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Chapter II

SEED STORAGE PROTEINS AS SOURCES OF BIOACTIVE PEPTIDES

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ABSTRACT

Of all the food proteins, animal proteins, especially milk proteins, have been well researched in terms of the possibility of releasing bioactive peptides.However, plant proteins, particularly seed storage proteins (SSP) have received less attention compared to animal proteins in this respect. Several studies have identified SSP as sources of bioactive peptides with various bioactivities including antihypertensive, antioxidative, cholesterol lowering, immunomodulating and opioid activities. These peptides are inactive within the sequence of the precursor proteins and can be released due to proteolysis.

Several studies have reported the ability of SSP to release bioactive peptides. Of these the potential of SSP to release ACEI peptides have been extensively studied. According to the amino acid composition of different bioactive peptides from SSP it is evident that certain amino acids are under-represented. SSP of soybeans are the most researched and they have been identified to release bioactive peptides with various physiological functions. However, published studies on bioactive peptides derived from other SSP are limited and the available studies are confined to a single bioactivity. Depending on the initial protein source, the enzyme employed, and processing conditions used, the biological activities of the peptides are different. Hydrolysis of same SSP with different enzymes gives rise to peptides with different bioactivities. The bioactivities of SSP derived peptides have been investigated mainly based on *in vitro* studies and in animal models. Human clinical studies to confirm such findings are very limited, also the understanding of the *in vivo* availability of bioactive peptides from food proteins in human digestive system. It is necessary to examine SSP of widely consumed grains and oilseeds to explore whether they could release bioactive peptides. Most of the identified

bioactive peptides are reactive due to its low molecular weight. However, their exact mechanism of activities is not clear. A highly challenging future need is to investigate the exact mechanism of the bioactive peptides on the physiological functions of interest.Findings of such studies will reveal the value of SSP beyond the currently accepted nutritional value. This chapter summarises the research findings on SSP-derived bioactive peptides in preventing lifestyle related diseases.

INTRODUCTION

The plant seeds are nutrient dense. They are rich in carbohydrates, lipids and proteins and contain less lignin and cellulose than other plant parts. The proteins of the seeds are mainly in the storage form, have no known enzymatic activity, and are biochemically well characterized [1]. Due to the abundance and versatility of seed storage proteins (SSP), scientists have endeavoured to exploit their various properties. Apart from providing amino acid requirement essential for human and animal nutrition, SSP function as a source of numerous bioactive peptides with antihypertensive, antioxidative, immunomodulating, opioid and other activities. Such peptides, which are hidden in the latent state within the SSP primary structure, may be released during *in vivo* digestion or food processing [2]. Many bioactive peptides have common structural properties including a relatively short residue length, hydrophobic amino acid residues, and the presence of Arg, Lys and Pro. It can be predicted that in future specific bioactive peptides will find many applications as functional ingredients in health promoting foods. The majority of the bioactive peptides studied are from animal protein sources including milk and few from plant proteins have been studied [3].This chapter discusses the bioactive peptides identified from SSP and their physiological functions.

1. SEED STORAGE PROTEINS - GENERAL DESCRIPTION

Seeds are the major plant tissues harvested by human kind. They act as a major source of dietary proteins with a protein content ranging from 10% (in cereals) to 40% (in certain legumes and oilseeds) of the seed weight [4]. Proteins in seeds are of two categories, namely storage proteins and housekeeping proteins. The storage proteins are in abundance. The housekeeping proteins are responsible for maintaining normal cell metabolism [5]. According to another classification, seed proteins are divided into storage, structural and biologically active proteins [6]. Of these, the structural proteins contribute to the structure (*e.g.*, cell wall) whereas biologically active proteins are part of cellular defence mechanism (*e.g.*, lections, enzymes and enzyme inhibitors). Structural- and biologically active proteins are in minor quantities. The non-enzyme proteins that are present in high amounts serve as a store of amino acids required for germination and seedling growth. These proteins are referred to as SSP [4]. SSP are the most abundantly consumed proteins by humans [7] and the major proteins in grains [8]. In the seed, primarily SSP are found in the protein storage vacuoles (PSV) as protein bodies. Mature seeds contain densely packed SSP deposits that entirely fill the PSV [9].

SSP represent a major fraction of human protein intake, either directly or indirectly. A vast majority of the global population depends on the proteins obtained from edible seeds

of cereals, legumes, and nuts to satisfy their dietary protein need and requirement [10].No single seed type contains the complete spectrum of essential amino acid requirement of human or farm animals. For example, cereal proteins are deficient in Lys and Trp and legume proteins are deficient in S-containing amino acids, Met and Cys [8,11].

Because of the abundance and economic importance SSP were among the earliest of all plant proteins to be characterized. Detailed studies on SSP ensued when Osborne [12] classified them into following groups based on their extraction and solubility. They are albumins (water extractable), globulins (extractable in dilute salt solutions), prolamins (extractable in aqueous alcohol) and glutelins (most difficult to solubilise but extractable by weakly acidic or alkaline or dilute sodium dodecyl sulphate solution). Of these, albumins and globulins comprise the storage proteins of dicots (*e.g*., pulses, Table 1), whereas prolamins and glutelins are the major storage proteins of monocots (*e.g*., cereals, Table 1) [8]. As SSP, serve as a reserve of nitrogen for the seedling, they are generally rich in Asn, Gln and Arg or Pro. Most of the SSP have high molecular weights and their water solubility is poor. This may play an important role in the deposition and higher order aggregation of storage proteins because of the osmotic conditions that exist in the drying and imbibing seed [13]. However, there are many examples of small water soluble SSP in dicot seeds like alfalfa, *Amaranthus*, castor bean, *Chenopodium*, mung bean, mustard, pea, and many other oil bearing seeds.

With an understanding of the relation between protein structure, genetic encoding and evolution, SSPare classified into families based on their amino acid sequences and elements of 3D structure. Some of these families are further grouped into large superfamilies, which have less similarity in sequence but share common structural features. The two major protein superfamilies identified are cupin and prolamin. The prolamin superfamily was first identified by Kreis and group [14] based on the existence of a conserved skeleton of eight Cys residues. The low molecular mass sulphur rich proteins (*e.g*., 2S albumins of dicot seeds, inhibitors of α-amylase and trypsin, puroindolines and grain softness proteins of cereals, hydrophobic protein of soybean, and non-specific lipid transfer proteins) and prolamines of SSP are included in the prolamin superfamily[15]. The cupin proteins are characterized by the "β-barrel" or "jelly-roll" like structure of the molecule ("cupa" is the latin term for small barrel). OfSSP, globulins (7S vicillin like globulins and 11S legumin like globulins) belong to the cupin superfamily [16].

1.1. Cereals

Cereal grains contain relatively little protein compared tolegume seeds, with an average of 10 to12% on a dry weight basis [17]. In mature cereal grains SSPaccountfor about 50% of the total proteins. They generally lack Lys, Thr and Trp. About 80 to 90% of cereal SSP is consisted of prolamins and glutelins whereas albumins and globulins account for the remainder. Prolamins and glutelins are present in approximately equal amounts in wheat, barley and rye. In sorghum and maize prolamins are in excess compared to glutelins. In oats, glutelins are excessive compared to prolamins. Rice is unique as its protein is largely consisted of glutelin[11]. The cereal prolamins are present as monomers or small aggregates whereas glutelins form large disulphide bonded aggregates.Table 1 provides the trivial names used for cereal proteins based on their latin generic names.

1.2. Legumes

Much of the proteins in legume seeds are salt soluble globulins (7S and 11S of cupin superfamily; Table 1). Legume proteins tend to be deficient in sulphur-containing amino acidsCys and Met and also in Trp. They are high inglutamic and aspartic acidamides and Arg.

Legume globulins are named as legumin (11S) and vicillin (7S). Vicillin consists of dicarboxyllic acids and their amides and small amounts of Met, Cys and Trp [8] and has no disulphide bonds.

1.3. Brassica and other Oilseeds

The major oilseeds include soybean (*Glycine max* (L.) Merr.), cottonseed (*Gossypium hirstum* L.), rapeseed/canola (*Brassica napus* L. *B. rapa* L. and *B. juncea*), sunflower seed (*Helianthus annus* L. var *marcocarpus* DC), peanut (*Arachis hypogae* L.), linseed or flaxseed (*Linum usitatissimum* L.), safflower (*Carthamus tinctorius*), sesame (*Sesamum indicum* L.) and coconut (*Cocus nucifera*) with protein contents ranging from 20 to 40% [24]. Unlike food legumes, most of the oilseed proteins are destined for animal feed after oil extraction. By far, soybean protein is the major seed protein utilized in foods and is available commercially in a variety of products. Furthermore, soybean protein has attracted more scientific research due to health benefits derived from soy protein consumption. The majority of the oilseeds contain 11S globulin and 2S albumin as the major SSP except for soybean (11S glycinin and 7S β-conglycinin) and flaxseed (12S linin and 2S conlinin) (Table 1).

2. BIOACTIVE PEPTIDES

Apart from providing the amino acid requirements, food proteins, including seed proteins have the ability to generate bioactive peptides possessing various physiological and health benefits. These bioactive peptides are defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health [25]. They are inactive within the sequence of the parent protein, and can be released during gastrointestinal (GI) digestion, hydrolysis by proteolytic microorganisms and through the action of proteolytic enzymes derived from micro organisms or plants [26]. Once liberated, bioactive peptides possess different activities *in vitro* and *in vivo* including antihypertensive, antimicrobial, antioxidative, antithrombotic and immunomodulatory [27]. Therefore, upon oral administration bioactive peptides may affect the major body systems namely the cardiovascular, digestive, immune and nervous systems depending on their amino acid sequence [26].

