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REVIEW ARTICLE

Antioxidative peptides: enzymatic production, in vitro and in vivo antioxidant activity and potential applications of milk-derived antioxidative peptides

O. Power · P. Jakeman · R. J. FitzGerald

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Abstract The beneficial effects of food-derived antioxidants in health promotion and disease prevention are being increasingly recognized. Recently, there has been a particular focus on milk-derived peptides; as a source of antioxidants, these peptides are inactive within the sequence of the parent protein but can be released during enzyme hydrolysis. Once released, the peptides have been shown to possess radical scavenging, metal ion chelation properties and the ability to inhibit lipid peroxidation. A variety of methods have been used to evaluate in vitro antioxidant activity, however, there is no standardised methodology, which hinders comparison of data. This review provides an overview on the generation of antioxidative peptides from milk proteins, the proposed mechanisms of protein/peptide induced antioxidant activity, in vitro measurement of antioxidant activity, in vivo evaluation of plasma antioxidant capacity and the bioavailability of antioxidative peptides. The understanding gained from other food proteins is referred to where specific data on milk-derived peptides are limited. The potential applications and health benefits of antioxidant peptides are discussed with a particular focus on the aging population. The regulatory requirements for peptide-based antioxidant functional foods are also considered.

Keywords Bioactive peptide · Antioxidant · Milk protein · Hydrolysate · Amino acids · Functional food

O. Power · R. J. FitzGerald (✉)
Food for Health Ireland, Department of Life Sciences, University of Limerick, Schrodinger Building, Limerick, Ireland
e-mail: dick.fitzgerald@ul.ie

P. Jakeman
Food for Health Ireland, Department of Physical Education and Sports Science, University of Limerick, Limerick, Ireland

Abbreviations

AAPH	2,2′-Azobis-2-methyl-propanimidamide dihydrochloride
ABAP	2,2-Azobis(2-aminopropane) hydrochloride
ABTS	2,2′-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid
α-LA	α-Lactalbumin
Ala	Alanine
AO	Antioxidant
AOC	Antioxidant capacity
AOPI	Antioxidative potency index
Arg	Arginine
Asp	Aspartic acid
AUC	Area under the curve
BHA	Butylated hydroxylanisole
BHT	Butylated hydroxyltoluene
β-CN	β-Casein
β-LG	β-Lactoglobulin
CA	Caffeic acid
Caco-2	Human adenocarcinoma colon cancer cell monolayer
Cap-e	Cell-based antioxidant protection assay
CCl_4	Carbon tetrachloride
CN	Casein
CAT	Catalase
CPP	Caseinphosphopeptide
Cys	Cysteine
DCFH-DA	Dichlorofluorescein diacetate
DH	Degree of hydrolysis
DMPO	5,5-Dimethyl-1-pyrroline N-oxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DSHEA	Dietary Supplements and Health Education Act
ECL	Enhanced chemiluminescence
E:S	Enzyme to substrate ratio

ESR	Electron spin resonance
EFSA	European Food Safety Authority
ET	Electron transfer
EWH	Egg white hydrolysate
EU	European Union
FDA	United States Food and Drug Administration
FOSHU	Foods for specific health use
FPH	Fish protein hydrolysate
FRAP	Ferric reducing antioxidant power
GSH	Glutathione
GPx	Glutathione peroxidase
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GSSG	Oxidised glutathione
HAT	Hydrogen atom transfer
HepG2	Human hepatocarcinoma
His	Histidine
$O\mathring{H}$	Hydroxyl radical
H_2O_2	Hydrogen peroxide
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
MDA	Malondialdehyde
Met	Methionine
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NDA	Dietetic products, nutrition and allergies
NLEA	Nutrition Labeling and Education Act
NO	Nitric oxide
NOX4	NADPH 4 oxidase
ORAC	Oxygen radical absorbance capacity assay
PAC	Plasma antioxidant capacity
Phe	Phenylalanine
Pro	Proline
QSAR	Quantitative structure activity relationship
RACI	Relative antioxidant capacity index
ROO·	Peroxyl radical
ROS	Reactive oxygen species
RP-HPLC	Reverse phase-high performance liquid chromatography
Ser	Serine
SHR	Spontaneously hypertensive rats
SOD	Superoxide dismutase
SOSA	Superoxide anion scavenging activity
O_2·	Superoxide radical
TAC	Total antioxidant capacity
TBARS	Thiobarbituric acid reactive substances
TEAC	Trolox equivalent antioxidant capacity
Thr	Threonine
TRAP	Total radical trapping antioxidant potential
Trp	Tryptophan
Tyr	Tyrosine
UF	Ultrafiltration
US	United States
Val	Valine
WPC	Whey protein concentrate
WPI	Whey protein isolate

Introduction

Milk is a complete food, containing a good balance of nutrients and a rich source of nitrogen and essential amino acids. The nutritional role of milk is clearly recognised, and for most mammals, milk provides the sole source of nutrition in the early stages of life. In addition to its fundamental nutritional role, the biofunctional potential of milk is now being recognised. Much of the bioactivity can be attributed to milk proteins and their constituent peptides (FitzGerald and Meisel 2003). These peptides are inactive or encrypted within the sequence of the native protein but enzymatic hydrolysis can release active peptides. They can have a beneficial effect on a variety of biological systems including the cardiovascular, gastrointestinal, immune and nervous systems (Murray and FitzGerald 2007). Several milk-derived peptides have been found to possess antioxidant (AO) activity (Pihlanto 2006). The ability of peptides to interact with radical species or to inhibit oxidative reactions could lead to the development of novel food ingredients relevant in health promotion and disease prevention. As the body ages, there is an increased requirement for dietary protein in order to prevent age-related sarcopenia (Paddon-Jones et al. 2008). Aging is associated with an increased production of reactive oxygen species (ROS; Kregel and Zhang 2007) and a decrease in plasma and cellular AOs (Gilca et al. 2007). Over time, this can place the body under increased oxidative stress, advancing the aging process and increasing the susceptibility to age-related diseases. However, consumption of an AO-rich protein source may prove an effective strategy to offset some of the deleterious effects of aging.

