction analysis by biochemistry

Previous studies on antithrombotic activity of bioactive peptides have been carried out using the titration (Markwardt 1970; Iyer and Fareed 1995), colorimetric (Chang, Knecht, and Braun 1983) and microplate reader (Yang, Wang, and Xu 2007) methods. The chromogenic substrate method (colorimetric method), a common way for the thrombin inhibitory assay, is used to evaluate the inhibition of amidolytic activity of thrombin by the bioactive peptides (Brahma et al. 2017; Deng et al. 2010; Dennis et al. 2000; Watanabe et al. 2011). The programmatic process of this experiment is as following: A set of bioactive inhibitory peptides with varied concentrations are pre-incubated with the thrombin in some certain concentration for a few minutes (e.g., 2 minutes), followed by addition of the enzymatic substrate for a while, then add S2238, a specific chromogenic substrate for thrombin from Chromogenix (Monza, Milano, Italy). The release of colored product p-nitroaniline (*p*-NA) was monitored at 405 nm for 10 min in a microplate reader. Percentage inhibition is calculated by taking the rate of increase in absorbance in the absence inhibitor as 100% activity. Dose-response curve is fitted using special software, such as Origin to calculate the IC_{50} and inhibition constant (K_i) (Table 3).

Although the accuracy of the colorimetric method is relatively higher than the titration approach, it requires expensive chromogenic substrate. Thus, the microplate reader method with low biological limit of detection and repeatability standard deviation like the colorimetric method is gradually gaining acceptance among more and more researchers (Jung and Kim 2009; Brahma et al. 2017; Athukorala et al. 2006; Sabbione et al. 2016b). According to Yang, Wang, and Xu (2007) method, we summarized the approach of a general structure in this review. 140 μL of fibrinogen solution (0.1%) and 40 mL thrombin inhibitors or blank buffer (Tris-HCl) were added into the plate wells, mixed and incubated for 5 min. 10 μL thrombin was then added to start the reaction. The fibrinogen, EWPH and thrombin were dissolved in 0.05 mol/L Tris-HCL buffer solution containing 0.12 mmol/L NaCl (pH 7.2). Microplate reader was set at a wavelength of 405 nm, at 37.8 °C.

Summary

Bioactive peptides and protein hydrolysates hold great promise as valuable functional ingredients in healthy diets to fight the global epidemic of non-communicable diseases. The anticoagulant peptides from diverse food sources have varying efficacy for inhibiting thrombosis. Numerous novel anti-thrombotic peptides agents are studied and applied in the clinical stage. However, several challenges (e.g., high cost, sources selection, multi-step isolation/purification procedures) remain to be solved to ensure anticoagulant peptides successful adoption as functional food ingredients that can prevent and cure thrombus. To provide a better understanding of the peptide structure-activity relationship at the molecular level, structural chemistry needs more attention. Standardized methodology for analysis and robust clinical trials to evaluate efficacy of anticoagulant peptides is of critical importance for quality assurance and justification of health claims.

As an important defense system of human, hemostasis involves vessels, platelets, coagulation system and fibrinolysis system. Currently, amounts of researches focus on the coagulation factors of the cascade to find an alternative drug for embolism. Moreover, the early embolus involved by platelets is the basic of thrombosis, thus, the interaction of bioactive peptides and platelets has already been paid attention gradually. Until now, to our knowledge, few groups have considered the role of vascular endothelial cell in thrombosis. Consequently, the exploration of the effect of bioactive peptides on vascular endothelial cell may provide new ideas for treating thrombosis. The combined utilization of advanced technologies, such as mass spectrometry, proteomics, crystallography, cryoelectron microscopy, and so on, would provide strong technical support for these studies.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Natural Science Foundation of China (31771926, 31730069), and the State Key Research and Development Plan "Modern Food Processing and Food Storage and Transportation Technology and Equipment (2017YFD0400201 and 2018YFD0400105)."

References

Aguilar-Toalá, J., L. Santiago-López, C. Peres, C. Peres, H. Garcia, B. Vallejo-Cordoba, A. González-Córdova, and A. Hernández-Mendoza. 2017. Assessment of multifunctional activity of bioactive peptides derived from fermented milk by specific *Lactobacillus plantarum strains*. *Journal of Dairy Science* 100 (1):65–75. doi:10.3168/jds.2016-11846.

Aisiku, O., C. G. Peters, C. K. De, C. C. Ghosh, J. R. Dilks, S. F. Fustolo-Gunnink, M. D. Huang, C. Dockendorff, S. M. Parikh, and B. Flaumenhaft. 2015. Parmodulins inhibit thrombus formation without inducing endothelial injury caused by vorapaxar. *Blood* 125 (12):1976–85. doi:10.1182/blood-2014-09-599910.

Atanassov, A., and B. Tchorbanov. 2009. Synthetic and natural peptides as antithrombotic agents-a view on the current development. *Biotechnology & Biotechnological Equipment* 23 (1):1109–14. doi:10.1080/13102818.2009.10817623.

Athukorala, Y., W. K. Jung, T. Vasanthan, and Y. J. Jeon. 2006. An anticoagulative polysaccharide from an enzymatic hydrolysate of *ecklonia cava*. *Carbohydrate Polymers* 66 (2):184–91. doi:10.1016/j.carbpol.2006.03.002.

Biela, A., M. Khayat, H. Tan, J. Kong, A. Heine, D. Hangauer, and G. Klebe. 2012. Impact of ligand and protein desolvation on ligand binding to the S_1 pocket of thrombin. *Journal of Molecular Biology* 418 (5):350–66. doi:10.1016/j.jmb.2012.01.054.

Bode, W., I. Mayr, U. Baumann, R. Huber, S. R. Stone, and J. Hofsteenge. 1989. The refined 1.9 Å crystal structure of human

α-thrombin: Interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. *The EMBO Journal* 8 (11):3467–75. doi:10.1002/j.1460-2075.1989.tb08511.x.

Bode, W., and R. Huber. 1992. Natural protein proteinase inhibitors and their interaction with proteinase. *European Journal of Biochemistry* 204 (2):433–51. doi:10.1111/j.1432-1033.1992.tb16654.x.