Table 1. Predominant storage proteintypes, their common names and storage sites in the seeds of important crops [18-23]

Family, species and common name	Predominant storage protein types and their common names	Primary storage site
Compositae		
Helianthus annus (Sunflower)	11S Helianthinin 2S Albumin	Cotyledons (embryo)
Cruciferae		
Brassica napus (Canola/Rapeseed)	11S Globulin (Cruciferin) 2S Albumin (Napin)	Cotyledons (embryo)
Graminae		
Avena sativa (Oats)	Prolamin (Avenin), 11S Globulin 7S Globulin	Starchy endosperm Embryo/Aleuron layer
Hordeum vulgare (Barley)	Prolamin (Hordein) 7S Globulin	Starchy endosperm Embryo/Aleuron layer
Oryza sativa (Rice)	11S Globulin (Glutelin), Prolamin 7S Globulin	Starchy endosperm Embryo
Triticum aestivum (Wheat)	Prolamin (Gliadin+Glutenin) 7S Globulin	Starchy endosperm Embryo/Aleuron layer Starchy
Zea mays (Maize)	Prolamin (Zein) 7S Globulin	endosperm Embryo
Leguminoceae		
Arachis hypogea (Peanut)	11S Arachin, 7S Conarachin	Cotyledons (embryo)
Glycine max (Soybean)	11S Glycinin, 7S β-Conglycinin	Cotyledons (embryo)
Phaseolus vulgaris (French bean)	2S Albumin	Cotyledons (embryo)
Pisum sativum (Field pea)	7S Phaseolin	Cotyledons (embryo)
Vicia faba (Broad bean)	11S Legumin, 7S Vicillin & Convicillin, 2S Albumin 7S Vicillin 11S Legumin	Cotyledons (embryo)
Linaceae		
Linum usitatissimum (Flaxseed)	12S Linin, 2S Conlinin	Cotyledons (embryo)
Palmae		
Cocos nucifera (Coconut)	11S and 7S Globulins, 2S Albumin	Endosperm
Elais guineenis (Oil palm)	7S Globulin	Endosperm
Pedialaceae		
Sesamum indicum (Sesame)	11S α-Globulin, 2S β-Globulin	Cotyledons (embryo)

Food-derived bioactive peptides commonly contain 2 to 9 amino acids [25], however, this range may be extended to 3 to 20 or more amino acid residues [28]. For example lunasin, a food-derived peptide with proven anticancer activity, contains 43 amino acids with a molecular weight of 5400 Da [29]. In order to perform biological functions, these peptides must cross the intestinal epithelium and enter into the blood circulation, or bind directly to specific epithelial cell surface receptor sites [3].

A significant proportion of dietary nitrogen is absorbed into the body in the form of small peptides. The human GI tract secrets number of peptidases that function synergistically to cleave polypeptide chains into amino acids and small peptides. The small intestine is the primary absorption site of these end products of protein digestion. Small (2 to 3 amino acids) and large (10 to 51 amino acids) peptides generated in the diet can be absorbed intact through the intestine and produce biological effects at the tissue level [30]. The results of electrophysiological studies carried out in 1970s and 1980s have suggested the existence of a peptide transport system in the intestinal epithelium by which peptides would be actively transported through the intestinal membrane under H^+ gradient [31]. However, this transport mechanism carries only di- and tri-peptides [32]. Oligopeptides with more than four residues transport through the intestinal epithelium via other routes such as pinocytosis or through para cellular channels [30, 33] depending on the molecular size and hydrophobicity. After absorption, the intestinal tract serum peptidase can further hydrolyse the peptide bonds. Therefore, resistance to peptidase degradation is a prerequisite to sustain the physiological effects of bioactive peptides following oral ingestion or intravenous infusion.

Bioactive peptides have been mainly isolated and studied from animal protein sources including milk. However, there is ample evidence to suggest that SSP are precursors of bioactive peptides of physiological importance. Thishas been confirmed by various *in vitro* and *in vivo* studies, which are discussed further herein.

3. PHYSIOLOGICAL FUNCTIONS OF SSP-DERIVED BIOACTIVE PEPTIDES

3.1. Antihypertensive Effect and Angiotensin I-Converting Enzyme (ACE) Inhibiting Peptides

Hypertension or high blood pressure is a major health problem all over the world, with 50% of individuals above 55 years in many industrialized countries are suffering. It is a major risk factor for coronary heart disease, congestive heart failure, stroke and renal disease [34]. In humans the systolic blood pressure (SBP) refers to the blood pressure at the active contraction phase of the cardiac cycle whereas diastolic blood pressure (DBP) is the pressure at the relaxation phase of the cardiac cycle [35]. A person is said to be hypertensive when SBP is $\geq$ 140 mmHg or DBP is $\geq$90 mmHg (140/90 mmHg) or taking medications for hypertension [36].

ACE plays a critical role in the regulation of blood pressure. It is a zinc metalloprotease, a component in the rennin-angiotensin system (RAS), and a dipeptidyl carboxypeptidase that catalyses hydrolysis of carboxy terminal dipeptides from oligopeptide substrates (Figure 1). ACE and other components of RAS coexist locally in various tissues including blood vessels,

kidney, adrenal glands, heart and brain in addition to circulation. High concentrations of ACE are found on epithelial surfaces such as intestinal, choroids plexus, and placental brush borders influencing the fluid and electrolyte balance at these fluid membrane interfaces [37]. ACE increases the blood pressure by catalysing the conversion of decapeptide angiotensin I into the potent vasoconstricting octapeptide angiotensin II, and also by catalysing the degradation of bradykinin, a blood pressure lowering nonapeptide. Angiotensin II leads to several effects that are central and link to a further increase in blood pressure (Figure 1). Therefore, inhibition of ACE results in an overall antihypertensive effect [38].

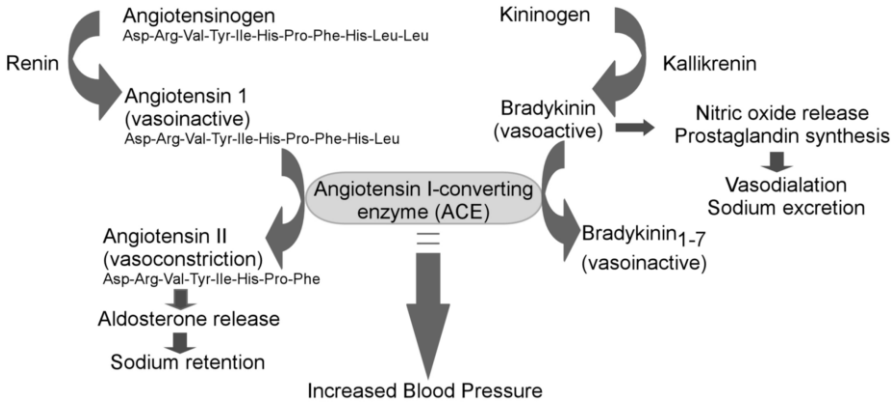


Figure 1. Summary of the effects of ACE on rennin-angiotensin system.

ACE has two domains (N and C), each of which contains an active site with a His-Glu-X-X-His. The His residues are considered to participate in Zn binding. Glu residue is involved in the catalysis by binding the activated water molecule, which initiates a nucleophilic attack on the susceptible peptide bond of the substrate. Of these, the C domain is the dominant angiotensin 1-converting site [39]. The first available competitive inhibitors of ACE were isolated from *Bothrops jararaca* snake venom as naturally occurring pentapeptide Glu-Lys-Trp-Ala-Pro and the nonapeptideGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro[40].Structure-activity correlation studies of analogs of these peptides indicated that their carboxy terminal tripeptide residues (Trp-Ala-Pro) play a dominant role in competitive binding to the active site of ACE [41] (Figure 2). Captopril, a highly potent ACE inhibitory (ACEI) drug, was developed based on the antihypertensive mechanism of these peptides. The sulphydryl group of captopril, which is coupled to the dipeptide Ala-Pro, binds strongly with the Zn atom in the active site of ACE and inhibits the enzyme activity (Figure 2). Numerous other ACE inhibitors such as enalapril and lisinopril have also been developed [42]. As stated by Cheung and others [41], Trp, Tyr, Phe, and Pro are the most potent C-terminal amino acids of these peptides that contribute most to substrate binding at the active site and thereby inhibit the activity of ACE.

From a database containing 168 dipeptides and 140 tripeptides Wu and others [44] identified that amino acid residues with bulky or hydrophobic side chains are preferred for ACEI dipeptides (Table 2). For tripeptides, the most favourable residues for the carboxyl terminus are found to be aromatic amino acids, while positively charged amino acids are preferred for the middle position, and hydrophobic amino acids are preferred for the amino terminus. At present, many ACE inhibitors have been discovered from enzyme catalysed

hydrolysis of proteins from animal sources including bovine casein, human casein and fish proteins [45] and also from plant sources such as enzymatic digests of SSP.

Brassica spp.

Seeds of canola/rapeseed (*Brassica napus*) have significant protein content which constitutes 20 to 25% of the dry seed weight whereas the meal that remains after oil extraction consists of approximately 40% protein. Four potent ACEI peptides, Ile-Tyr, Arg-Ile-Tyr, Val-Trp and Val-Trp-Ile-Ser, have been isolated from the Subtilisin® digest of rapeseed protein [46].Among thesepeptides, Arg-Ile-Tyr and Val-Trp-Ile-Ser have exhibited IC_{50} (50% inhibitoryconcentration) values of 28 and 30 μM (0.013 and 0.016 mg/mL), respectively. All these peptides lowered blood pressure of spontaneously hypertensive rats (SHR) following oral administration. However, the Arg-Ile-Tyr peptide has also reduced blood pressure of old rats in which ACE is less effective indicating that the mechanism of blood pressure reduction by Arg-Ile-Tyr may not be via ACE inhibition.