Oxidative stress and antioxidants

Aerobic metabolism is essential for cell survival. Consequently, by-products of oxidative metabolism, ROS and free radicals are inherent. A free radical is defined as any species with an unpaired electron capable of independent existence (Halliwell and Gutteridge 1989). Biological radicals can be classified as exogenous or endogenous. Exogenous radicals are derived from external or

environmental origins such as air pollutants, tobacco smoke and radiation. Endogenous radicals are synthesized within the body as by-products of metabolic or inflammatory reactions. They are principally oxygen or nitrogen centred such as the peroxyl radical (ROO·), the hydroxyl radical (OH·), nitric oxide (NO) and the superoxide radical (O_2·). Through evolution, aerobic organisms have developed an AO defence system to protect against the damaging effects of reactive species. In healthy individuals, equilibrium exists between oxidative species and the AO defences allowing the body to defend itself against low levels of oxidative stress. The body's AO defence is dynamic and determined by the dietary intake of AOs (and their precursors), endogenous synthesis of AOs and the rate of AO excretion (Maxwell 2000). During periods of oxidative stress, this metabolic equilibrium may be disturbed, as pro-oxidants overwhelm the AO defences. Oxidative stress is characterised by a number of changes including an increase in formation of radicals, a decrease in production of AOs, a disturbance in cellular redox balance and oxidative damage to cellular components. This may damage cell membranes, through oxidation of proteins, membrane lipid or through DNA damage. There are four classes of biomarkers used to assess oxidative stress including measurement of radical production (i.e. OH·, O_2·), AOs (i.e. glutathione (GSH), ascorbate), oxidation products (i.e. protein carbonyls) and the AO/pro-oxidant balance (Powers and Jackson 2008). Over time, oxidative stress compromises cellular function and increases the susceptibility to diseases such as diabetes, arthritis and atherosclerosis. While over exposure to ROS can have a damaging effect on biological functions, free radicals also play important roles within the body. They are involved in cell signalling and protection against infection (Dröge 2002). The theory of hormesis suggests that there can be a favourable biological response when exposed to low levels of a stressor (Finley et al. 2011) making complete removal or eradication of ROS undesirable.

Biological AOs are defined as substances, present at relatively low concentrations that can delay or inhibit oxidation of a substrate (Frankel and Meyer 2000). They are classified, based on source, as enzymatic or non-enzymatic. They boost the body's oxidative defence and confer their protective effects by acting as preventative AOs or scavenging AOs. There are a number of enzymes central to the body's AO defence system including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Many of these enzymes function as preventative AOs; that is, they intercept oxidising species before damage to various cellular biomolecules can occur. Many of the AO enzymes work cooperatively in vivo.

The majority of nutrient-based AOs act as chain breaking AOs in which they stop or slow the oxidative process once it has begun. They can behave as *sacrificial* AOs by reacting with a radical before vital biomolecules are damaged or as donor AOs in which they are oxidised to products with insufficient reactivity to propagate the radical chain reaction (i.e. uric acid, vitamin C, E). The physicochemical properties of an AO molecule can dictate its action and efficacy in vivo. Hydrophilic AOs, such as ascorbic acid and uric acid, scavenge free radicals in the aqueous phase. Lipophilic AOs, such as vitamins E and K, quench radical species present in cell membranes or lipid bilayers and provide protection against lipid peroxidation of polyunsaturated fatty acids.

Proteins, peptides and amino acids also contribute to the body's AO defence. Proteins present in blood can on average scavenge 20 % (range 10–50 %) of the peroxyl radical scavenging ability of plasma (Wayner et al. 1987). GSH, a thiol containing tripeptide (Cysteine–Glutamic acid–Glycine; Cys–Glu–Gly), is synthesized predominantly within the liver from the amino substrates and is present in the liver at concentrations between 5 and 7 mM. The most important AO function of GSH is to scavenge free radicals and ROS. It can interact directly with radicals by hydrogen atom transfer (HAT). It also acts as a substrate for GPx which catalyses the removal of hydrogen peroxide (H_2O_2) producing the oxidised glutathione (GSSG). GSH also has a role in reducing some AO-derived radicals within the cell including the vitamin E radical and semidihydroascorbate radical thereby recycling vitamins E and C (Powers and Jackson 2008). GSH can be regenerated from GSSG by GSH reductase. The ratio of GSSG:GSH is often used as an indicator of cellular redox state (Wu et al. 2004). Carnosine, a histidine (His) containing dipeptide (β-Alanine–His; β-Ala–His) found in skeletal muscle, can act as a radical scavenger and metal ion chelator (Chan et al. 1994).

There is an emerging body of scientific research that has established, in vitro, the AO properties of peptides derived from food protein hydrolysates. These protein-derived AOs offer a number of potential benefits. They are considered safer alternatives to synthetic AOs such as butylated hydroxylanisole (BHA), butylated hydroxyltoluene (BHT) and propyl gallate. Some of these peptides in turn possess multiple biological activities, which may increase their physiological effectiveness and further reduce the risk of disease development (Meisel 2004). Furthermore, peptides can act synergistically with non-peptide AOs enhancing their protective effect (Kitts and Weiler 2003).

Enzymatic hydrolysis

Enzymatic hydrolysis is an effective means of liberating bioactive peptides from intact protein sequences

(Korhonen and Pihlanto 2006). Hydrolysis is a chemical reaction, which involves cleavage of the peptide bond fragmenting proteins into smaller peptides or free amino acids. Hydrolysis can be achieved using enzymes or chemicals (acid/alkali). Chemical hydrolysis particularly damages the protein substrate through racemization and potentially yields toxic substances such as lysino-Ala (Clemente 2000). The destruction of some amino acids and peptides during acid hydrolysis also leads to hydrolysis products with reduced nutritional properties (Neklyudov et al. 2000).

Enzyme-catalysed hydrolysis uses milder conditions, making it less damaging to the protein substrate, and is an ideal means of generating food-grade protein hydrolysates. Enzymatic hydrolysis of milk proteins can modify both the techno-functional and bio-functional properties of the resultant hydrolysate. This strategy has been employed in the past to generate hydrolysates with increased solubility (Slattery and FitzGerald 1998), decreased viscosity and enhance foaming capacity (Panyam and Kilara 1996), immunostimulatory/antimicrobial properties (Meisel 1998) and reduced allergenicity (Ena et al. 1995). In general, the larger molecular weight peptides are associated with techno-functional benefits (Silvestre 1997) and lower molecular weight peptides with bioactive effects. Bioactive peptides typically contain 2–20 amino acid residues and have molecular weight $< 6{,}000$ Da (Sarmadi and Ismail 2010).

The composition of the hydrolysate will be dependent on the protein substrate, the proteolytic enzyme, enzyme to substrate ratio (E:S), physicochemical conditions (pH, pre-heat treatment, hydrolysis time and temperature of reaction), the degree of hydrolysis (DH) achieved, post-hydrolysis modifications and the interactive effects between these parameters. A variety of different enzymes have been used to hydrolyse milk proteins generating hydrolysates with differing degrees of hydrolysis, containing a diverse array of peptides and different AO activity (Table 1). A number of studies have reported potent AO activity of Alcalase® derived hydrolysates (Peng et al. 2010). Compared to other enzyme hydrolysates, Alcalase® derived hydrolysates contain a higher yield of AO peptides with shorter amino acid sequences and appear to be more resistant to digestive enzymes (Sarmadi and Ismail 2010).