Brahma, R. K., G. Blanchet, S. Kaur, R. M. Kini, and R. Doley. 2017. Expression and characterization of haemathrins, madanin-like thrombin inhibitors, isolated from the salivary gland of tick *haemaphysalis bispinosa* (acari: Ixodidae). *Thrombosis Research* 152:20–29. doi:10.1016/j.thromres.2017.01.012.

Brochier, B. V., and V. Ravault. 2016. High throughput development of a non-protein a monoclonal antibody purification process using mini-columns and bio-layer interferometry. *Engineering in Life Sciences* 16 (2):152–9. doi:10.1002/elsc.201400244.

Caliceti, C., P. Rizzo, R. Ferrari, F. Fortini, G. Aquila, E. Leoncini, L. Zambonin, B. Rizzo, D. Calabria, P. Simoni, et al. 2017. Novel role of the nutraceutical bioactive compound berberine in lectin-like OxLDL receptor 1-mediated endothelial dysfunction in comparison to lovastatin. *Nutrition, Metabolism & Cardiovascular Diseases* 27 (6):552–63. doi:10.1016/j.numecd.2017.04.002.

Capriotti, A. L., C. Cavaliere, S. Piovesana, R. Samperi, and A. Laganà. 2016. Recent trends in the analysis of bioactive peptides in milk and dairy products. *Analytical and Bioanalytical Chemistry* 408 (11):2677–85. doi:10.1007/s00216-016-9303-8.

Català-Clariana, S., F. Benavente, E. Giménez, J. Barbosa, and V. Sanz-Nebot. 2013. Identification of bioactive peptides in hypoallergenic infant milk formulas by CE-TOF-MS assisted by semiempirical model of electromigration behavior. *Electrophoresis* 34 (13):1886–94. doi:10.1002/elps.201200547.

Chang, J. Y., R. Knecht, and D. G. Braun. 1983. A new method for the selective isolation of cysteine-containing peptides. Specific labelling of the thiol group with a hydrophobic chromophore. *Biochemical Journal* 211 (1):163–71. doi:10.1042/bj2110163.

Choi, J. H., S. J. Kim, and S. Kim. 2016. A novel anticoagulant protein with antithrombotic properties from the mosquito *Culex pipiens pallens*. *International Journal of Biological Macromolecules* 93:156–66. doi:10.1016/j.ijbiomac.2016.08.055.

Ciesielski, G. L., V. P. Hytönen, and L. S. Kaguni. 2016. Biolayer interferometry: A novel method to elucidate protein-protein and protein-DNA interactions in the mitochondrial DNA replisome. *Methods in Molecular Biology* 1351:223–31. doi:10.1007/978-1-4939-3040-1-17.

Clemetson, K. J., and J. M. Clemetson. 2001. Platelet collagen receptors. *Thrombosis and Haemostasis* 86 (1):189–97. doi:0.1055/s-0037-1616217.

Cheung, I. W. Y., S. Nakayama, M. N. K. Hsu, A. G. P. Samaranayaka, and E. C. Y. Li-Chan. 2009. Angiotensin-I converting enzyme inhibitory activity of hydrolysates from oat (*Avena sativa*) proteins by in silico and in vitro analyses. *Journal of Agricultural and Food Chemistry* 57 (19):9234–42. doi:10.1021/jf9018245.

Daliri, B. M., D. H. Oh, and B. H. Lee. 2017. Bioactive peptides. *Foods* 6 (5):32. doi:10.3390/foods6050032.

Daliri, E. B., B. H. Lee, and D. H. Oh. 2017. Current trends and perspectives of bioactive peptides. *Critical Reviews in Food Science and Nutrition* 57:1–12. doi:10.1080/10408398.2017.1319795.

Dallas, D. C., A. Guerrero, E. A. Parker, R. C. Robinson, J. Gan, J. B. German, D. Barile, and C. B. Lebrilla. 2015. Current peptidomics: Applications, purification, identification, quantification, and functional analysis. *Proteomics* 15 (5–6):1026–38. doi:10.1002/pmic.201400310.

Davie, E. W., and O. D. Ratnoff. 1964. Waterfall sequence for intrinsic blood clotting. *Science* 145 (3638):1310–2. doi:10.1126/science.145.3638.1310.

Davie, E. W., K. Fujikawa, and W. Kisiel. 1991. The coagulation Cascade: Initiation, maintenance, and regulation. *Biochemistry* 30 (43):10363–70. doi:10.1021/bi00107a001.

Deng, L., Q. He, T. Kang, H. Yin, X. Jin, H. Li, W. Gan, C. Yang, J. Hua, Y. Wu, et al. 2010. Identification of an anticoagulant peptide that inhibits both FXIa and FVIIa/tissue factor from the blood-feeding nematode *Ancylostoma caninum*. *Biochemical and Biophysical Research Communications* 392 (2):155–9. doi:10.1016/j.bbrc.2009.12.177.

Dennis, M. S., C. Eigenbrot, N. J. Skelton, M. H. Ultsch, L. Santell, M. A. Dwyer, M. P. O'Connell, and R. A. Lazarus. 2000. Peptide exosite inhibitors of factor VIIa as anticoagulants. *Nature* 404 (6777):465–70. doi:10.1038/35006574.

Díazgómez, J. L., F. Castorenatorres, R. E. Preciadoortiz, and S. Garcíalara. 2017. Anti-cancer activity of maize bioactive peptides. *Frontiers in Chemistry* 5:1–8. doi:10.3389/fchem.2017.00044.

Digiandomenico, A., P. Warrener, M. Hamilton, S. Guillard, P. Ravn, R. Minter, M. M. Camara, V. Venkatraman, R. S. Macgill, J. Lin, et al. 2012. Identification of broadly protective human antibodies to *Pseudomonas aeruginosa* exopolysaccharide psl by phenotypic screening. *The Journal of Experimental Medicine* 209 (7):1273–87. doi:10.1084/jem.20120033.

Eikelboom, J. W., and J. I. Weitz. 2010. New anticoagulants. *Circulation* 121 (13):1523–32. doi:10.1161/CIRCULATIONAHA.109.853119.