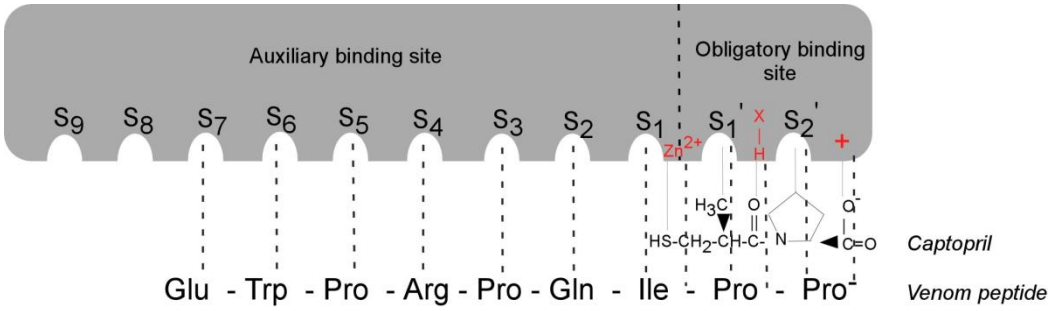


Figure 2. Proposed model for binding of venom nonapeptide and captopril on to the active sites of ACE.

Wu and others [47] have identified ACEI activity in canola protein hydrolysed with Alcalase® with an IC_{50} of 0.19 mg protein/mL. These peptides also exhibited stability against GI protease incubation. The fraction with most potent ACE inhibition (IC_{50} of 0.014 mg protein/mL) had peptides of <870 Da molecular mass as the predominant (87%) molecules. Aromatic amino acids contributed up to 37% of the total amino acids of these peptides. ACEI activity exhibited by Alcalase® hydrolysed rapeseed protein [48] and defatted canola meal [49] showed that SSP in canola protein are good sources of ACEI peptides upon hydrolysis by food grade microbial enzymes. According to Wu and others [49] the IC_{50} for ACEI activity of hydrolysed heat-treated and untreated canola meals were 0.027 to 0.028 mg/mLand 0.035 to 0.044 mg/mL,respectively. Heat denaturation of protein may play a role in generating more ACEI peptides because of the increased susceptibility to hydrolysis. These peptides retained their activity upon *in vitro* GI digestion with pepsin and pancreatin. Two ACEI peptides, Val-Ser-Val (IC_{50}: 0.15 μM or 0.05×10^{-3}mg/mL) and Phe-Leu (IC_{50}:1.33 μM or 0.39×10^{-3}mg/mL) were purified from the Alcalase® hydrolysed canola meal with sequence reference to the napin and cruciferin. Interestingly, total protein of canola and isolated napin and cruciferin when hydrolysed with Alcalase® showed IC_{50} of 0.015, 0.035 and 0.029 mg/mL, respectively. However, reliable evidence on the exact canola SSPthat generate ACEI peptides is lacking [50]. Other *Brassica* spp showing ACEI activity upon hydrolysis include

Brassica carinata (Ethiopean mustard) which showed ACEI activity upon sequential hydrolysis by trypsin, chymotrypsin and carboxypeptidase A (IC_{50} of 0.338 mg protein/mL) [51].

Table 2. Prediction and location of ACEI peptides in some seed storage proteins [44]

Peptide (known)	Position	Parent protein	Predicted log IC_{50}	Observed log IC_{50}
FW	f150-151	Legumin A2 precursor; garden pea	0.60	0.77
	f149-150	Legumin A precursor; garden pea		
WW	f150-151	Glycinin G1 precursor; soybean	0.52	1.91
	f147-148	Glycinin G2 precursor; soybean		
YW	f219-220	Legumin J precursor; garden pea	0.92	1.64
	f153-154	Legumin K; garden pea		
	f3-4	Conglycinin, chain precursor; soybean		
	f27-28	Glycinin G3 precursor; soybean		
	f147-148	Glycinin G4 precursor; soybean		
	f156-157	Glycinin precursor; soybean		
VRF	f4-6	Conglycinin, chain precursor; soybean	0.14	1.38
IKP	f265-267	Glycinin G1 precursor; soybean	0.37	0.44
	f279-281	Legumin J precursor; garden pea		
	f6-8	Vicilin 47 kDa protein; garden pea		
LRW	f377-379	Legumin A2 precursor, garden pea	-0.11	-0.64
	f374-376	Legumin A precursor; garden pea		

Buckwheat

Buckwheat (*Fagopyrum esculentum* Moench) is a pseudo cereal and has crude protein content of 12% [52]. The potential health benefits arising from buckwheat include strong

hypocholesterolaemic activity [53] and blood pressure-reducing effect [54]. Buckwheat SSP are rich in Asp, Arg and Lysbut lower in Glu and Pro than cereal proteins [55]. The major storage protein of buckwheat seed is a 13S globulin that resembles legumin in structure [56].

Enzymatic hydrolysis of buckwheat globulin with Thermolysin® has shown production of ACE inhibitors with an IC_{50} value of 0.043 mg protein/mL[57]. Li and others [58] showed that *in vitro* digestion of buckwheat flour with GI proteases can enhance the ACEI activity of the digest (IC_{50} of 0.14 mg protein/mL) when compared with intact buckwheat (IC_{50} of 3 mg/mL). When fed to SHR, buckwheat digest was able to lower SBP. The tripeptides Tyr-Gln-Tyr and Pro-Ser-Tyr have been identified as the main ACE inhibitors in the buckwheat digest. Ma and others [59] have isolated and purified an ACEI peptide from buckwheat protein extract without enzymatic digestion. The identified ACE inhibitor was the tripeptide Gly-Pro-Pro that exhibited IC_{50} value of 6.25×10^{-3}mg protein/mL. Proline was the C-terminal amino acid of ACEI peptides identified in buckwheat [41, 59].

Chickpea

The crude protein content of chickpea (*Cicer arietinum* L.) varies from 12.6 to 30.5% [60]. Chickpea is considered as a good source of vegetable proteins because of the well-balanced amino acid composition, high protein bioavailability, and relatively low level of associated antinutritional factors[61]. The main storage protein of chickpea is legumin, which accounts for 64% of the total protein content and 97% of the total globulins in the seed [62]. Pedroche and others [63] reported that the hydrolysate prepared by treating chickpea protein isolate with Alcalase®, possesses ACEI activity. Four ACEI peptides have been purified from the hydrolysate. Two of them were competitive inhibitors of ACE while the other two were uncompetitive inhibitors. Yust and others [27] isolated six ACEI peptides (Met-Asp, Asp-Phe-Leu-Ile, Met-Phe-Asp-Leu, Met-Asp-Leu, Met-Asp-Leu-Ala and amino acid Met) with IC_{50} values ranging from 0.011 to 0.021 mg/mL released due to the Alcalase® treatment of isolated legumin. Methionine was the most abundant amino acid in all these peptides, although hydrophobic amino acids were also in abundance. Among these, Yust and others [27] have identified an ACEI peptide which consists exclusively of Met. ACEI peptides with Met residues were also purified from β-lactoglobulin [64], sardine muscle [65] and human α_{s1} casein [66]. No *in vivo* studies are available on ACEI peptides generated by chickpea proteins.

Field Pea

The protein content of field pea (*Pisum sativum* L.) varies from 15.6 to 32.5% depending on the cultivar and the conditions prevailing in the growing season [67]. Pea protein is mainly composed of globulins (11S and 7S) and heterogeneous albumins (sulphur-rich PA1 albumin and the larger PA2 albumin) [68]. The globulin fraction consists of about 80% of the total proteins in the mature pea seeds [69]. Several *invitro* studies have shown the possibility of pea proteins to act as a precursor of ACEI peptides. Vermeirssen and others [70] subjected isolated pea protein to simulated GI digestion and investigated theACEI activity of the hydrolysate that exhibited IC_{50} values of 0.076 mg/mL. *In silico* GI digestion of vicilin and albumin PA2 proteins revealed that pea SSP have the ability of releasing a number of potent ACEI peptides [71]. In another study, Vermeirssen and others [72] subjected pea proteins to fermentation with*Lactobacillus helveticus*and *Saccharomyces cerevisiae* and then to

simulated GI digestion.When the IC_{50} value of non-fermented and fermented pea proteins were compared at the end of GI digestion the former was found to have the low IC_{50} value suggesting that GI digestion is the predominant factor that controls release of ACEI peptides from pea proteins.Therefore, the hydrolysis of pea proteins by microbial proteases may hinder the action of GI proteases on the pea proteins to release more potent ACEI peptides.Vermeirssen and others [73] have fractionated the GI digest of pea protein isolates using ultrafiltration and reverse phase chromatography to increase the ACEI activity. They found that ACEI activity was increased by four times and all the peptides liberated from pea have similar ACE inhibiting ability. Pea protein isolate digested with GI proteases has exerted a strong hypotensive effect showing a reduction in the mean arterial blood pressure by 44.4 mmHg in SHR after intravenous administration of 50 mg protein/kg body weight. However, only a fraction of ACEIpeptides of the pea protein digest was transported through Caco–2 cells, which are sources of small intestinal peptidases. As the Caco-2 cells are tighter than the intestinal cells of mammals sufficient absorption of these peptides might occur under actual *in vivo* conditions [74].