The rate at which a proteinase will catalyse a hydrolysis reaction is dependent on reaction conditions; the key variables being pH, temperature, time, presence of activators or inhibitors and any pre-hydrolysis modifications. Heat treatment assists the unfolding of the protein's globular structure making hydrophobic regions more accessible to the enzyme and may alter the profile of peptides released during hydrolysis. It has been shown that the AO potential of whey protein hydrolysates can be improved by heat treatment prior to hydrolysis (Pena-Ramos and Xiong 2001). Hydrolysis is a dynamic process, with peptides continuously being formed and degraded; therefore, careful selection and optimisation of the parameters discussed above are essential to maximise the release of AO peptides.

Ultrafiltration (UF) is a simple non-denaturing membrane concentration and fractionation technique, which separates proteins/peptides based on differences in size. UF is widely used within the dairy industry for concentration and fractionation of milk proteins and some studies have used UF post-hydrolysis to enrich the AO activity of crude protein hydrolysates (Table 1). Bioactive peptides can also be purified by a combination of chromatographic techniques including reverse phase high performance liquid chromatography (RP-HPLC), gel filtration, ion exchange and selective adsorption (Etzel 2004). Affinity chromatography has been exploited for purification of copper-chelating peptides from sunflower protein (Megías et al. 2008) and chickpea (Megías et al. 2007) hydrolysates. Following affinity purification, the hydrolysates generated by gastric proteinases (pepsin and pancreatin) had a 2.5-fold higher AO activity than the parent protein. The increase in AO activity was attributed to a fourfold (2.6–11 %) increase in the His content which binds to copper via its imidazole ring.

In vitro assays

In vitro measurement of AO activity is central in evaluating the AO potential of hydrolysed protein or peptide-enriched preparations. Due to the complex nature of AOs, there is no single technique to measure the total AO activity of a food system. Therefore, a variety of analytical techniques are employed. Mechanistically, radical scavenging assays can be divided into two categories based on the chemical principles of electron transfer (ET) or HAT (Huang et al. 2005) from the AO compound to the radical species (Table 2). The end result, stabilising a free radical, of each mechanism is the same but the kinetics of each reaction can vary. These mechanisms can occur simultaneously and the dominant mechanism is determined by the structure of the AO compound, its solubility in the system, ionisation potential and bond dissociation energy (Prior et al. 2005).

ET-based assays are non-competitive systems involving a single redox reaction where the test AO transfers one electron to reduce an oxidisable probe. A change in spectrophotometric absorbance is used to quantify the reducing capacity of the AO. The trolox equivalent AO capacity (TEAC) assay is the most popular ET-based assay (Zulueta et al. 2009). The mechanism is based on scavenging of the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid; ABTS) radical cation ($ABTS^{\cdot+}$) by an AO present in the

Table 1 Antioxidative peptides derived from bovine and human milk protein following enzyme hydrolysis

Substrate	Hydrolysis conditions	Sequence	Fragment	Antioxidative activity	References
Bovine milk	n/a	β-Casein fragment	High molecular weight fraction ~24 kDa	ORAC, TEAC	Clausen et al. (2009)
Milk protein concentrate	Trypsin, chymotrypsin, 45 °C, pH 8, 2 h, 25 % (w/v) protein Ultrafiltration 6 kDa, Sephadex G15 gel filtration fractionation	Molecular weight <6 kDa, fraction 3 from Sephadex G-15	ND	Hydroxyl radical scavenging, superoxide radical scavenging, total antioxidative capacity (Fe^{3+} reduction)	Pan et al. (2011)
Milk protein isolate	Validase®, 55 °C, 6 h, E:S 2 %, pH 7 DH 35 %, UF Fraction 1–3 kDa	ND	ND	ORAC, 240 μmol TE/g peptide DPPH Lipid peroxidation	Hogan et al. (2009)
Casein	Pepsin, pH 2, 37 °C, 24 h Purification gel filtration (fractions 300–5,000 Da) and ion exchange		αs1-CN f(144–149)	Superoxide scavenging activity (SOSA) and DPPH scavenging activity SOSA IC_{50}:	Suetsuna et al. (2000)
		Tyr–Phe–Tyr–Pro–Glu–Leu		79 μM	
		Phe–Tyr–Pro–Glu–Leu		127.5 μM	
		Tyr–Pro–Glu–Leu		189.3 μM	
		Pro–Glu–Leu		306 μM	
		Glu–Leu		63 μM	
Casein	Trypsin, pH 7.8, room temperature, 24–28 h, post hydrolysis ion exchange fractionation			Inhibition of lipid peroxidation, DPPH radical scavenging	Rival et al. (2001)
		Val–Lys–Glu–Ala–Met–Pro–Lys	β-CN(98–105)	0.095 DPPH radical scavenging activity(RSA)	
		Ala–Val–Pro–Tyr–Pro–Gln–Arg	β-CN f(177–183)	1.0 DPPH RSA	
		Lys–Val–Leu–Pro–Val–Pro–Glu–Lys	β-CN f(169–176)	0.99 DPPH RSA	
		Val–Leu–Pro–Val–Pro–Glu–Lys	β-CN f(170–176)	1.05 DPPH RSA	
Casein	Trypsin pH 8, 50 °C, 2 h. Ultrafiltration 10 kDa	Molecular weight <10 kDa	ND	Inhibition of TBARS, ORAC	Díaz and Decker (2004)
Casein	Caseinate, mammalian, bacterial and plant food grade enzymes	ND	ND	FRAP assay17-32 μM, cellular AO status (GSH, SOD and CAT activity)	Phelan et al. (2009)
Whey protein	Chymotrypsin, 37 °C, E:S 1 %, 5 h, pH 7.8, post-hydrolysis ultrafiltration (10, 5, 3 kDa)	ND	ND	TEAC	Salami et al. (2010)