Eriksson, B. I., D. J. Quinlan, and J. I. Weitz. 2009. Comparative pharmacodynamics and pharmacokinetics of oral direct thrombin and factor Xa inhibitors in development. *Clinical Pharmacokinetics* 48 (1):1–22. doi:10.2165/0003088-200948010-00001.

Feng, L., M. Tu, M. Qiao, F. Fan, H. Chen, W. Song, and M. Du. 2018. Thrombin inhibitory peptides derived from *mytilus edulis*, proteins: Identification, molecular docking and in silico prediction of toxicity. *European Food Research and Technology* 244 (2):207–17. doi:10.1007/s00217-017-2946-7.

Ferri, M., J. Graen-Heedfeld, K. Bretz, F. Guillon, E. Michelini, M. M. Calabretta, M. Lamborghini, N. Gruarin, A. Roda, A. Kraft, et al. 2017. Peptide fractions obtained from rice by-products by means of an environment-friendly process show in vitro health-related bioactivities. *PLoS One* 12 (1):e0170954. doi:10.1371/journal.pone.0170954.

Figueiredo, A. C., S. D. De, and P. J. Pereira. 2013. The tick-derived anticoagulant madanin is processed by thrombin and factor Xa. *PLoS One* 8:e71866. doi:10.1371/journal.pone.0071866.

Figueiredo, A. C., S. D. De, R. Gutiérrezgallego, T. B. Cereija, S. Macedoribeiro, P. Fuentesprior, and P. J. Barbosapereira. 2012. Unique thrombin inhibition mechanism by anophelin, an anticoagulant from the malaria vector. *Proceedings of the National Academy of Sciences of the United States of America* 109:E3649–58. doi: 10.1073/pnas.1211614109.

Flaumenhaft, R. 2013. Protein disulfide isomerase as an antithrombotic target. *Trends in Cardiovascular Medicine* 23 (7):264–8. doi:10.1016/j.tcm.2013.03.001.

Fosgerau, K., and T. Hoffmann. 2015. Peptide therapeutics: Current status and future directions. *Drug Discovery Today* 20 (1):122–8. doi:10.1016/j.drudis.2014.10.003.

Francischetti, I. M., J. G. Valenzuela, and J. M. Ribeiro. 1999. Anophelin: Kinetics and mechanism of thrombin inhibition. *Biochemistry* 38 (50):16678–85. doi:10.1021/bi991231p.

Gallagher, W. H., and C. K. Woodward. 1989. The concentration dependence of the diffusion coefficient for bovine pancreatic trypsin inhibitor: A dynamic light scattering study of a small protein. *Biopolymers* 28 (11):2001–24. doi:10.1002/bip.360281115.

Gandhi, P. S., Z. Chen, and E. D. Cera. 2010. Crystal structure of thrombin bound to the uncleaved extracellular fragment of PAR1. *Journal of Biological Chemistry* 285 (20):15393–8. doi:10.1074/jbc.M110.115337.

Grabherr, M. G., J. Pontiller, E. Mauceli, W. Ernst, M. Baumann, T. Biagi, R. Swofford, P. Russell, M. C. Zody, F. D. Palma, et al. 2011. Exploiting nucleotide composition to engineer promoters. *PLoS One* 6 (5):e20136. doi:10.1371/journal.pone.0020136.

Handley, L. D., N. A. Treuheit, V. J. Venkatesh, and E. A. Komives. 2015. Thrombomodulin binding selects the catalytically active form of thrombin. *Biochemistry* 54(43):6650–8. doi:10.1021/acs.biochem.5b00825.

He, R., A. T. Girgih, E. Rozoy, L. Bazinet, X. R. Ju, and R. E. Aluko. 2016. Selective separation and concentration of antihypertensive peptides from rapeseed protein hydrolysate by electrodialysis with

ultrafiltration membranes. *Food Chemistry* 197:1008–14. doi:10.1016/j.foodchem.2015.11.081.

Herrera, C. F., J. C. Ruiz Ruiz, A. D. Betancur, J. J. Acevedo Fernández, and M. R. Segura Campos. 2016. The hypolipidemic effect and antithrombotic activity of *Mucuna pruriens* protein hydrolysates. *Food & Function* 7 (1):434–44. doi: 10.1039/c5fo01012h.

Herrera, C. F., J. C. Ruiz-Ruiz, D. Betancur-Ancona, and M. R. Segura-Campos. 2016. Potential therapeutic applications of *Mucuna pruriens* peptide fractions purified by high-performance liquid chromatography as angiotensin-converting enzyme inhibitors, antioxidants, antithrombotic and hypocholesterolemic agents. *Journal of Medicinal Food* 19(2):187–95. doi:10.1089/jmf.2015.0098.

Hirsh, J., M. O'Donnell, and J. I. Weitz. 2005. New anticoagulants. *Blood* 105 (2):453–63. doi:10.1182/blood-2003-12-4195.

Ho, T. Y., C. C. Li, H. Y. Lo, F. Y. Chen, and C. Y. Hsiang. 2017. Corn silk extract and its bioactive peptide ameliorated lipopolysaccharide-induced inflammation in mice via the nuclear factor-κB signaling pathway. *Journal of Agricultural and Food Chemistry* 65 (4):759–68. doi:10.1021/acs.jafc.6b03327.

Huang, J., Q. Liu, B. Xue, L. Chen, Y. Wang, S. Ou, and X. Peng. 2016. Angiotensin-I-converting enzyme inhibitory activities and *in vivo* antihypertensive effects of sardine protein hydrolysate. *Journal of Food Science* 81 (11):H2831–40. doi:10.1111/1750-3841.13508.

Huang, X., R. Chen, H. Xu, W. Lai, and Y. Xiong. 2016. Nanospherical brush as catalase container for enhancing the detection sensitivity of competitive plasmonic ELISA. *Analytical Chemistry* 88(3):1951–8. doi:10.1021/acs.analchem.5b04518.

Huang, Y. L., M. F. Ma, C. J. Chow, and Y. H. Tsai. 2017. Angiotensin I-converting enzyme inhibitory and hypocholesterolemic activities: Effects of protein hydrolysates prepared from *Achatina fulica* snail foot muscle. *International Journal of Food Properties* 20 (12):3102–11. doi:10.1080/10942912.2016.1274904.