Maize

The major proteins of maize (*Zea mays*) seeds are zein and glutelins (Table 1), which represent approximately 60% and 30%, respectively of the total seed proteins [75]. Zein is deficient in Lys and Trp and also to a lesser extent in Arg, His and Met whereas glutelin has a nutritionally balanced amino acid composition [76]. Therefore, the nutritional value of maize protein is said to be poor. There are four main types of zeins (α,β, γ and δ) classified according to their solubility properties. Of these proteins α-zein displays more hydrophobic properties [77]. ACEI tripeptides Leu-Arg-Pro, Leu-Ser-Pro and Leu-Gln-Pro, having IC_{50} values of 0.27, 1.7, and 1.9 M (110, 590 and 745 mg/mL), respectively have been isolated from α-zeinhydrolysate of Thermolysin® hydrolysis. The hypotensive activity of Leu-Arg-Pro on SHR exhibited a significant decrease in blood pressure (15 mmHg) after a 30 mg/kg intravenous injection [78]. Furthermore, orally administered unpurified Thermolysin® hydrolysate of α-zein also reduced the blood pressure level of SHR. Yano and others [79] hydrolysed urea-denatured α-zein into small peptides by digestion with Thermolysin®. The ACEI activity (IC_{50}) of this Thermolysin® digest of α-zein was 24.5 μg/mL (0.024 mg/mL). Thirty-six ACEI peptides, including 5 dipeptides, 14 tripeptides, 9 tetrapeptides, 5 pentapeptides, and 3 hexapeptides, were isolated from this hydrolysate. Although zein is a protein with low nutritional quality, its ability to release ACEI peptides upon enzymatic hydrolysis reveals the value of zein as a source of bioactive peptides.

Mung Bean

Mung bean (*Phaseolus radiatus* L.) which has a protein content ranging from 20 to 33% has accepted anti-inflammatory, anti-tumor, cholesterol lowering, detoxifying and diuretic properties. The seeds are processed and consumed as cooked whole beans or splits, sprouts, mature seeds and flour [80]. Mung bean protein has been identified as a good source of ACEI peptides when hydrolysed *in vitro* with the protease Alcalase® [81]. An increase of ACEI activity of the mung bean hydrolysates when treated with GI proteases *in vitro* indicated the presence of pro-drug type peptides in the hydrolysate. A significant decrease in SBP has been reported when this hydrolysate was orally administered to SHR [81]. The presence of high

levels of hydrophobic amino acids in mung bean protein explains its' potential to generate ACEI peptides. It was confirmed that tripeptides composed of hydrophobic amino acids in the C and N- terminals have potent ACEI activity. Three ACEI peptides have been isolated from mung bean protein hydrolysate treated with Alcalase®; Lys-Asp-Tyr-Arg-Leu, Val-Thr-Pro-Ala-Leu-Arg and Lys-Leu-Pro-Ala-Gly-Thr-Leu-Phe with IC_{50} values of 26.5 μM, 82.4 μM and 13.4 μM (0.02, 0.06, and 0.01 mg/mL), respectively. The presence of hydrophobic amino acids Leu and Phe at the C-terminal position of Lys-Asp-Tyr-Arg-Leu and Lys-Leu-Pro-Ala-Gly-Thr-Leu-Phe, respectively may contribute to the ACEI activity of these peptides. In addition, the peptide Lys-Leu-Pro-Ala-Gly-Thr-Leu-Phe shares the C-terminal dipeptide with α-lactalbumin derived Tyr-Gly-Leu-Phe and β lactoglobulin derived Tyr-Leu-Leu-Phe those possess strong ACEI activity *in vitro* [82]. Meisel [83] suggested that the positive charge on the ε-amino group of Arg and Lys in the C-terminal also contribute to the ACEI potency of peptides; and provides an explanation for the ACEI of Val-Thr-Pro-Ala-Leu-Arg. However, the mung bean SSPthose act as the precursors of ACEI peptides have not been identified.

Sesame

Sesame (*Sesamum indicum* L.) seeds contain 40 to 50% protein (rich in S-amino acids) on a fat free basis. Sesame is used in confectionery, personal care products and fortification of soft drinks and juices while the protein hydrolysates are found in soups, sauces, gravies, snacks, meat products, and other savory applications [84]. Besides oil and protein, sesame contains characteristic lignans. Ochi and others [85] isolated three peptides with ACEI activity from a Thermolysin® hydrolysate of sesame seed proteins: Met-Leu-Pro-Ala-Tyr, Val-Leu-Tyr-Arg-Asp-Gly and Ileu-Val-Tyr. Each peptide showed antihypertensive effect in SHR when orally administered at the dose of 100 mg/kg. Nakano and others [86] isolated and identified six ACEI peptides from commercially available sesame peptide powder. They are Leu-Val-Tyr, Leu-Gln-Pro, Leu-Lys-Tyr, Leu-Ser-Ala, Ileu-Val-Tyr and Val-Ileu-Tyr. Among these the tripeptides with Tyr and Pro at the C- terminal and Leu at the N-terminal have shown noticeable ACEI activity. These results could be supported by the substrate specificity for ACE as reported by Cheung and others [41]. The peptides derived from sesame peptide powder have also shown to reduce SBP in SHR.

Soybeans

Soybean (*Glycine max* L. Merr.) proteins are used in a variety of ways and consist mainly of glycinin (11S) and β-conglycinin (7S), but also contain several minor proteins [87].Several peptides derived from soy protein have exhibited ACEI activity in chemical assays and mouse models. Soy peptides with ACEI activity have been reported to contain Gly, nonpolar (Ala or Leu), aromatic (Phe), polar (Asn, Gln or Pro), or negatively charged (Asp or Glu) amino acid residues at the carboxyl terminal and Gly, non-polar (Ile or Val), aromatic (Tyr or Phe), polar (Gln), positively charged (His), or negatively charged (Asp) amino acids at the amino terminal. Val was the most frequently observed residue in ACEI soy peptides, followed by these amino acids in decreasing order of occurrence; Leu, Phe, Asp, Pro, Gln, Gly, Ala, Ile, Asn, Glu, His, Thr, Arg, Met, and Lys [88]. It has been reported that soybean peptide fractions, His-His-Leu (HHL) isolated from fermented soybean paste exerted ACEI activity *in vitro* with an IC_{50} value of 2.2 mg/mL. Moreover, the synthetic tripeptide HHL resulted in a significant decrease of ACE activity in the aorta and led to lower SBP in SHR [89]. ACEI

peptides derived from treatment of soy protein with Alcalase® had a significant hypotensive effect on SHR at 100 mg/kg of body weight/day administration level demonstrating its bioactivity *in vivo*. However, the ACEI activity of soy peptide was found to be significantly lower than that of clinical hypotensive drug, captopril [90]. The ACEI peptides Asp-Leu-Pro and Asp-Gly with IC_{50} values of 4.8 μM (1.82×10^{-3} mg/mL) and 12.3 μM (2.5×10^{-3} mg/mL), respectively derived from hydrolysis of soy protein by Alcalase®, were stable to GI enzyme digestion *in vitro* [91]. The ACEI peptides generated by peptidic digestion of soy proteins have been characterized and identified as Ile-Ala (IC_{50}: 153 μM or 0.03 mg/mL), Tyr-Leu-Ala-Gly-Asn-Gln (IC_{50}: 14 μM or 0.01 mg/mL), Phe-Phe-Leu (IC_{50}: 37 μM or 0.01 mg/mL), and Ile-Tyr-Leu-Leu (IC_{50}: 42 μM or 0.02 mg/mL). These peptides were investigated for reducing blood pressure of SHR when given orally at a dose of 2g/kg body weight [92]. The mean SBP of SHR was reduced to 17.6 mmHg in 2h upon oral administration and this antihypertensive effect was continued for 6h [92]. According to Lo and Chan [88] sequential *in vitro* digestion of soy isolate with pepsin and pancreatin generated peptides with ACEI activity suggesting the potential of soy proteins to release peptides with such activity under the physiological conditions. However, a reduction of ACEI activity of peptides in peptic hydrolysate was reported upon further hydrolysis by pancreatin. Digestion of soy hydrolysate and the fermented products such as *natto* and *tempeh* with a variety of endoproteases (pronase, trypsin, Glu C protease, plasma proteases and kidney membrane proteases) have demonstrated that ACEI peptides were mostly derived from glycinin, the highly expressed soy protein, and were found mainly in the pronase, kidney membrane proteases and plasma proteases digests of the fermented soy products [93]. Among the soy proteins, β-conglycinin was found to be resistant to proteolytic attack even by the multi-enzyme systems, plasma proteases and kidney membrane proteases [94], stable to acid hydrolysis, and resistant to tryptic proteolysis [93]. According to Lo and others [95] isolated soy proteins have the potential to release ACEI peptides under conditions that simulated the upper GI tract. As there could be a reduction of ACEI activity upon pancreatin digestion [88], studies simulating only the upper GI tract are not sufficient to predict the potential of soy proteins as precursors of ACEI peptides. Though soy protein has been extensively studied for production of ACEI peptides further clinical studies are needed to confirm that such peptides are released and active under physiological conditions.

Sunflower

Defatted sunflower (*Helianthus ananus* L.) seed meal contains about 30% proteins, which can be used as a food ingredient. Megias and others [96] have isolated an ACEI peptide from the pepsin and pancreatin digest of sunflower seed protein isolate. The peptide has a sequence of Phe-Val-Asn-Pro-Gln-Ala-Gly-Ser (IC_{50} 5.7×10^{-3} mg/mL) and it corresponded to a fragment of helianthinin (11S globulin, Table 1). Hydrolysis of isolated sunflower protein with Alcalase® followed by Flavourzyme® also has generated ACEIpeptides [97]. The ACEI fractions of the hydrolysates were rich in Asp, Ser, Gly, Thr and Val compared to the original protein hydrolysate whereas some fractions were rich in Ala, Met, Ile, Leu or Phe residues supporting the fact that ACEI peptides are rich in hydrophobic amino acids. The lowest IC_{50}value (0.08×10^{-3} mg/mL) wasresulted in for Flavourzyme®-generated fractions and was much lower than that of ACEI peptides released

by *in vitro* GI digestion of sunflower protein as reported by Megias and others [96]. There is no *in vivo* evidence on the ACEI activity of sunflower peptides.