Table 1 continued

Substrate	Hydrolysis conditions	Sequence	Fragment	Antioxidative activity	References
Whey protein concentrate	Alcalase®, Protamex® or Neutrase® 45 °C, pH 6–8, 3 h, E:S 1 %	ND	ND	ABTS, TRAP	Dryáková et al. (2010)
	Alcalase®hydrolysate DH 11.3 %			50 % ABTS activity at 0.1 mg/ml	
	Neutrase®/Protamex® hydrolysate DH 6 %			50 % ABTS activity at 0.1 mg/ml	
Whey protein concentrate enriched in β-LG	Thermolysin, 80 °C, pH 8, 8 h, ES 0.1 (w/w), 3 kDa membrane permeate			ORAC-FL;	Contreras et al. (2011)
		Leu–Gln–Lys–Trp	β-LG f(58–61)	2.5 μmol TE/mg protein	
		Leu–Asp–Thr–Asp–Tyr–Lys–Lys	β-LG f(95–101)		
	Corolase PP®, 37 °C, pH 7.5, ES 0.1 (w/w)	ND	ND	1.1 μmol TE/mg protein	
Whey protein isolate	Alcalase®, pre heated 95 °C, 5 min, hydrolysis 5 h, pH 8.5, E:S 0.5 %, DH 35 %, molecular weight 0.1–2.8 kDa	ND	ND	Radical scavenging; FRAP, DPPH, ESR activity	Peng et al. (2009)
β-LG	Corolase PP®, 37 °C, 24 h			ORAC radical scavenging activity	Hernandez-Ledesma et al. (2005)
				2.1 μmol TE/mg protein	
		Trp–Tyr–Ser–Leu–Ala–Met–Ala–Ala–Ser–Asp–Ile	β-LG f(19–29)	ORAC; 2.6 μmol TE/μmol peptide	
		Met–His–Ile–Arg–Leu	β-LG f(145–149)	ORAC; 0.3 μmol TE/μmol peptide	
		Tyr–Val–Glu–Glu–Leu	β-LG f(42–46)	ORAC; 0.8 μmol TE/μmol peptide	
α-LA	Corolase PP®, 37 °C, 24 h	ND	ND	ORAC; 2.9 μmol TE/mg protein	
Human milk	Pepsin (pH 3.5. 37 °C, 30 min) and pancreatin (pH 7, 37 °C, 60 min), post-hydrolysis ultrafiltration 3 kDa	Trp–Ser–Val–Pro–Glu–Pro–Lys	β-Casein f(154–160)	TEAC; 1.7 μmol TE/μmol peptide	Hernández-Ledesma et al. (2007)
Human milk	Pepsin (pH 4, 37 °C, 30 min, pH 6 for 30 min) and pancreatin (pH 7, 37 °C, 2 h) digest	RP-HPLC fractionation		ORAC; 127 μM TE/g	Tsopmo et al. (2009)
		Fraction 23		5,274 μM TE/g	
	Post-hydrolysis ultra-filtration; 3 kDa permeate				
	Synthetic peptides	Tyr–Gly–Tyr–Thr–Gly–Ala		5,169 μM TE/mM	
		Ile–Ser–Glu–Leu–Gly–Trp		4,479 μM TE/mM	

ND not determined

Table 2 Summary of the main analytical features of in vitro assays used to evaluate food protein/peptide antioxidant properties

Assay	Free radical generator	Radical species	Conditions	Measurement	References
Oxygen radical absorbance capacity (ORAC)	AAPH	ROO·	37 °C, phosphate buffer pH 7	Fluoresence kinetics (Ex: 485 nm, Em: 520 nm) Net area under curve expressed as trolox equivalents	Cao et al. (1993) Prior et al. (2003)
Total radical trapping AO potential (TRAP)	ABAP or APPH	ROO·	37 °C, phosphate buffer, pH 7	Oxygen consumption or fluoresence Lag time expressed as trolox equivalents	Wayner et al. (1985) Ghiselli et al. (1995)
Trolox equivalence AO capacity (TEAC)	ABTS	$ABTS^{o+}$	Metmyglobin, H_2O_2, phosphate buffer pH 7	Absorbance 600 nm Endpoint assay expressed a trolox equivalents	Miller et al. (1993) Re et al. (1999)
2,2 Diphenyl-1 picrylhydrazyl (DPPH)	DPPH	DPPH·	Organic medium, methanol	% DPPH scavenged EC_{50} T_{EC50} $1/(EC_{50}*T_{EC50})$	Brand-Williams et al. (1995)
Ferric reducing AO power (FRAP)	2,4,6-tripyridyl-*s*-triazine complex $[Fe(III)\text{-}(TPTZ)_2]^{3+}$		Aqueous pH 3.6	Δ 593 nm Rate of formation of the $[Fe(II)\text{-}(TPTZ)_2]^{2+}$ ferrous complex	Benzie and Strain (1996)
Enhanced chemiluminesence (ECL)	Para-iodophenol-luminol	E-O· intermediate to L·	H_2O_2, pH 8, horseradish peroxidase	Luminescence, Time of maximum slope of recovery curve	Robinson et al. (1997)
Electron spin resonance (ESR)				ESR spectroscopy, % scavenged relative to control,	Peng et al. (2009)
Direct	DPPH	DPPH·	In ethanol	Relative scavenging activity (RSA)	
Indirect	n/a	OH·	pH 7.4, DMPO $Fe(II)/H_2O_2$ EDTA, DMPO	$(RSA) = (H_0-H)/H_0 \times 100$ %	
		O_2·			

test sample. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical, with an unpaired electron at one atom of the nitrogen bridge. The DPPH radical has a deep violet colour and is widely used to monitor the radical scavenging ability of various AOs (Brand-Williams et al. 1995). The decolourisation assay is typically monitored by a change in absorbance but can also be monitored by electron spin resonance (ESR; Calliste et al. 2001).

HAT assays monitor competitive reactions between test compound, free radical generator and an oxidisable probe.

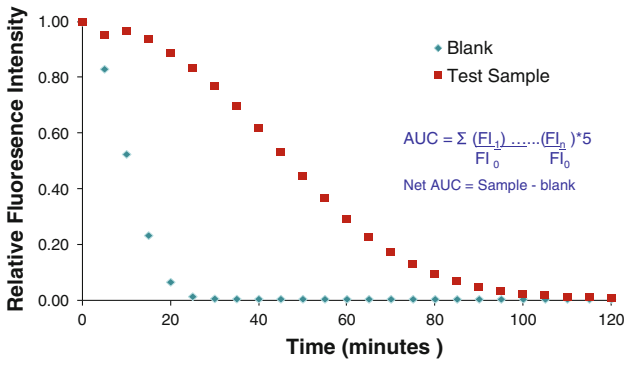

Fig. 1 Typical fluoresence decay curve for a test sample and blank determined by the oxygen radical absorbance capacity (ORAC) assay. *AUC* area under the curve, *FL* fluroesence

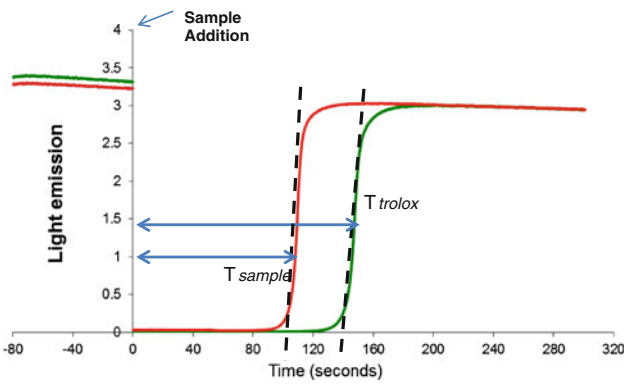

Fig. 2 Typical kinetics of light emission from the enhanced chemiluminescence reaction of trolox and a test sample

The ability of the AO to quench the free radicals, by hydrogen donation, will inhibit the oxidation of the probe. The total radical trapping AO potential (TRAP) assay is based on measuring oxygen consumption during a controlled lipid oxidation reaction (Wayner et al. 1985). The water soluble, azo-compound 2,2-azobis (2-aminopropane) hydrochloride (ABAP) acts as a radical generator by extracting a hydrogen atom to initiate lipid peroxidation. The presence of an AO in the test sample interferes with the initiation of lipid peroxidation.