Herbert, N. 2012. The role of structural information in the discovery of direct thrombin and factor Xa inhibitors. *Trends in Pharmacological Sciences* 33:279–88. doi:10.1016/j.tips.2012.03.004.

Indumathi, P., and A. Mehta. 2016. A novel anticoagulant peptide from the Nori hydrolysate. *Journal of Functional Foods* 20:606–17. doi:10.1016/j.jff.2015.11.016.

Iyer, L., and J. Fareed. 1995. Determination of specific activity of recombinant hirudin using a thrombin titration method. *Thrombosis Research* 78 (3):259–63. doi:10.1016/0049-3848(95)90877-1.

Jasuja, R., F. H. Passam, D. R. Kennedy, S. H. Kim, L. V. Hessem, L. Lin, S. R. Bowley, S. S. Joshi, J. R. Dilks, B. Furie, et al. 2012. Protein disulfide isomerase inhibitors constitute a new class of antithrombotic agents. *Journal of Clinical Investigation* 122 (6):2104–13. doi:10.1172/JCI61228.

Ji, W., C. Zhang, and H. Ji. 2017. Two novel bioactive peptides from antarctic krill with dual angiotensin converting enzyme and dipeptidyl peptidase IV inhibitory activities. *Journal of Food Science* 82 (7):1742–9. doi:10.1111/1750-3841.13735.

Jo, H. Y., W. K. Jung, and S. K. Kim. 2008. Purification and characterization of a novel anticoagulant peptide from marine echiuroid worm, *Urechis unicinctus*. *Process Biochemistry* 43(2):179–84. doi:10.1016/j.procbio.2007.11.011.

Jung, W. K., and S. K. Kim. 2009. Isolation and characterisation of an anticoagulant oligopeptide from blue mussel, *Mytilus edulis*. *Food Chemisrty* 117(4):687–92. doi:10.1016/j.foodchem.2009.04.077.

Jung, W. K., H. Y. Jo, Z. J. Qian, Y. J. Jeong, S. G. Park, I. W. Choi, and S. K. Kim. 2007. A novel anticoagulant protein with high affinity to blood coagulation factor Va from *Tegillarca granosa*. *BMB Reports* 40 (5):832–8. doi:10.5483/BMBRep.2007.40.5.832.

Kameyama, K., and A. Minton. 2006. Rapid quantitative characterization of protein interactions by composition gradient static light scattering. *Biophysical Journal* 90 (6):2164–9. doi:10.1529/biophysj.105.074310.

Kastelowitz, N., R. Tamura, A. Onasoga, T. J. Stalker, O. R. White, P. N. Brown, G. L. Brodsky, L. F. Brass, B. R. Branchford, P. J. Di, et al. 2017. Peptides derived from MARCKS block coagulation complex assembly on phosphatidylserine. *Scientific Reports* 7 (1):17. doi:10.1038/s41598-017-04494-y.

Khiari, Z., D. Rico, A. B. Martin-Diana, and C. Barry-Ryan. 2014. Structure elucidation of ACE-inhibitory and antithrombotic peptides isolated from mackerel skin gelatine hydrolysates. *Journal of the Science of Food and Agriculture* 94(8):1663–71. doi:10.1002/jsfa.6476.

Koehbach, J., C. W. Gruber, C. Becker, D. P. Kreil, and A. Jilek. 2016. MALDI TOF/TOF-based approach for the identification OFD-amino acids in biologically active peptides and proteins. *Journal of Proteome Research* 15 (5):1487–96. doi:10.1021/acs.jproteome.5b01067.

Koh, C. Y., M. Kazimirova, P. A. Nuttall, and R. M. Kini. 2009. Noncompetitive inhibitor of thrombin. *Chembiochem* 10 (13):2155–8. doi:10.1002/cbic.200900371.

Koh, C. Y., M. Kazimirova, A. Trimnell, P. Takac, M. Labuda, P. A. Nuttall, and R. M. Kini. 2007. Variegin, a novel fast and tight binding thrombin inhibitor from the tropical bont tick. *Journal of Biological Chemistry* 282 (40):29101–13. doi:10.1074/jbc.M705600200.

Koh, C. Y., S. Kumar, M. Kazimirova, P. A. Nuttall, U. P. Radhakrishnan, S. Kim, J. Jagadeeswaran, T. Imamura, J. Mizuguchi, S. Iwanaga., et al. 2011. Crystal structure of thrombin in complex with s-variegin: Insights of a novel mechanism of inhibition and design of tunable thrombin inhibitors. *PLoS One* 6 (10):e26367. doi:10.1371/journal.pone.0026367.

Kong, Y., S. Li, Y. Shao, Z. L. He, M. M. Chen, X. Ming, and J. F. Wei. 2014. Antithrombotic peptides from *Scolopendra subspinipes mutilans* hydrolysates. *International Journal of Peptide Research and Therapeutics* 20 (2):245–52. doi:10.1007/s10989-013-9387-3.

Korhonen, H., and A. Pihlanto. 2006. Bioactive peptides: Production and functionality. *International Dairy Journal* 16 (9):945–60. doi:10.1016/j.idairyj.2005.10.012.

Koyama, T., K. Noguchi, Y. Aniya, and M. Sakanashi. 1998. Analysis for sites of anticoagulant action of plancinin, a new anticoagulant peptide isolated from the starfish *Acanthaster planci*, in the blood coagulation cascade. *General Pharmacology* 31 (2):277–82. doi:10.1016/S0306-3623(97)00443-6.

Lahrichi, S. L., M. Affolter, I. S. Zolezzi, and A. Panchaud. 2013. Food peptidomics: Large scale analysis of small bioactive peptides-a pilot study. *Journal of Proteomics* 88:83–91. doi:10.1016/j.jprot.2013.02.018.

Lai, R., H. Takeuchi, J. Jonczy, H. H. Rees, and P. C. Turner. 2004. A thrombin inhibitor from the ixodid tick, *Amblyomma hebraeum*. *Gene* 342 (2):243–9. doi:10.1016/j.gene.2004.07.012.