Wheat

Wheat (*Triticumaestivum* L.) grain has a low protein content, which ranges between 9 to 16% of the dry weight. Wheat grain proteins play a major role in processed food such as bread, biscuits, breakfast cereals and pasta [98]. The major wheat storage proteins are alcohol soluble gliadins and glutelins. Wheat germ (WG), a byproduct of the flour milling industry is an excellent source of vitamins, minerals, dietary fiber, proteins and calories. The defatted wheat germ contains >30% protein that is rich in the essential amino acids, especially Lys, Met, and Thr, which many of the cereal grains are deficient of [99]. Hydrolysate of WG obtained from *Bacillus licheniformis* alkaline protease hydrolysis has demonstrated ACE inhibition *in vitro.* Sixteen peptides having 2 to 7 amino acids with the IC_{50} value less than 20 μM have been isolated from the hydrolysate. The major peptide possessing the most powerful ACEI activity (IC_{50} 0.48 μM or 0.21×10^{-3} mg/mL) was Ile-Val-Tyr [100]. Simulated GI digestion of WG hydrolysate has further increased ACEI ability indicating that there is production of new ACEI peptides due to GI protease activity. Motoi and Kodama [101] isolated ACEI peptide from wheat gliadin hydrolysate prepared with acid proteases. The amino acid sequence of this peptide was identified as Ile-Ala-Pro (IC_{50} 2.7 μM or 0.91×10^{-3} mg/mL). This peptide inhibited the hypertensive activity of angiotensin II with intravenous injection, and decreased the blood pressure significantly with intraperitoneal administration when tested using SHR.

Other SSP Derived Peptides with ACEI Activity

There are several other SSP derived peptides with identified ACEI activity. They include protein hydrolysates of peanut [102], kidney bean [103], red bean [104] and Thermolysin® hydrolysed flaxseed meal [105]. The amino acid residues and their sequence arrangement in bioactive peptides determine the bioactivities. Therefore biological activities of peptides can be strengthened by replacing the amino acid residues. Transgenic rice with antihypertensive activity has been developed by introducing antihypertensive ovokinin like peptide structure obtained from egg albumen into the hypervariable regions of glutelin, the major storage protein in rice [106].

3.2. Opioid Activity

Human endogenous opioidergic system is consisted of opioid receptors and their ligands, endogenous opioids with alkaloid or peptide structure, which exert their activity upon binding with opioid receptors. Opioid receptors are located in the central and peripheral nervous system, in the immune system and in the endocrine system of mammals [107]. Specific organ tissues that resemble opioid activity include the spinal chord, adrenal gland, digestive tract, pituitary gland and hypothalamus. Of the three types of opioid receptors,μ-, κ- and δ-, the μ-type receptors are responsible for emotional behaviour and suppression of intestinal motility. The δ-type receptor is associated with emotions and reward behaviour whereas the κ-receptors are important for sedation and food intake [3]. The endogenous opioid peptides

include enkephalins, endorphin and dynorphins [3]. They possess agonistic as well as antagonistic effects on opioid receptors in the human body and elicit effects in all cells or tissues where opioids are known to be active [3]. Basically the opioid peptides have an effect on the nervous system and GI functions [108]. Also the fixation of opioid peptides to blood vessel opioid receptors is considered as an alternative mechanism of reducing blood pressure by bioactive peptides [109]. There is much evidence on involvement of opioid peptides in food intake regulation and obesity in humans and experimental animals [110]. Agonists to opioid receptors induce a positive energy balance and obesity, whereas antagonists at these receptors reduce food intake andbody weight in rodent obesemodels [111, 112]. Interestingly, the inhibitory effects of opioid receptor antagonistson food intake and body weight appear most pronounced in obeseanimals or when animals were fed a highly palatable diet. Opiates are well recognized to have variety of interactions with the monoamines in the central nervous system, which influences the feeding behaviour. The sensory pleasure response of foods is also largely brought about by the release of the endogenous opioid peptides in the brain. Therefore, blockade of opioid receptors by opioid antagonists could reduce the taste preferences, and diminish consumption of preferred foods. Especially the preferences for sweet taste or dietary fat are under the control of endogenous opioids. Therefore, abnormalities of endogenous opioid system could increase the consumption of sweet and fatty foods that leads to overweight and obesity [113].

Aseries of food protein derived opioid peptides (exogenous opioid peptides), which act as exogenous supplements for the endogenous opiodergic system have been identified [107]. These food derived opioid peptides are found to be important for the human body. One reason is the stability of food-derived opioid towards enzymatic degradation because they are released after intestinal enzymatic action as opposed to the susceptible endogenous opioid peptides. The other is food-derived opioid peptides usually possess weaker activity than endogenous ones, which makes them less likely to cause the adverse side effects often associated with opioids such as dependence, tolerance and addiction [25]. Structurally, both exogenous and endogenous opioid peptides vary in the N-terminal sequence. The endogenous peptides have the same N-terminal sequence of Gly-Gly-Phe whereas several food derived opioid peptides have been found to have a Tyr residue at the amino terminal (*e.g.*, Tyr-X-Phe, Tyr-X1-X2-Phe) [25; 108]. At present several food-derived opioid peptides of animal and plant origin have been identified. Interestingly, opioid peptides derived from animal proteins are mostly μ-receptor selective, while those of plant origin are mostly δ-receptor selective [114]. The bovine β casein fragment was the first food protein that was identified as an opioid receptor ligand, which later led to the identification of several other opioid agonists, and antagonists from milk proteins [107]. Of the plant protein derived opioids, hydrolysates of RuBisCo in spinach leaves [115], alfalfa white protein concentrate [109] and several SSP have been studied for opioid agonist and antagonist activities (Table 3) [107].

Of the SSP-derived opioid peptides the most extensively studied are gluten exorphins (GEs). These are opioid peptides isolated from enzymatic digests of wheat gluten. Digestion of wheat proteins releases peptides that act on opioid receptors in the gut. Zioudrou and others [116] have identified that peptides in the peptic hydrolysate of wheat gluten have morphine like opioid activity *in vitro*. GEs are classified into three groups according to their structure, namely, GEA, GEB, and GEC. There are two members of GEA: GEA5 (Gly-Tyr-Tyr- Pro-Thr)and GEA4 (Gly-Tyr-Tyr-Pro) and two members of GEB: GEB5 (Tyr-Gly-Gly-Trp-Leu)and GEB4 (Tyr-Gly-Gly-Trp).

Table 3. SSP studied for opioid activity [107]

Seed	Protein	Materials with opioid agonist or antagonist activity		
		Looked for	Found	Identified
Barley	Hordein	+	+	-
Maize	Zein	+	+	-
Oats	Avenin	+	-	-
Rice	Albumin	+	+	+
Rye	Secalin	+	-	-
Soy	α Protein	+	-	-
Wheat	Gluten	+	+	-
	Gliadin	+	+	-

Among the gluten derived peptides, GEB5 has the most potent *in vitro* activity as agonist on both μ- and δ- receptors [117] and is also the most potent food-derived δ-opioid peptide that has been reported to date [114]. GEB5 has been identified to be released *in vivo* after gluten ingestion and also crosses the intestinal barrier fully intact. It stimulates prolactin release in rats by acting through opioid receptors in the nerves outside the blood brain barrier [117]. Schusdziarra [118] had administered the digested gluten into the stomach of dogs and noted a more rapid rise in peripheral vein insulin and glucagon levels than when an equivalent amount of undigested protein was administered. Additional studies have suggested that the effect may be related to activation of opiate receptors by exorphins. Fukudome and others [119] reported that GEA5 can stimulate insulin release in rats upon both oral and intravenous administration. Insulin has an appetite suppressing effect in humans. Therefore GEA5, via stimulation of insulin release, may prevent excess energy intake and development of obesity. However, the appetite suppressing effect of insulin is found among lean subjects and not in the obese subjects [120]. No study is available to prove GEs can exert similar opioid activity in human subjects.

The same GEA5 peptide has exhibited the capability of suppressing the endogenous pain inhibitory system, and has facilitated to acquire/consolidation process of learning/memory in mice [121]. GEs have also been studied for GI functions. Hydrolysed gluten has prolonged the intestinal transit time in healthy volunteers, which can be reversed by the administration of the opiate blocker naloxone. The naloxone-reversible increase in plasma somatostatin-like activity may be responsible for the delayed transit time [122]. However, the authors have not found any effect of GEs on appetite regulation of the volunteers.

Cholecystokinin (CCK) is produced in the brain and enteroendocrine cells and is an important physiologic endocrine factor in appetite control. The food intake suppression by CCK is well established [123]. Pupovac and Anderson [124] identified that ingestion of soy hydrolysate by rats lead to satiety as they provided satiety signals through opioid and CCK receptors. Peptic hydrolysate of soyβ-conglycinin is a potent stimulator of CCK release from intestinal mucosal cells and inhibits food intake and gastric emptying through the CCK release [123]. The fragment of β-conglycinin that binds with the rat intestinal membrane to stimulate CCK release has been found to possess the sequence of Gly-Arg-Gly-Arg-Gly-Arg-

Gly. The f51-63 of β-conglycinin was the appetite suppressant and the multiple Arg residues in this fragment may have been involved in manifesting this effect (123).

Oryzatensin (Gly-Tyr-Pro-Met-Tyr-Pro-Leu-Pro-Arg), a bioactive peptide isolated from tryptic digest of rice albumin [124] was able to contract longitudinal muscle strips of the guinea pig ileum. Oryzatensin showed a biphasic ileum contraction, which was characterized by a rapid contraction followed by a slower one. The latter was mediated by the cholinergic nervous system. Although oryzatensin showed weak affinity for μ-opioid receptors, the apparent anti-opioid activity seemed to be associated with the slower contraction. Upon oral administration, the ACEI peptide Arg-Ile-Tyr isolated from rapeseed has also decreased food intake and gastric emptying in mice via stimulating CCK release [125]. This reveals that a peptide with a given sequence of amino acid can perform several bioactivities. Althoughthese studies have encouraging findings on the effect of SSP-derived peptides on opoid activity, long term clinical studies are needed to identify whether such peptides could exert the same effects in humans.