Many assays use a stable radical generated chemically which do not occur in vivo; therefore, it may be more biologically relevant to use a radical source that occurs in vivo. The oxygen radical absorbance capacity assay (ORAC) is one such assay (Ou et al. 2001). In this assay, the peroxyl radicals formed by decomposition of 2,2′-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) react with a fluorescent probe to form a non-fluorescent product. The protective effects of the AO inhibit probe oxidation and thus fluorescence is maintained. The AO capacity is determined by a decrease in the rate and amount of fluorescent product formed over time, with values expressed as net (sample–blank) area under the fluorescence decay curve (Fig. 1). Trolox, a water soluble derivative of vitamin E, is used to quantify the AO capacity of the test compounds and ORAC values are expressed as trolox equivalents. This assay has been developed to allow high throughput analysis using 96-well plates (Huang et al. 2002). Area under the curve (AUC) calculations account for the kinetics of the reaction including lag time, initial rate and total extent of inhibition and allow comparison of AOs with and without a lag phase (Magalhães et al. 2008). The ORAC assay is the assay of choice in the food and pharmaceutical industries and is often quoted in product literature (Huang et al. 2002). The United States (US) Department of Agriculture has untaken a comprehensive analysis of the AO capacity of nearly 300 fruits and vegetables using this methodology (Haytowitz and Bhagwat 2010).

Radical scavenging AOs can also function as reducing agents. The ferric reducing antioxidant power (FRAP) assay measures the ability of an AO to reduce the ferric ion, ferric 2,4,6-tripyridyl-*s*-triazine complex $[Fe(III)\text{-}(TPTZ)_2]^{3+}$ to the $[Fe(II)\text{-}(TPTZ)_2]^{2+}$ ferrous complex at pH 3.6 (Benzie and Strain 1996). Luminescence is the emission of photons, which occurs when a molecule in an excited state returns to the ground state. The enhanced chemiluminescence (ECL) assay involves horse radish peroxidise catalysed oxidation of luminol by hydrogen peroxide (Robinson et al. 1997). This system includes an enhancer *p*-iodophenol which ensures a more intense, prolonged and stable light output. The rate-limiting step in the reaction sequence is the continuous emission of light from the free radical intermediates of luminol, *p*-iodophenol and oxygen. Light emission can be quenched by the addition of a radical scavenging AO (Fig. 2). The AOs act as electron donors to reduce the phenoxy radicals preventing them from reacting with luminol. The lag time, period of light suppression, is proportional to the concentration of the AO present. AO activity can be expressed as trolox equivalents.

ESR spectroscopy is a technique that directly detects chemical species with an unpaired electron. It measures the interaction between unpaired electrons and an applied magnetic field. There are two main types of ESR, direct ESR uses a stable radical species such as DPPH and ESR spin trapping which uses an exogenous spin trapping molecule such as 5,5-dimethyl-1-pyrroline N-oxide (DMPO) to measure short lived radical species (i.e. $HO\cdot, O_2^{\cdot -}$). This technique has been used to evaluate free radical scavenging capacity of AOs in food and nutraceutical research (Yu and Cheng 2008). ESR has also been used to evaluate the radical scavenging activity of fermented marine blue mussel-derived peptides. These peptides displayed superoxide, hydroxyl, carbon centred and DPPH scavenging capacity as measured by ESR (Rajapakse et al. 2005b). ESR is a highly sensitive technique and has been used to detect AO activity of whey protein isolate (WPI) hydrolysates (Peng et al. 2009).

While these in vitro assays evaluate the AO potential of the test substance, it must be remembered that they are indirect measures of AO activity. Activities measured by these systems are assay specific and only reflect the chemical reactivity in the conditions being tested (Huang et al. 2005). Standardisation of the various methodologies, although desirable, has proven difficult making the comparison of data from different assays difficult. Due to the complex chemical nature of AOs, the use of multiple assays in combination has been proposed to generate an AO profile for the food system under investigation (Prior et al. 2005). A number of indices have been proposed and applied for analysis of a variety of foods. The relative antioxidative capacity index (RACI) is a statistical means of integrating the antioxidative capacity data from a number of different in vitro chemical assays (Sun and Tanumihardjo 2007). Results from each assay are expressed as a standardised score (*z*-score; raw data-mean/standard deviation). The RACI is created for each substrate by averaging the standard scores for each method. RACI does not reflect a single mechanism, and foods with a similar RACI may have different quality and quantity of AO present and therefore, cannot be assumed to have equivalent AO potency. The RACI may be useful as a screen of antioxidative potential in order to select samples for further investigation. The antioxidative potency composite index (AOPI) is based on similar principals and uses results from

a number of different chemical assays (Seeram et al. 2008). The best score for each assay is assigned a value of 100 and an index score for each sample is expressed relative to this value (sample score/best score × 100). All assays carry equal weight, and the average score for all assays is used to generate the AOPI for each substrate.

In spite of the shortcomings, in vitro assays are valuable research tools and combined with bioavailability data, and biomarker assays can provide excellent assessment of the AO activity of various food systems under investigation (Huang et al. 2005).

Antioxidant peptides

Peptides generated from digests of food proteins have been shown to possess AO activity. Both plant and animal-derived substrates contain peptides with AO properties. Some examples of plant protein sources of AO peptides include soy (Moure et al. 2006; Park et al. 2010), chickpea (Yust et al. 2012), sunflower (Megías et al. 2008), alfalfa leaf (Xie et al. 2008), algae protein waste (Sheih et al. 2009), peanut (Hwang et al. 2010), rice endosperm (Zhang et al. 2010) and maize zein (Tang et al. 2010). Animal protein sources of AO peptides include egg white (Davlos et al. 2004; Rao et al. 2012), yellowfin sole (Jun et al. 2004), mussel (Rajapakse et al. 2005a, b), hoki (Kim et al. 2007b), pacific hake (Cheung et al. 2012), gelatin derived from pacific cod skin (Himaya et al. 2012), bullfrog skin (Qian et al. 2008), royal jelly (Guo et al. 2009), water buffalo horn (Liu et al. 2010), shrimp (Faithong et al. 2010), squid (Rajapakse et al. 2005a), bovine liver (Di Bernardini et al. 2011), yak milk casein (CN; Mao et al. 2011) and bovine milk protein which is the particular focus of the following section. As already indicated, where data on milk-derived peptides is limited, the understanding gained from some of the food proteins listed above is drawn upon.