Lemes, A. C., L. Sala, J. D. C. Ores, A. R. C. Braga, M. B. Egea, and K. F. Fernandes. 2016. A review of the latest advances in encrypted bioactive peptides from protein-rich waste. *International Journal of Molecular Sciences* 17 (6):950. doi:10.3390/ijms17060950.

Li, J., A. Schantz, M. Schwegler, and G. Shankar. 2011. Detection of low-affinity anti-drug antibodies and improved drug tolerance in immunogenicity testing by octet®, biolayer interferometry. *Journal of Pharmaceutical and Biomedical Analysis* 54(2):286–94. doi:10.1016/j.jpba.2010.08.022.

Liu, Y., W. Luo, H. Yang, W. Fang, T. Xi, Y. Li, and J. Xiong. 2015. Stimulation of nitric oxide production contributes to the antiplatelet and antithrombotic effect of new peptide pENW (pGlu-Asn-Trp). *Thrombosis Research* 136 (2):319–27. doi:10.1016/j.thromres.2015.05.016.

Liu, R., W. Zheng, J. Li, L. Wang, H. Wu, X. Wang, and L. Shi. 2015. Rapid identification of bioactive peptides with antioxidant activity from the enzymatic hydrolysate of *Mactra veneriformis*, by UPLC-Q-TOF mass spectrometry. *Food Chemistry* 167:484–9. doi:10.1016/j.foodchem.2014.06.113.

Macedo-Ribeiro, S., C. Almeida, B. M. Calisto, T. Friedrich, R. Mentele, J. Stürzebecher, P. Fuentes-Prior, and P. J. Pereira. 2008. Isolation, cloning and structural characterisation of boophilin, a multifunctional Kunitz-type proteinase inhibitor from the cattle tick. *PLoS One* 3 (2):e1624. doi:10.1371/journal.pone.0001624.

Macfarlane, R. G. 1964. An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. *Nature* 202 (4931):498–9. doi:10.1038/202498a0.

Mackman, N. 2008. Triggers, targets and treatments for thrombosis. *Nature* 451 (7181):914–8. doi:10.1038/nature06797.

Maraganore, J. M., P. Bourdon, J. Jablonski, K. L. Ramachandran, and J. W. Fenton. 1990. Design and characterization of hirulogs: A novel class of bivalent peptide inhibitors of thrombin. *Biochemistry* 29 (30):7095–101. doi:10.1021/bi00482a021.

Marcone, S., O. Belton, and D. J. Fitzgerald. 2017. Milk-derived bioactive peptides and their health promoting effects: A potential role in atherosclerosis. *British Journal of Clinical Pharmacology* 83 (1): 152–62. doi:10.1111/bcp.13002.

Markwardt, F. 1970. Hirudin as an inhibitor of thrombin. *Methods in Enzymology* 19:924–32. doi:10.1016/0076-6879(70)19082-3.

Martínez-Sánchez, S. M., A. Minguela, D. Prieto-Merino, M. P. Zafrilla-Rentero, J. Abellán-Alemán, and S. Montoro-García. 2017. The effect of regular intake of dry-cured ham rich in bioactive peptides on inflammation, platelet and monocyte activation markers in humans. *Nutrients* 9 (4):321–11. doi:10.3390/nu9040321.

McNicol, A., and S. J. Israels. 2003. Platelets and anti-platelet therapy. *Journal of Pharmacological Sciences* 93 (4):381–96. doi:10.1254/jphs.93.381.

Mechmeche, M., F. Kachouri, H. Ksontini, and M. Hamdi. 2017. Production of bioactive peptides from tomato seed isolate by *Lactobacillus plantarum* fermentation and enhancement of antioxidant activity. *Food Biotechnology* 31 (2):94–113. doi:10.1080/08905436.2017.1302888.

Mejri, L., R. Vásquez-Villanueva, M. Hassouna, M. L. Marina, and M. C. García. 2017. Identification of peptides with antioxidant and antihypertensive capacities by RP-HPLC-Q-TOF-MS in dry fermented camel sausages inoculated with different starter cultures and ripening times. *Food Research International* 100:708–16. doi:10.1016/j.foodres.2017.07.072.

Melanie, S., M. P. Daniel, M. Kara, Y. Guo, E. Mia, O. Richard, D. S. Sarah, and M. Chris. 2016. Comparison of enzyme-linked immunosorbent assay, surface plasmon resonance and biolayer interferometry for screening of deoxynivalenol in wheat and wheat dust. *Toxins* 8 (4):103. doi:10.3390/toxins8040103.

Minkiewicz, P., Dziuba. J. 2008a. Food peptidomics. *Food Technology and Biotechnology* 4:1–10.

Minkiewicz, P., J. Dziuba, A. Iwaniak, M. Dziuba, and M. Darewicz. 2008b. BIOPEP database and other programs for processing bioactive peptide sequences. *Journal of AOAC International* 91:965–980.

Minkiewicz, P., J. Dziuba, and J. Michalska. 2011. Bovine meat proteins as potential precursors of biologically active peptides-A computational study based on the BIOPEP database. *Food Science and Technology International* 17 (1):39–45. doi:10.1177/1082013210368461.

Nasri, R., I. B. Amor, A. Bougatef, N. Nedjar-Arroume, P. Dhulster, J. Gargouri, M. K. Châabouni, and M. Nasri. 2012. Anticoagulant activities of goby muscle protein hydrolysates. *Food Chemistry* 133 (3):835–41. doi:10.1016/j.foodchem.2012.01.101.

Neves, A. C., P. A. Harnedy, M. B. O'Keeffe, and R. J. Fitzgerald. 2017. Bioactive peptides from Atlantic salmon (*Salmo salar*) with angiotensin converting enzyme and dipeptidyl peptidase IV inhibitory, and antioxidant activities. *Food Chemistry* 218:396–405. doi:10.1016/j.foodchem.2016.09.053.

New, R., M. Bogus, G. S. Bansal, M. Dryjska, K. Zajkowska, and M. Burnet. 2017. Efficacy of bioactive cyclic peptides in rheumatoid arthritis: Translation from in vitro to in vivo models. *Molecules* 22 (10):1613–8. doi:10.3390/molecules22101613.