3.3. Antioxidant Activity

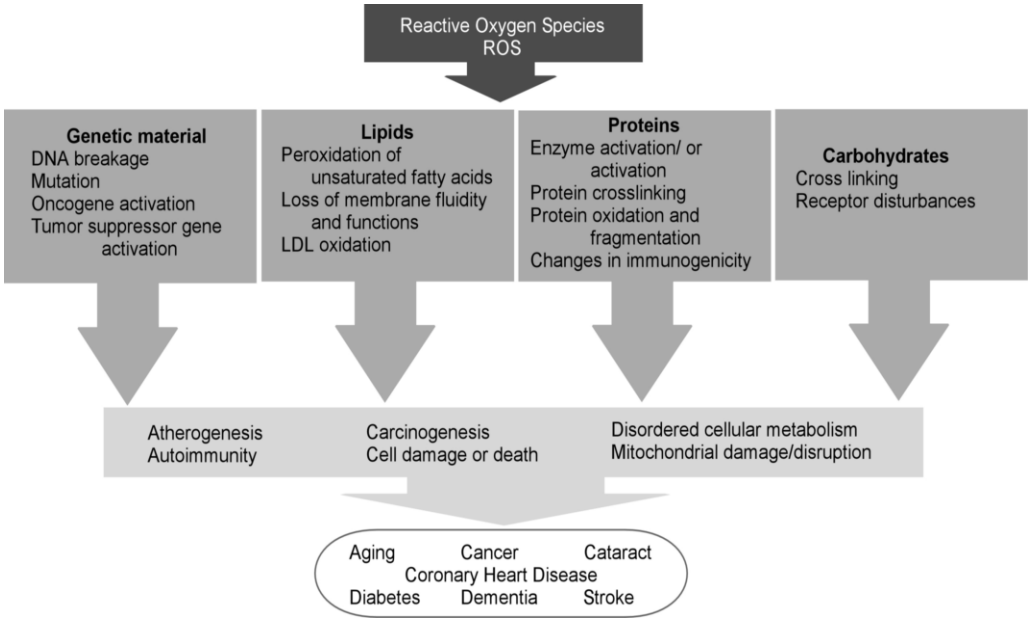
Free radicals such as superoxide, nitric oxide,and hydroxylandother oxygen-derived species (hydrogen peroxide, and hypochlorous acid) are formed constantly in the human body during metabolism of oxygen. This is in addition to the exposed oxidants such as air pollutants, ozone, oxides of nitrogen, tobacco smoke, and motor vehicle exhaust. These reactive oxygen species (ROS) are highly reactive as they contain an unpaired electron in the outer orbit. Molecules with unpaired electrons such as superoxide are capable of initiating chemical chain reactions through free radical generation [126]. For example superoxide ($O_2^{\cdot}$**)** together with H_2O_2 can form the reactive hydroxy radical ($OH^{\cdot}$) via the Haber-Weiss reaction, which proceeds quickly when metal ions such as Fe^{2+} or Cu^{+} are present (Fenton reaction when iron is the catalyst) [127]. Such chain reactions contribute to lipid peroxidation, DNA damage and protein degredation during oxidatively stressful events [126]. The antioxidant defense systems of the human body consist of a variety of enzymes (catalase, and superoxide dismutase) and antioxidant compounds, to protect against these ROS, but these defenses are not completely efficient [128]. When ROS are generated excessively or the antioxidative defences are depressed, a number of pathological events including aging, cellular injury and DNA degradation take place. Uncontrolled production of free radicals is associated with the onset of many diseases such as cancer, rheumatoid arthritis and atherosclerosis (Figure 3). Therefore antioxidants in the human diet are of great interest as possible protective agents to help the human body to reduce oxidative damages [129]. Antioxidants can prevent or inhibit oxidation by preventing generation of ROS or by inactivating ROS (Table 4).

Several naturally occurring antioxidant peptides such as glutathione (GSH: Glu-Cys-Gly), carnosine (β-Ala-L-His), anserine (β-Ala-3 methyl-L-His), homocarnosine (γ aminobutyryl-L –His), with free radical scavenging activity have been identified in the human body. Of these, GSH scavenge free radicals by donating its H atomand by acting as a co-substrate in reduction of H_2O_2 and other hydroperoxides by GSH peroxidase (Figure 4) [130]. Amongst the His containing peptides, carnosine was first identified in 1900 in beef extract [131]. It is one of the most abundant (1-20 mM) nitrogenous compounds present in the non-

protein fraction of vertebrate skeletal muscle and certain other tissues, including olfactory epithelium. In addition to OH· scavenging, carnosine has also been reported to be a scavenger of O_2· and a chelator of Cu, which in turn prevent Haber-Weiss reaction [132].

Peptides of antioxidant activities have been also identified from several seed protein hydrolysates. Six antioxidant peptide fragments, which are active against lipid peroxidation, have been isolated from soy β-conglycininenzymatic digest. These peptides are consisted of 5 to 16 amino acid residues including hydrophobic amino acids such as Leu or Valat the N-terminal position and His, Proand Tyr in the sequences [133]. Chen and others [134] have developed 22 synthetic peptides based on the smallest peptide of soy digest, which was Leu-Leu-Pro-His-His, and revealed that the His-containing peptides can act as a metal-ion chelator, an active oxygen quencher, and a OH· scavenger. In the peptide sequence, His and Pro played important roles in the antioxidant activity and, among the peptides tested, Pro-His-His had the most antioxidant activity [134]. Oligopeptides isolated from soy hydrolysates, fermented soy and papain hydrolysed soy protein have exhibited antioxidant activity [135]. However, these results are contradictory to those of Pena-Ramos and Xiong [136] who found that papain hydrolysis of soy protein does not generate antioxidant activity indicating that genotype has an effect on the antioxidant peptide release.

Saito and others [138] have constructed peptide libraries using antioxidative tripeptides isolated from soy proteins and have measured the antioxidative activity of the peptides using several *in vitro* methods. Among the antioxidative tripeptides Tyr-His-Tyr showed a strong synergistic effect with phenolic antioxidant inspite of having a marginal reducing activity and a moderate peroxynitrite scavenging activity. Tripeptides with Cys had the strong peroxynitrite scavenging activity whereas tripeptides with Trp or Tyr at the C-terminus had strong radical scavenging activity and weak peroxynitrite scavenging activity [138].



Adopted from [127].

Figure 3. Damage to the biological molecules by reactive oxygen species leading to increased risk of diseases.

Table 4. Mechanism of action of antioxidants [127]

	Action	Examples
Prevention	Protein binding/ inactivation of metal ions	Transferrin, Ferritin, Ceruloplasmin, Albumin
Enzymatic diversion/neutralization	Specific channelling of ROS into harmless products	Superoxide dismutase, Catalase Glutathione peroxidase
Scavenging	Sacrificial interaction with ROS by expandable (replaceable or recyclable) substrates	Ascorbic acid, α-Tcopherol, Uric acid, Bilirubin, Glutathione
Quenching	Absorption of electrons and/or energy	α-Tocopherol, β-Carotene

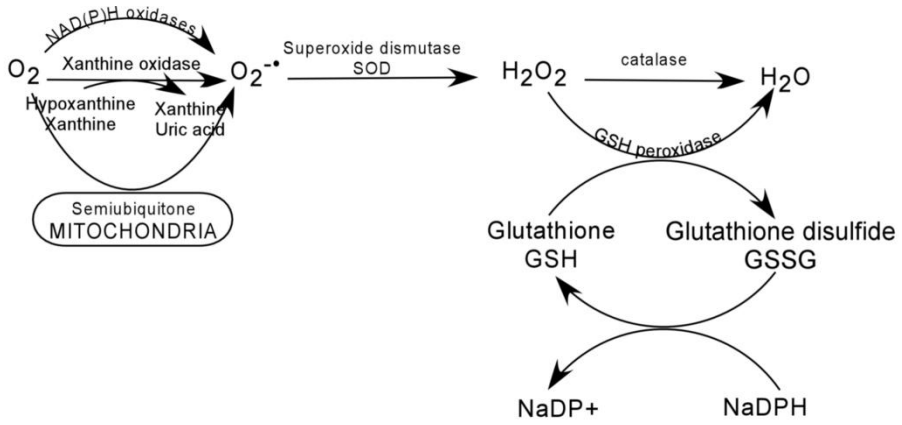
Wheat germ protein hydrolysates (WGPH) obtained from Alcalase® catalysed hydrolysis had an antioxidant activity close to that of α-tocopherol in a linoleic acid emulsion system [139]. WGPH showed scavenging activity against free radicals such as DPPH, $O_2^{\cdot}$, and $OH^{\cdot}$. Alcalase®-hydrolysed zein has exhibited an antioxidant activity close or comparable to those of butylated hydroxyanisole (BHA), α-tocopherol, and ascorbate by acting as a metal ion chelator or a hydrogen donor, as well as a radical stabilizer to inhibit lipid oxidation [140].

These data suggest the occurrence of antioxidant peptide sequences in SSP, which can be released during enzymatic hydrolysis. The antioxidant mechanism of the released peptides varies with the hydrolysis conditions, structure/amino acid sequence of the peptides, which is determined by the enzyme used to catalyse protein hydrolysis. All these studies show possibility of SSP-derived peptides to act as exogenous antioxidants.