Milk protein-derived antioxidant peptides

The principle protein components of milk CN (80 %) and whey (20 %) have both been used to generate AO peptides. CN is the predominant phosphoprotein in milk and is composed of αs1, αs2, β, κ-CN (Fox and McSweeney 2003). CN's contain a large amount of proline (Pro) residues, which disrupts the formation of α-helical and β-sheet secondary structure (Swaisgood 1992). CN's have a relatively open or disordered structure which makes them highly susceptible to proteolysis. Whey has a globular structure and is composed of five major fractions: β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin, immunoglobulins and a number of minor proteins such as lactoferrin and lactoperoxidase. Each whey protein faction has different physicochemical properties, which can be modified by enzymatic hydrolysis (Walzem et al. 2002). Whey, CN and other milk proteins are excellent precursors of biologically active peptides (Haque et al. 2009). Highly potent AO peptides can be generated by enzymatic hydrolysis of milk proteins (Table 1).

Intact CN has been shown to possess AO activity against thiobarbituric acid reactive substances (TBARS) and in a model linoleic acid oxidation system (Laakso 1984). The proposed mechanism of AO activity is the quenching of free radicals by the oxidation of amino acids in CN. Free amino acids were not as effective as the intact CN, suggesting that the primary structure of the CN molecule is a determinant of AO functionality (Laakso 1984). A peptic digest of CN (αs_1-CN) produced a tyrosine (Tyr) containing hexapeptide (Tyr–Phe–Tyr–Pro–Glu–Leu) with strong superoxide anion scavenging activity (SOSA; Suetsuna et al. 2000). A synthetic peptide with the same sequence was used to investigate the mechanism of AO activity. In addition to SOSA activity, the peptide had hydroxyl and DPPH radical scavenging activity. Removal of amino acid residues (Tyr, Phenylalanine (Phe), Tyr) from the hexapeptide resulted in a loss of AO activity and the most potent activity was found in the Glu–Leu dipeptide portion of the sequence. Various enzyme digests and sub-fractions of bovine CN have been shown to have AO activity (Rival et al. 2001). Tryptic and subtilisin digests of CN and tryptic digests of β-casein (β-CN) were shown to have the highest inhibition of linoleic acid oxidation (Rival et al. 2001). The tryptic digest of β-CN had greater activity than the undigested CN. Radical scavenging activity was related to the presence of specific amino acid residues such as methionine (Met), Tyr, arginine (Arg) and Pro.

Caseinphosphopeptides (CPPs) have been reported to have AO activity (Kitts 2005). CPPs are derived from enzymatic hydrolysis of CN and are rich in phosphoserine residues. The proposed mechanism of AO activity of CPP's is linked to the presence of phosphate groups originating from the phosphoserine residues in close proximity to the peptide chain. This creates a polar and anionic domain which can sequester cationic metal ions. CPP's contain the functional domain, SerP–SerP–SerP–Glu–Glu. CPP's have been shown to possess ABTS· radical scavenging (Chiu and Kitts 2003), hydroxyl, peroxyl and metal chelating activity (Kim et al. 2007a). The AO activity of enriched CPP preparations and low molecular weight CN hydrolysates were evaluated (Díaz and Decker 2004). The low molecular weight CN hydrolysates were more effective peroxyl radical scavengers, whereas the enriched CPP's had greater metal chelation properties. High amounts of CPP were pro-oxidative, whereas CN hydrolysates had only AO activity. This AO activity of CN hydrolysates may

be related to their amino acid content as they had ~2–4-fold higher concentrations of His, Tyr, Pro and lysine (Lys) residues than intact CPP.

Whey proteins, their hydrolysates and peptides derived from them have also been shown to possess potent AO activity (Pihlanto 2006). Hydrolysis of β-LG and α-LA with a variety of commercial proteinases produced peptides with AO (ORAC) activity (Hernandez-Ledesma et al. 2005). A Corolase PP® digest of β-LG produced the most potent peptide (f19–29; Trp–Tyr–Ser–Leu–Ala–Met–Ala–Ala–Ser–Asp–Ile). Synthetic β-LG f19–29 had a higher radical scavenging ability than BHA (2.62 µmol Trolox/µmol peptide vs. 2.43 µmol Trolox/µmol BHA). The AO activity was attributed to the presence of tryptophan (Trp), Tyr and Met residues in the peptide. The radical scavenging ability of another β-LG peptide (f42–46; Tyr–Val–Glu–Glu–Leu) was compared to an equimolar mixture (Tyr + Val + 2(Glu) + Leu) of amino acids and the peptide was more potent (0.8 µmol Trolox/µmol peptide vs. 0.4 µmol Trolox/µmol amino acid mixture). This suggests that in some instances the peptide bond or structural conformation of the peptide can enhance AO activity. The mechanism(s) of whey protein-induced AO activity have been investigated and these have been attributed to the synergistic action of sulfhydryl groups, free radical scavenging by specific amino acids and chelation of iron (Tong et al. 2000). Hydrolysates of WPI have been shown to possess AO activity. Five hour digestion with Alcalase® produced an hydrolysate with strong reducing power (FRAP). When fractionated on the basis of molecular mass, the low molecular weight fraction (0.1–2.8 kDa) was most potent (Peng et al. 2009). This study concluded that the mechanism of radical scavenging activity was dependent on the size and composition of the peptides.

A number of statistical tools and models can be used in predicting the AO properties of peptides. Stepwise multiple linear regression, correlation and principle component analysis have all been used to study the properties of WPI hydrolysates (Peña-Ramos et al. 2004). The statistical analysis showed that hydrolysates with the highest AO activity contained low molecular weight peptides with a specific amino acid composition, in particular, a high concentration of His and hydrophobic amino acids. These statistical tools could prove useful in designing a targeted enzyme hydrolysis strategy to release AO peptides. The AO potential of milk protein hydrolysates and milk protein-derived peptides is determined by many factors, which can interact both synergistically and antagonistically; examining any one factor in isolation could prove misleading. Response surface modelling is also a means of exploring the relationship between several independent variables and one or more dependent variables. This model has been successfully applied to maximise the AO activity of a whey protein concentrate (WPC) derived hydrolysates (Contreras et al. 2011).