Nimalaratne, C., N. Bandara, and J. Wu. 2015. Purification and characterization of antioxidant peptides from enzymatically hydrolyzed chicken egg white. *Food Chemistry* 188:467–72. doi:10.1016/j.foodchem.2015.05.014.

Noeske-Jungblut, C., B. Haendler, P. Donner, A. Alagon, L. Possani, and W.-D. Schleuning. 1995. Triabin, a highly potent exosite inhibitor of thrombin. *Journal of Biological Chemistry* 270 (48):28629–34. doi:10.1074/jbc.270.48.28629.

Nongonierma, A. B., and R. J. Fitzgerald. 2017. Strategies for the discovery and identification of food protein-derived biologically active peptides. *Trends in Food Science and Technology* 69:289–305. doi:10.1016/j.tifs.2017.03.003.

Nurdiani, R., T. Vasiljevic, T. Yeager, T. K. Singh, and O. N. Donkor. 2017. Bioactive peptides with radical scavenging and cancer cell cytotoxic activities derived from flathead (*Platycephalus fuscus*) by-products. *European Food Research and Technology* 243 (4):627–37. doi:10.1007/s00217-016-2776-z.

Pripp, A. H., T. Isaksson, L. Stepaniak, and T. Sørhaug. 2004. Quantitative structure-activity relationship modelling of ACE-inhibitory peptides derived from milk proteins. *European Food Research and Technology* 219 (6):579–83. doi:10.1007/s00217-004-1004-4.

Qiao, M., M. Tu, Z. Wang, F. Mao, H. Chen, L. Qin, and M. Du. 2018. Identification and antithrombotic activity of peptides from blue mussel (*Mytilus edulis*) protein. *International Journal of Molecular Sciences* 19 (1):138–12. doi:10.3390/ijms19010138.

Rafiq, S., N. Huma, I. Pasha, M. Shahid, and H. Xiao. 2017. Angiotensin-converting enzyme-inhibitory and antithrombotic activities of soluble peptide extracts from buffalo and cow milk cheddar cheeses. *International Journal of Dairy Technology* 70 (3):380–8. doi:10.1111/1471-0307.12373.

Rajapakse, N., W. K. Jung, E. Mendis, S. H. Moon, and S. K. Kim. 2005. A novel anticoagulant purified from fish protein hydrolysate inhibits factor XIIa and platelet aggregation. *Life Science* 76 (22): 2607–19. doi:10.1016/j.lfs.2004.12.010.

Rawlings, N. D., A. J. Barrett, and A. Bateman. 2012. MEROPS: The database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Research* 40(D1):D343–50. doi:10.1093/nar/gkr987.

Richardson, J. L., B. Kröger, W. Hoeffken, J. E. Sadler, P. Pereira, R. Huber, W. Bode, and P. Fuentes-Prior. 2000. Crystal structure of the human α-thrombin-haemadin complex: An exosite II-binding inhibitor. *The EMBO Journal* 19 (21):5650–60. doi:10.1093/emboj/19.21.5650.

Rizzello, C. G., A. Lorusso, V. Russo, D. Pinto, B. Marzani, and M. Gobbetti. 2017. Improving the antioxidant properties of quinoa flour through fermentation with selected autochthonous lactic acid bacteria. *International Journal of Food Microbiology* 241:252–61. doi:10.1016/j.ijfoodmicro.2016.10.035.

Rodríguez-Díaz, J. C., L. E. Kurozawa, F. M. Netto, and M. D. Hubinger. 2011. Optimization of the enzymatic hydrolysis of blue shark skin. *Journal of Food Science* 76 (7):C938–49. doi:10.1111/j.1750-3841.2011.02318.x

Rojas-Ronquillo, R., A. Cruz-Guerrero, A. Flores-Nájera, G. Rodríguez-Serrano, L. Gómez-Ruiz, J. P. Reyes-Grajeda, J. Jiménez-Guzmán, and M. García-Garibay. 2012. Antithrombotic and angiotensin-converting enzyme inhibitory properties of peptides released from bovine casein by *Lactobacillus casei Shirota*. *International Dairy Journal* 26 (2):147–54. doi:10.1016/j.idairyj.2012.05.002.

Gustafsson, D., J.-E. Nyström, S. Carlsson, U. Bredberg, U. Eriksson, E. Gyzander, M. Elg, T. Antonsson, K.-J. Hoffmann, A.-L. Ungell, et al. 2001. The direct thrombin inhibitor melagatran and its oral prodrug H376/95: Intestinal absorption properties, biochemical and pharmacodynamic effects. *Thrombosis Research* 101 (3):171–81. doi:10.1039/b001793k.

Rusconi, C. P., E. Scardino, J. Layzer, G. A. Pitoc, T. L. Ortel, D. Monroe, and B. A. Sullenger. 2002. RNA aptamers as reversible antagonists of coagulation factor IXa. *Nature* 419 (6902):90–94. doi:10.1038/nature00963.

Sabbione, A. C., A. E. Nardo, M. C. Añón, and A. Scilingo. 2016. Amaranth peptides with antithrombotic activity released by simulated gastrointestinal digestion. *Journal of Functional Foods* 20: 204–14. doi:10.1016/j.jff.2015.10.015.

Sabbione, A. C., G. Rinaldi, M. C. Añón, and A. A. Scilingo. 2016. Antithrombotic effects of *Amaranthus hypochondriacus* proteins in rats. *Plant Foods for Human Nutrition* 71 (1):19–27. doi:10.1007/s11130-015-0517-2.

Sabbione, A. C., A. Scilingo, and M. C. Añón. 2015. Potential antithrombotic activity detected in amaranth proteins and its hydrolysates.

LWT - Food Science and Technology 60 (1):171–7. doi:10.1016/j.lwt.2014.07.015.

Salzet, M., V. Chopin, J. Baert, I. Matias, and J. Malecha. 2000. Theromin, a novel leech thrombin inhibitor. *Journal of Biological Chemistry* 275 (40):30774–80. doi:10.1074/jbc.M000787200.