3.4. Cholesterol Lowering Ability

Dietary proteins have been known to reduce serum cholesterol. Soy protein, in particular, has been demonstrated to have cholesterol lowering properties in various populations of children [141] and renal patients [142]. However, the mechanism responsible for plasma cholesterol lowering ability of soy protein remained questionable until recently. There is evidence to conclude that the bile acid binding ability of these peptides contributes to blood cholesterol level reduction. Bile acids are acidic steroids synthesized in the liver from cholesterol. They are secreted into the duodenum to participate in the digestion process and are actively reabsorbed from the terminal ileum to undergo enterohepatic circulation [143]. Studies on the hypocholesterolemic effect of soy proteins have resulted in the hypothesis that a peptide with high bile acid binding ability could inhibit the reabsorption of bile acid in the ileum and stimulate cholesterol transformation into bile acids in plasma and liver, ultimately reducing plasma cholesterol level (Figure 5) [144]. Such peptides could also decrease the micellar solubility of cholesterol in the small intestinal epithelial cells and decrease the blood cholesterol level. Soy isoflavones have been considered as the components that contribute to the cholesterol lowering effect of soy protein products. However, Sirtori and others [145] have demonstrated that a marked plasma cholesterol reduction can be obtained using isoflavone–poor soy protein products. Lovati and others [146] have conducted an experiment in which HepG2 cells were exposed to synthetic peptides corresponding to specific sequences

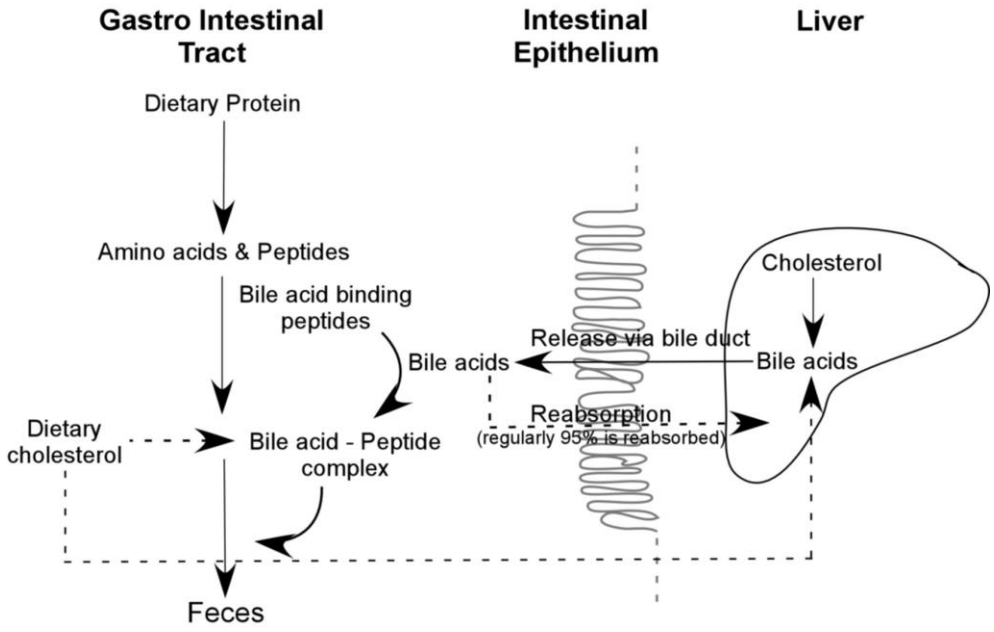
of β-conglycinin or to peptides of the *in vitro* digestion of the commercial isoflavone-poor soy protein concentrate. These authors have demonstrated that increased LDL uptake and degradation resulted in after Hep-G2 cell incubation with the synthetic peptide whereas incubation with the digest of soy protein concentrate exhibited a significant up regulation of LDL-receptors. The findings of this study indicated that the low molecular weight peptides released by soy proteins have cholesterol lowering properties. Leu-Pro-Tyr-Pro-Arg, which is a fragment of soybean glycinin, has reduced serum cholesterol in mice after oral administration at a dose of 50 mg/kg for 2 days (25.4% reduction in total cholesterol and 30.6% LDL-cholesterol) [147]. In this study, the excretion of fecal cholesterol and bile acids did not increase indicating that the mechanism responsible for a hypocholesterolemic activity might not be the binding of bile acids. However, there are studies supporting the bile acid binding action of soy glycinin derived peptides. Soy 11S globulin has exhibited higher hypocholesterolemic ability than 7S soy globulin and casein. The peptide Ile-Ala-Val-Pro-Gly-Glu-Val-Ala isolated from the pepsin hydrolysate of 11S globulin was able to bind cholic and deoxycholic acids which lead to hypocholesterolemic effect [144]. Of the five subunits of glycinin (subunit group I: A1aBIb, A1bB2, and A2B1a; subunit group II: A3B4, and A5A4B3), Choi and others [148] have identified a potential bile acid binding peptide sequence (Val-Ala-Trp-Trp-Met-Tyr) in the acidic polypeptide A1a of the A1aB1b subunit. Incorporation of nucleotide sequence encoding this peptide in to the DNA coding of A1a polypeptide exhibited an enhanced bile acid binding ability of glycinin [149].



Adopted from Ref. [137].

Figure 4. Pathways of reactive oxygen species (ROS) generation and clearance by the tripeptide glutathione; GSSG.

Soy protein peptic hydrolysate containing phospholipids (SPHP) has shown significantly greater *in vitro* bile acid binding capacity than that of soy protein peptic hydrolysate without phospholipids (SPH) [150]. The cholesterol micelles containing SPHP and SPH significantly suppressed cholesterol uptake by Caco-2 cells compared to cholesterol micelles containing casein tryptic hydrolysate. The *in vivo* rat feeding studies conducted have indicated that the fecal excretion of total steroids was significantly greater in rats fed with SPHP than SPH.



Based on Ref. [144].

Figure 5. Hypocholesterolemic action of bile acid binding peptides. Bile acids are synthesized from cholesterol, conjugated and then excreted into the bile ducts. Released bile acids are reabsorbed via intestinal epithelium facilitating the dietary cholesterol absorption. Dietary protein derived bile acid binding peptides enhance bile acid excretion with feces, reducing the bile acid re-absorption.

3.5. Modulation of Immune Function

The function of the immune system is based on the activities of different cell types such as monocytes/macrophage, platelets, erythrocytes, polymorphoneuclear leukocytes (eosinophils, neutrophils, basophils and mast cells), T and B-lymphocytes to protect the body against foreign microbes such as bacteria, viruses and parasites. A number of plant protein derived bioactive peptides, which modulate the immune function have been identified. Among these the major plant protein source is soy. Yoshikawa and others [147] have isolated a peptide soymetide-13 (Met-Ile-Thr-Leu-Ala-Ile-Pro-Val-Asn-Lys-Pro-Gly-Arg) derived from tryptic digest of the alpha subunit of β-conglycinin that stimulates phagocytosis in human polymorphoneuclear leukocytes. Methionine residue of the amino terminus was found to be essential for this activity. The peptide His-Cys-Gln-Arg-Pro-Arg isolated from tryptic digest of soy glycinin was also found to be immuno-stimulating,activating phagocytosis of human neutrophils, and stimulating tumor necrosis factor (TNF) when orally administered to mice [151].

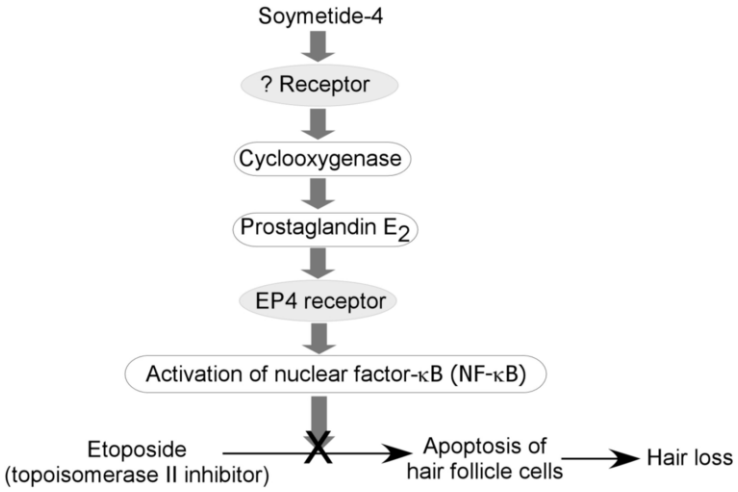
Soymetide is the first food derived peptide agonist of the N-formyl-methionyl-leucyl-phenylalanine (fMLP). fMLP is the synthetic form of N-formylmethionyl peptide that is strongly chemotactic for human neutrophils and macrophages thereby stimulates the immune system. The surface of neutrophils and macrophages carry specific fMLP receptors that mediate the generation of ROS from neutrophils and macrophages as well as the phagocytosis stimulating activity of the neutrophils. These functions lead to a rapid response for bacterial

infection as bacterial proteins have an N-formylmethionine residue at their N-termini. Such response leads to bacterial death by phagocytosis and ROS induced bactericidal effects. Soymetides have a weak affinity to the fMLP receptors. Therefore, following ingestion of soy proteins the immune system receives a signal that is similar to what received after a bacterial infection [151]. Soymetide-4 is a tetrapeptide and is the shortest peptide that stimulates phagocytosis. Soymetide–4 did not induce ROS formation by neutrophils *in vitro*, may be due to its lower affinity to fMLP receptors than that of fMLP. Therefore, soymetide-4 could be theoretically safe as an immunostimulating agent without causing any inflammation. Orally administration of soymetide–4 has also prevented hair loss (anti-alopecia mechanism) induced by the anti-cancer agent etoposide [151]. Of the soymetides isolated, the soymetide-4 is more desirable as an immunostimulating peptide due to its short length, which facilitates its absorption in the digestive tract.