Structure activity relationship

The structure–function relationship and the mechanism of peptide-induced AO activity have not been fully elucidated. Peptides have been shown to act as radical scavengers, metal chelators, reducing agents and inhibitors of lipid peroxidation (Table 1). Preliminary mechanistic studies suggest that the AO activity of proteins and peptides is related to their amino acid composition, sequence/structure and hydrophobicity (Chen et al. 1998). The mechanism of AO activity can be categorised by the physicochemical properties of the amino acid. For example, residues with an aromatic ring structure (Tyr, Trp and Phe) can donate a proton to electron deficient radicals (Chen et al. 1998). Hydrophobic residues (valine (Val), leucine (Leu) and Tyr) can enhance the solubility of peptides in a lipid matrix improving the accessibility to hydrophobic radical species or polyunsaturated fatty acids (Qian et al. 2008). In addition, the AO activity of peptides in an emulsion system may be related to physical interaction. Peptides can partition to the oil–water interface and potentially form a barrier that will block interaction between the free radical and lipid thereby preventing lipid peroxidation. A whey protein membrane was used to improve the oxidative stability of emulsified menhaden oil (Donnelly et al. 1998). Quantitative structure activity relationship (QSAR) is a statistical modelling approach that has been used to identify the structural characteristics of AO peptides. This approach found that N and C terminus amino acids of the peptide are important predictors of AO activity (Li et al. 2011). The hydrophobic properties of N terminal amino acids are important and will increase the AO activity of the peptide. Comparing the AO peptides derived from milk protein (Table 1), more than half of the peptides identified to date have a hydrophobic residue at the N terminus. The electronic charge properties (i.e. net charge, molecular polarity) of the C terminal amino acid are also an important predictor of AO activity. This position is a polar domain and is affected by its electrostatic potential with the amino acids Trp, Glu, Leu, isoleucine (Ile), Met, Val, and Tyr frequently present. The second amino acid adjacent to the C terminus is suggested to be the major contributor to AO activity and if this amino acid has large hydrogen bonding and steric properties and low hydrophobicity, this will increase the potential AO activity. This makes acidic or basic amino acid residues (Asp, Glu, His, Arg, Lys) or hydrophilic amino acids (Ser, Thr) preferable in this position. Of the potent CN-derived AO peptides, the second amino acid adjacent to the C terminus

is typically Glu, Pro, Leu and β-LG derived peptides typically have Glu, Arg, or Asp in this position (Table 1).

The sulfhydryl (R-SH) group in Cys has a unique AO activity and interacts with the radical species by hydrogen donation from the SH group (Elias et al. 2008). The imidazole group of His has been linked to its metal chelating, hydrogen donating and lipid peroxyl trapping ability (Chan et al. 1994). The AO activity of His-containing peptides is influenced by the physical environment; in particular, the presence of hydrophobic compounds can increase accessibility to hydrophobic radical species (Murase et al. 1993). Caffeic acid (CA) peptide conjugates were used to study the effect of His dipeptides on the AO activity of a natural AO. His-containing dipeptides enhanced the AO activity of CA and a CA, Pro; His amide (CA–Pro–His–NH_2) had the greatest radical scavenging (DPPH) and lipid peroxidation (linoleic acid) inhibitory activity (Seo et al. 2010).

AO activity is not solely dependent on the presence of specific amino acid residues. The location of the amino acids within the sequence of the protein/peptide will determine their accessibility and susceptibility to proteolytic release. During hydrolysis, the native structure of the protein will be altered and bioactive functional groups particularly within hydrophobic regions become exposed. As peptide bonds are cleaved, there can be an increased availability of free amino acids, thus providing a source of protons or electrons to maintain a high redox potential. Proteinase specificity will influence the characteristics of the resultant hydrolysate. For example; chymotrypsin has been used to generate hydrolysates of whey protein with AO activity. This enzyme is specific toward bonds containing aromatic or hydrophobic residues at the C-terminus and generates hydrolysates with AO amino acids at the C-terminus (Pihlanto 2006). Pepsin hydrolysis will expose hydrophobic residues as pepsin is specific towards hydrophobic and aromatic amino acids. Hydrophobic residues become exposed which facilitates electron transfer from these peptides (Zhu et al. 2008).

Surface exposure of amino acid residues affects their oxidation kinetics and determines why some amino acids are preferentially oxidised relative to others. For a given amino acid or peptide to act as an AO, it must be preferentially oxidised compared to other compounds in the food matrix. The oxidative reactivity of a variety of amino acids was evaluated by photochemical oxidation with a OH· and it was found that amino acid reactivity was in the following order Cys > Trp, Tyr > Met > Phe > His > Ile > Leu > Pro (Sharp et al. 2003). Synthetic peptides have been used to explore the mechanism of AO activity. A series of synthetic peptides were generated based on the structure of an AO peptide (Leu–Leu–Pro–His–His) derived from a proteolytic digest of soy protein. It was reported that the AO activity was dependent on the His–His segment of the peptide and removal of a C-terminal His residue reduced AO activity by approximately 50 % while removal of the N-terminal Leu had no effect on AO activity (Chen et al. 1996). Both His and Pro were crucial for activity; of the 28 peptides evaluated, Pro–His–His was the most potent. This peptide also showed a synergistic action with the lipophilic AOs, BHA and tocopherol. Tripeptide libraries have been constructed in order to determine which amino acids are essential for AO activity and to predict the most potent peptides (Saito et al. 2003). One tripeptide library contained 108 peptide sequences containing either two His or Tyr residues and the other was composed of 114 peptides structurally related to Pro–His–His. Different assays were used to establish the radical scavenging, reducing, peroxynitrite scavenging and lipid peroxidation potential. It was concluded that amino acid sequence influenced the AO activity and tripeptides containing Trp or Tyr at the C-terminus had strong radical scavenging ability (Xaa–Xaa–Trp/Tyr). The tripeptide, Tyr–His–Tyr, had synergistic action with polyphenols and a Cys containing tripeptide (Xaa–Xaa–Cys) had peroxynitrite scavenging activity.

Overall, it is likely that the AO activity of food protein-derived peptides is determined by the combined and synergistic action of some or all of the mechanisms detailed above rather than a single predominant factor. Synergistic action may arise due to interaction of AO compounds with different mechanisms of action or interaction of AO peptides with different polarities, which may be distributed in different phases of a food matrix (Laguerre et al. 2007). Therefore, when characterising the AO activity of protein hydrolysates, evaluating each type of activity may be important to gain a better understanding of the mechanisms involved.

In vitro cell culture models

Cell culture models are a useful tool to evaluate the potential health effects of AO peptides and hydrolysates in vitro. The results from in vitro chemical assays discussed above are somewhat limited as the assay systems may not reflect cellular physiological conditions. However, cell culture models can be used to evaluate bioavailability, metabolism and biological activity.

The human adenocarcinoma colon cancer cell monolayer (Caco-2) is the most widely accepted in vitro model of intestinal permeability due to the similarities with intestinal endothelium cells. When cultured under specific conditions, Caco-2 cells form a continuous monolayer with a structural arrangement that serves as a model of both paracellular and transcellular movement. The bioactive

compound can be added to the apical or basolateral side of the monolayer and over time, the rate of transport evaluated. The apparent permeability coefficient from Caco-2 permeability studies has been shown to correlate with human intestinal absorption (Irvine et al. 1999). This model has been used to evaluate the permeability of AO peptides in a fish protein hydrolysate (FPH). FPH fractions had different Caco-2 cell permeability values and it was estimated that ~5 % of the peptide material had passed through the cell (Samaranayaka et al. 2010).