Scharf, M., J. Engels, and D. Tripier. 1989. Primary structures of new 'iso-hirudins. *FEBS Letters* 255 (1):105–10. doi:10.1016/0014-5793(89)81070-1.

Schechter, I., and A. Berger. 1967. On the size of the active site in proteases. I. Papain. *Biochemical and Biophysical Research Communications* 27 (2):157–62. doi:10.1016/S0006-291X(67)80055-X.

Shimizu, M., N. Sawashita, F. Morimatsu, J. Ichikawa, Y. Taguchi, Y. Ijiri, and J. Yamamoto. 2009. Antithrombotic papain-hydrolyzed peptides isolated from pork meat. *Thrombosis Research* 123 (5):753–7. doi:10.1016/j.thromres.2008.07.005.

Silva, F. G. D. E., B. Hernández-Ledesma, L. Amigo, F. M. Netto, and B. Miralles. 2017. Identification of peptides released from flaxseed (*Linum usitatissimum*) protein by alcalase® hydrolysis: Antioxidant activity. *LWT-Food Science and Technology* 76:140–6. doi:10.1016/j.lwt.2016.10.049.

Singh, B. P., and S. Vij. 2017. Growth and bioactive peptides production potential of Lactobacillus plantarum, strain C2 in soy milk: A LC/MS-MS based revelation for peptides biofunctionality. *LWT - Food Science and Technology* 86:293–301. doi:10.1016/j.lwt.2017.08.013.

Skrzypczak, K., W. Gustaw, D. Szwajgier, E. Fornal, and A. Waśko. 2017. κ-casein as a source of short-chain bioactive peptides generated by *Lactobacillus helveticus*. *Journal of Food Science and Technology* 54 (11):3679–88. doi:10.1007/s13197-017-2830-2.

Song, R., R. Wei, B. Zhang, and D. Wang. 2012. Optimization of the antibacterial activity of half-fin anchovy (*Setipinna taty*) hydrolysates. *Food and Bioprocess Technology* 5 (5):1979–89. doi:10.1007/s11947-010-0505-3.

Steiner, V., R. Knecht, K. O. Boernsen, E. Gassmann, S. R. Stone, F. Raschdorf, J. M. Schlaeppi, and R. Maschler. 1992. Primary structure and function of novel O-glycosylated hirudins from the leech *Hirudinaria manillensis*. *Biochemistry* 31 (8):2294–8. doi:10.1021/bi00123a012.

Sun, T., F. Reid, Y. Liu, Y. Cao, P. Estep, C. Nauman, and Y. Xu. 2013. High throughput detection of antibody self-interaction by bio-layer interferometry. *Mabs* 5 (6):838–41. doi:10.4161/mabs.26186.

Tanaka-Azevedo, A. M., K. Morais-Zani, R. J. Torquato, and A. S. Tanaka. 2010. Thrombin inhibitors from different animals. *Journal of Biomedicine and Biotechnology* 2010:1–9. doi:10.1155/2010/641025.

Tornesello, A. L., L. Buonaguro, M. L. Tornesello, and F. M. Buonaguro. 2017. New insights in the design of bioactive peptides and chelating agents for imaging and therapy in oncology. *Molecules* 22:1–21. doi:10.3390/molecules22081282.

Tu, M., L. Feng, Z. Wang, M. Qiao, F. Shahidi, W. Lu, and M. Du. 2017. Sequence analysis and molecular docking of antithrombotic peptides from casein hydrolysate by trypsin digestion. *Journal of Functional Foods* 32:313–23. doi:10.1016/j.jff.2017.03.015.

Udenigwe, C. C. 2014. Bioinformatics approaches, prospects and challenges of food bioactive peptide research. *Trends Food Science and Technology* 36 (2):137–43. doi:10.1016/j.tifs.2014.02.004.

Valenzuela, J. G., I. M. Francischetti, and J. M. Ribeiro. 1999. Purification, cloning, and synthesis of a novel salivary anti-thrombin from the mosquito *Anopheles albimanus*. *Biochemistry* 38 (34):11209–15. doi:10.1021/bi990761i.

Van, D. L. A., M. T. Stubbs, W. Bode, T. Friedrich, C. Bollschweiler, W. Höffken, and R. Huber. 1996. The ornithodorin-thrombin crystal structure, a key to the TAP enigma? *The Embo Journal* 15 (22):6011–7.

Velazquezcampoy, A., S. A. Leavitt, and E. Freire. 2004. Characterization of protein-protein interactions by isothermal titration calorimetry. *Methods in Molecular Biology* 261:35–54. doi:10.1385/1-59259-762-9:035.

Vercruysse, L., G. Smagghe, A. V. D. Bent, A. V. Amerongen, M. Ongenaert, and J. V. Camp. 2009. Critical evaluation of the use of bioinformatics as a theoretical tool to find high-potential sources of ACE inhibitory peptides. *Peptides* 30 (3):575–82. doi:10.1016/j.peptides.2008.06.027.

Viskov, C., M. Just, V. Laux, P. Mourier, and M. Lorenz. 2009. Description of the chemical and pharmacological characteristics of a new hemisynthetic ultra-low-molecular-weight heparin, AVE5026. *Journal of Thrombosis and Haemostasis* 7 (7):1143–51. doi:10.1111/j.1538-7836.2009.03447.x.

Wada, Y., and B. Lönnerdal. 2014. Bioactive peptides derived from human milk proteins-mechanisms of action. *The Journal of Nutritional Biochemistry* 25 (5):503–14. doi:10.1016/j.jnutbio.2013.10.012.

Wallner, J., G. Lhota, D. Jeschek, A. Mader, and K. Vorauer-Uhl. 2013. Application of bio-layer interferometry for the analysis of protein/liposome interactions. *Journal of Pharmaceutical and Biomedical Analysis* 72:150–4. doi:10.1016/j.jpba.2012.10.008.

Wang, X. Q., H. H. Yu, R. Xing, and P. C. Li. 2017. Characterization, preparation, and purification of marine bioactive peptides. *BioMed Research International* 2017:1–16. doi:10.1155/2017/9746720.