Oryzatensin (Gly-Tyr-Pro-Met-Tyr-Pro-Leu-Pro-Arg) obtained from tryptic digestion of rice albumin has also been identified as an immuno-modulatory peptide. Comparatively short C-terminal fragments of oryzatensin demonstrating similar activity were also reported. Oryzatensin also induced phagocytosis and production of superoxide anions in human leukocytes *in vitro* [26]. Horiguchi and others [152] have identified the effect of wheat gluten hydrolysate (hydrolysed with protease and amylase) on the immune system of healthy human volunteers. Intake of 3 g of wheat gluten hydrolysate for 6 days led to a significant increase in natural killer (NK) cell activity of the test group compared to the placebo. NK cells play a critical role in immune surveillance against tumour development and viral infections. Hence NK cell activation is effective in patients with autoimmune disease or cancer and in elderly people who usually have low levels of NK cell activity [152]. The immuno-enhancing activity of wheat gluten hydrolysate might be caused by immuno-stimulating peptides present in the hydrolysate. According to Clare and others [153] ACEI peptides can stimulate the immune system via inactivation of bradykinin. Bradykinin stimulates macrophages to enhance lymphocyte migration and to increase secretion of lymphokines.

3.6. Calmodulin Binding Activity

Calmodulin (CaM) is an important soluble protein in humans that bind with Ca^{2+} and regulates the activity of many cellular enzymes [154], including adenylyl cyclase, cyclic neucleotide phosphodiesterase, Ca^{2+}-Mg^{2+} ATPase, calcinurin, nitric oxide synthase, andseveral protein kinases. By regulating these enzymes CaM acts as a Ca^{2+} dependent regulator ofcyclic nucleotide metabolism, Ca^{2+} transport, protein phosphorylation-dephosphorylation cascades, ion transport, cytoskeletal function and cell proliferation [155]. Excessive activity of CaM dependent enzymes such as protein kinase II leads to increased phosphorylation of various cellular proteins related to pathogenic chronic diseases. Therefore, any compound capable of reducing the activity of CaM can inhibit these reactions and suppress disease progression.Since CaM is negatively charged with an exposed hydrophobic surface in its active site, peptides having basic amino acids that give net positive charge or peptides with hydrophobic surfaces have the potential to inhibit CaM [156]. A number of peptides in insect venom with these properties have been reported to bind with CaM and inhibit CaM activated phosphodiesterase. The most potent of these peptides also found to have an alpha helical structure [157]. Peptides with CaM binding activity have been identified in the protein hydrolysates of several SSP.



Adapted from Ref. [151].

Figure 6. Hypothetical mechanism of anti-alopecia effect of orally administered soymetide-4: Activation of cycloxygenase by soymetide-4 releases PGE2 which then activates nuclear factor-κB (NF-κB) and suppress apoptosis of hair follicle cells and etoposide induced alopecia.

Pea proteins hydrolysate of Alcalase®-catalysis that was rich in positively charged amino acids such as Lys and Arg and contained short peptides that bind CaM and reduced the activity of CaM dependent protein kinase II *in vitro* [156]. Hydrolysis of flaxseed protein isolate by the same enzyme has yielded peptides that can bind to CaM and inhibit CaM dependent neuronal and endothelial nitric oxide synthase [158]. No research evidence is available for the possibility of other SSP generating CaM binding peptides or the action of such peptides *in vivo*.

3.7. Anticancer Activity

Peptides isolated from hydrolysed SSP have demonstrated cancer preventive properties both *in vitro* and *in vivo*. Xiao and group [159] have reported that diets containing soy protein isolate inhibit tumourgenesis in rats by enhancing somatostatin, which is a known antiproliferative agent for colon cancer cells. Part of this anticancer activity may be attributed to the bioactive peptides generated during soy protein digestion. Peptides obtained by Thermolase® hydrolysis of defatted soy protein showed *in vitro* cytotoxicity (IC_{50} value of 0.16 mg/mL) in mouse monocyte macrophage cell line. Further purification of this hydrolysate yielded a nonapeptide, X-Met-Leu-Pro-Ser-Tyr-Ser-Pro-Tyr [160]. Lunasin, a chemopreventive bioactive peptide from 2S albumin of soybean, can reduce carcinogen induced cell transformation in mice [161], and skin tumour incidences [162]. Exogenous application of the lunasin peptide inhibited carcinogen-induced transformation of murine fibroblast cells to cancerous foci *in vitro*. Lunasin is a unique, naturally occurring peptide of 43-amino acids and contains nine Asp residues at its carboxyl end preceded by Arg-Gly-Asp, the cell adhesion motif. It acts as a chemopreventive agent that functions possibly via binding with chromatin. Lunasin has been able to inhibit core histone acetylation by binding to non-

acetylated H3 and H4 histones. This mechanism is believed to be responsible for the anticancer property of this chromatin binding peptide [162]. Lunasin has also been isolated from barley (*Hordeum vulgare* L), wheat [161] and amaranth (*Amaranthus hypochondriacus*) [163] seed proteins. The crude and partially purified lunasin from barley has suppressed colony formation in stably *ras*-transfected mouse fibroblast cells. These fractions also inhibited histone acetylation in mouse fibroblast and human breast cells in the presence of the histone deacetylase inhibitor sodium butyrate [30]. According to Jeong and others [159], the bioactive lunasin could be extracted from liver of rats fed with a diet rich in lunasin of wheat. This indicates that lunasin remains intact and biologically active upon GI digestion. In amaranth seeds, the highest concentration of lunasin was observed in the glutelin fraction (3.0 µg/g). Lunasin was also found in albumin, prolamin and globulin fractions and even in popped amaranth seeds [163].

4. PREDICTION OF BIOACTIVITIES OF SSP DERIVED PEPTIDES USING PEPTIDE DATABASES

SSP are encoded by families of polymorphic genes by a regulated process. For example, in maize 30 to 100 genes are involved in encoding zein. According to research findings the amino acid composition and sequence in bioactive peptides are the major factors that determine their bioactivities. For example most of the opioid peptides contain Tyr and Phe residues [164]whereas the majority of the ACEI peptides contain Pro residues at the C-terminus [45]. Therefore the primary structure of precursor proteins is a major determinant of the capability of a protein to generate bioactive peptides.

Computer assisted databases are available for predicting bioactivities of peptides located within the parent protein and also to obtain information on isolated bioactive peptides. These peptide libraries include information on peptides constructed via chemical synthesis or genetic engineering. Peptide databases allow comparison of experimental peptide mass and amino acid sequence against the molecular mass and amino acid sequence of peptides in the database and enable the users to find details on the peptide of interest. At present several databases are available (Table 5) to predict the precursor protein of a bioactive peptide with a known amino acid sequence. Such databases also provide information on genes responsible for coding a specific peptide. BIOPEP [165] is a database, which classifies food proteins as potential sources of bioactive peptides. As the database indicates, antihypertensive peptide fragments commonly occur in most of the food proteins [166,167]. This database identifies bovine β-casein and rice prolamin as the best precursors of antihypertensive peptides. Bioactive fragments as well as their surroundings are also found to be hydrophilic and mostly localized at random coil structures of the proteins [167]. According to a database search performed using BIOPEP by Wang and Mejia [168], soy proteins have sequences with antiamnestic, antihypertensive, antioxidative, antithrombotic, dipeptidyl peptidase IV inhibitory, immunostimulating and opioid activities. The 7S and 11S soy proteins were the sources of bioactive peptides while 2S protein has also exhibited some potential. Several opioid peptide sequences in soyproteins, which have not been previously reported, were revealed due to this matching.

Table 5. Databases available to search for peptides with bioactivities

Database	Accessible website	Information available
UniProt KB/SwissProt	http://www.expasy.org/sprot/	Protein sequence and function, Post-translational modification(s). Domains and sites, Similarities to other proteins, Disease(s) associated with deficiencies in the protein, Sequence conflicts, and variants
BioPep	http://www.uwm.edu.pl/biochemia	Potential prediction of bioactive peptides released from proteins
Bioactive polypeptide database (BioPD)	http://biopd.bjmu.edu.cn/help.asp	Basic information and structure of peptide, related gene information, Interactions between the peptide and other proteins, Information about diseases and the peptide if available
Antimicrobial peptide database (APD)	http://aps.unmc.edu/AP/main.php	Information on antifungal, antiviral and anticancer peptides. Statistical information on peptide sequence, structure and function to use in novel peptide design.
SwePep	http://www.swepep.org	Information on endogenous bioactive peptides, Molecular mass, modifications, Precursor information, and organism affiliation of neuropeptides, Hormones, Characterized and uncharacterized bioactive peptides and potential bioactive peptides
ANTIMIC: a database of antimicrobial sequences	http://research.i2r.a-star.edu.sg/Templar/DB/ANTIMIC/	Comprehensive information on natural antimicrobial peptides (AMPs), both known and putative, Facilitates efficient extraction of data and its analysis at molecular level, and search for new AMPs.
JenPep: Peptide binding database	http://www.jenner.ac.uk/Jenpep/	Quantitative binding data for immunological protein-peptide interactions

Dziuba and others [169] carried out abioinformatic-aided analysis of the biologically active fragments and the bonds of food proteins that are susceptible to the action of endopeptidases of known specificity.According to this analysis, wheat gliadins were the mostsusceptible for bioactive peptides release. These peptides showed antihypertensive, dipeptidylpeptidase inhibitory, opioid and antioxidative effect, and were released due to activity of chymotrypsin, elastase, ficin or pepsin. Wu and others [44] have constructed a database of di- and tripeptides having ACEI activity based on the published literature and have predicted the IC_{50} value of ACEI peptides from SSP (of pea and soybean) (Table 2). The other important online databases of bioactive peptides are BioPD [170]and SwePep. In addition to these the antimicrobial peptide database (APD) provides information on antibacterial, antifungal and antiviral peptides.

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