Wartchow, C. A., F. Podlaski, S. Li, K. Rowan, X. Zhang, D. Mark, and K. S. Huang. 2011. Biosensor-based small molecule fragment screening with biolayer interferometry. *Journal of Computer-Aided Molecular Design* 25 (7):669–76. doi:10.1007/s10822-011-9439-8.

Watanabe, R. M., A. M. Tanaka-Azevedo, M. S. Araujo, M. A. Juliano, and A. S. Tanaka. 2011. Characterization of thrombin inhibitory mechanism of rAaTI, a kazal-type inhibitor from *Aedes aegypti* with anticoagulant activity. *Biochimie* 93(3):618–23. doi:10.1016/j.biochi.2010.12.006.

Weitz, J. I., and S. M. Bates. 2005. New anticoagulants. *Journal of Thrombosis and Haemostasis* 3 (8):1843–53. doi:10.1111/j.1538-7836.2005.01374.x.

Weitz, J. I., and J. Hirsh. 1999. New antithrombotic agents. *Lancet* 353(9162):1431–6. doi: 10.1016/S0140-6736(98)09233-2.

Wolowacz, S. E., N. S. Roskell, J. M. Plumb, J. A. Caprini, and B. I. Eriksson. 2009. Efficacy and safety of dabigatran etexilate for the prevention of venous thromboembolism following total hip or knee arthroplasty. A meta-analysis. *Thrombosis and Haemostasis* 101 (01):77–85. doi: 10.1160/TH08-07-0493.

Wu, J., and R. E. Aluko. 2007. Quantitative structure-activity relationship study of bitter di and tripeptides including relationship with angiotensin I-converting enzyme inhibitory activity. *Journal of Peptide Science* 13 (1):63–69. doi: 10.1002/psc.800.

Wu, J., R. E. Aluko, and S. Nakai. 2006. Structural requirements of angiotensin I-converting enzyme inhibitory peptides: Quantitative structure-activity relationship study of di-and tripeptides. *Journal of Agricultural and Food Chemistry* 54 (3):732–8. doi: 10.1021/jf051263l.

Yamada, T., K. Kurihara, Y. Ohnishi, T. Tamada, K. Tomoyori, K. Masumi, I. Tanaka, R. Kuroki, and N. Niimura. 2013. Neutron and X-ray crystallographic analysis of the human α-thrombin-bivalirudin complex at pD 5.0: Protonation states and hydration structure of the enzyme-product complex. *Biochimica et Biophysica Acta* 1834 (8):1532–8. doi: 10.1016/j.bbapap.2013.05.014.

Yang, L. J., L. T. Zhang, L. H. Yan, H. F. Zheng, P. F. Lu, J. J. Chen, J. Dai, H. B. Sun, Y. Xu, and T. Yang. 2017. Stability assessment of a new antithrombotic small peptide, Arg-Gly-Asp-Trp-Arg (RGDWR), and its derivative. *Biotechnology Letters* 39 (8):1183–90. doi:10.1007/s10529-017-2346-x.

Yang, W. G., Z. Wang, and S. Y. Xu. 2007. A new method for determination of antithrombotic activity of egg white protein hydrolysate by microplate reader. *Chinese Chemical Letters* 18 (4):449–51. doi: 10.1016/j.cclet.2007.02.014.

Ye, X., M. Chen, Y. Chen, X. Su, Y. Wang, W. Su, and Y. Kong. 2015. Isolation and characterization of a novel antithrombotic peptide from enzymatic hydrolysate of *Agkistrodon acutus*, venom. *International Journal of Peptide Research and Therapeutics* 21 (3):343–51. doi:10.1007/s10989-015-9463-y.

Yu, G., F. Wang, B. Zhang, and J. Fan. 2016. In vitro inhibition of platelet aggregation by peptides derived from oat (*Avena sativa* L.), highland barley (*Hordeum vulgare* Linn. var. nudum Hook. f.), and buckwheat (*Fagopyrum esculentum* Moench) proteins. *Food Chemistry* 194:577–86. doi:10.1016/j.foodchem.2015.08.058.

Zanfardino, A., G. Criscuolo, L. B. Di, E. Pizzo, M. L. Ciavatta, E. Notomista, A. Carpentieri, A. Pezzella, and M. Varcamonti. 2017. Identification of a new small bioactive peptide from *Lactobacillus gasseri* supernatant. *Beneficial Microbes* 8 (1):133–41. doi:10.3920/BM2016.0098.

Zhang, H., W. H. Yokoyama, and H. Zhang. 2012. Concentration-dependent displacement of cholesterol in micelles by hydrophobic rice bran protein hydrolysates. *Journal of the Science of Food and Agriculture* 92 (7):1395–401. doi:10.1002/jsfa.4713.

Zhang, N., C. Zhang, Y. Chen, and B. Zheng. 2016. Purification and characterization of antioxidant peptides of *Pseudosciaena crocea* protein hydrolysates. *Molecules* 22 (1):57. doi:10.3390/molecules220100

Zhang, S. B. 2016. In vitro antithrombotic activities of peanut protein hydrolysates. *Food Chemistry* 202:1–8. doi:10.1016/j.foodchem.2016.01.108.

Zheng, Y., Y. Li, Y. Zhang, X. Ruan, and R. Zhang. 2017. Purification, characterization, synthesis, in vitro ACE inhibition and in vivo anti-hypertensive activity of bioactive peptides derived from oil palm kernel glutelin-2 hydrolysates. *Journal of Functional Foods* 28:48–58. doi:10.1016/j.jff.2016.11.021.

Zhou, Y. 2014. The potential biomedical application of cyclopeptides from marine natural products. *Current Organic Chemistry* 18 (7):918–24. doi:10.2174/1385272818071405151547 36.

Zvereva, I., G. Dudko, and M. Dikunets. 2018. Determination of GnRH and its synthetic analogues' abuse in doping control: Small bioactive peptide UPLC-MS/MS method extension by addition of in vitro and in vivo metabolism data; evaluation of LH and steroid profile parameter fluctuations as suitable biomarkers. *Drug Testing and Analysis* 10 (4):711–22. doi:10.1002/dta.2256.