Prior to human consumption, it is also important to establish that the potential AO peptide is non-toxic. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay evaluates the ability of functional mitochondria to catalyse a reduction reaction. This reaction will only proceed when cells are living, giving an indication of metabolic activity/viability of the cells. Interestingly, pre-treatment an AO peptide can enhance cell survival. A peptide (His–Phe–Gly–Asp–Pro–Phe–His) derived from fermented marine blue mussel had DPPH (72 %) radical scavenging activity, superoxide (98 %) scavenging activity at 200 μg/ml and enhanced cell viability by 76 % at a dose of 75 μg/ml (Rajapakse et al. 2005b).

Cellular AO activity of a test compound can be assessed via an intracellular oxidative challenge (Wolfe and Liu 2007). In this model, human hepatocarcinoma (HepG2) cells are pre-treated with the test AO compound. Dichlorofluorescein diacetate (DCFH-DA) is added and will diffuse into the cell. DCFH-DA can undergo intracellular hydrolysis producing DCFH. DCFH will react with a peroxyl radical (produced from decomposition of ABAP) to form a fluorescent compound (DCF). In the presence of an AO, the fluorescence intensity will be reduced as the formation of DCF is inhibited. This methodology has been used to evaluate the AO activity of a novel dipeptide (Pro–Arg) derived from protamine hydrolysis. The presence of the dipeptide was shown to reduce cellular oxidative damage (Wang et al. 2009). Human Jurkat T cells have membrane markers that resemble normal T cells and are used to evaluate T cell signalling in bioactivity studies and in response to cellular oxidative stress. These cells have been used to evaluate changes in endogenous AO biomarkers (GSH, CAT and SOD) on exposure to a range of CN hydrolysates for 24 h. Some CN hydrolysates enhanced GSH content (+165 %) and CAT activity (+309 %) relative to untreated cells (Phelan et al. 2009).

Antioxidative compounds have been shown to regulate gene expression of AO enzymes. Resveratrol, a well-characterised AO found in grapes, reduced endothelial oxidative stress by down regulation of NADPH 4 oxidase (NOX4) and enhancing the expression of SOD and GPX (Spanier et al. 2009). Hen egg yolk (phosvitin) derived peptides have been shown to up regulate the AO genes GPX3 and SOD2. Furthermore, simulated gastrointestinal digestion of phosvitin phosphopeptide reduced pro-inflammatory interleukin 8 secretion in Caco-2 cells and highlights the potential of the peptide to prevent oxidative stress and promote gut health (Young et al. 2011).

There are some drawbacks of using immortalised cell lines in AO research. Some immortalised cell lines including HepG2 cells show altered functional responses to oxidative stress (Honzel et al. 2008). Some cell lines may also undergo asymmetric divisions in culture making them dysfunctional and apoptotic which may lead to the formation of ROS, potentially confounding the data. The novel cell-based AO protection assay in erythrocytes (Cap-e) has been developed to overcome some of these factors. The erythrocyte model is favoured because it is an inert cell type, i.e., they do not engage in complex inflammatory reactions and are not capable of generating ROS as a result of stress or death (Honzel et al. 2008). In this assay, cells are exposed to the test compound in physiological saline. The cells are allowed time to absorb the test compounds and any compounds not absorbed are removed by washing. A precursor dye (DCFH-DA) is added and when exposed to oxidative challenge (AAPH or H_2O_2), the fluorescent DCF is produced. The fluorescence intensity is related to the amount of oxidative damage, and a reduction in fluorescent intensity is proportional to the AO protection provided by the test sample. The ORAC assay and the Cap-e assay were used to evaluate the AO protection of a novel bovine colostrum extract, Immunel™. Immunel™ had AO activity in the ORAC assay (18 μmol TE/g) and also showed a dose-dependent inhibition (~25 % at 10 mg/ml) of oxidative damage in the Cap-e assay. This suggests that the Immunel™ contained AO compounds that entered the cells (Honzel et al. 2008).

The Cap-e assay was also used to evaluate bioavailability of AO from acai berry juice in vitro and in vivo. In vitro, the acai berry juice had a dose-dependent (0.016–10 mg/ml) increase in AO protection. This suggests that the AO compounds were able to cross the erythrocyte cell membrane and provide protection from oxidative damage. Following on from this a double blind, placebo controlled, human study evaluated serum AO status following ingestion of the acai berry juice (120 ml) using the Cap-e assay. There was a 12 % increase in serum AO status 2 h post-ingestion ($p < 0.05$) of an acai berry-rich juice (Jensen et al. 2008).

In vivo animal models

To date, only a small number of studies have evaluated the efficacy of AO proteins/peptides using animal models. Intact dairy proteins have been shown to protect against

intestinal tumours in male Sprague–Dawley rats. The rats fed a diet of whey protein or CN had fewer tumours and a reduced tumour mass compared to animals fed red meat or soy. Tumour mass was lowest in the whey-fed animals. The protective effect of the protein was attributed to intracellular (liver) concentrations of GSH which were significantly higher in whey and CN-fed rats (McIntosh et al. 1995).

Recently, the AO activity of WPC and a marine alga (spirulina) has been evaluated in vitro and in vivo (Gad et al. 2011). In vitro, WPC with and without spirulina was shown to increase DPPH scavenging and metal chelating activity in a dose-dependent manner (20–100 mg/ml). The most potent in vitro dose (100 mg/ml) was evaluated in a chronic in vivo study. Over a 30-day period, rats were fed 50 mg/day of control or treatment WPC, spirulina alone or in combination and with or without the hepatotoxin carbon tetrachloride (CCl_4). CCl_4 was used to induce liver damage in the rats, and therefore assess the protective effect of WPC with/without spirulina. Treatment with WPC or spirulina alone decreased serum cholesterol (59, 69, 82, 208 mg/dL; WPC, spirulina, control and CCl_4) and increased liver tissue total AO capacity (TAC; 52, 54, 47, 27 μmol/g liver tissue; WPC, spirulina, control and CCl_4) compared to control and CCl_4 treated groups. A combination of WPC and spirulina increased TAC (58 μmol/g liver tissue) and decreased cholesterol (58 mg/dL) and malondialdehyde (MDA; a marker of lipid peroxidation). The protective effect of the WPC and spirulina was evident as both agents prevented liver damage induce by CCl_4 and both treatments improved biochemical markers of oxidative stress and the histological profile of the rats. The AO activity of the WPC was attributed to the Cys content and the ability to elevate cellular GSH and the AO activity of spirulina related to its β-carotene, tocopherol and phycocyanin content.

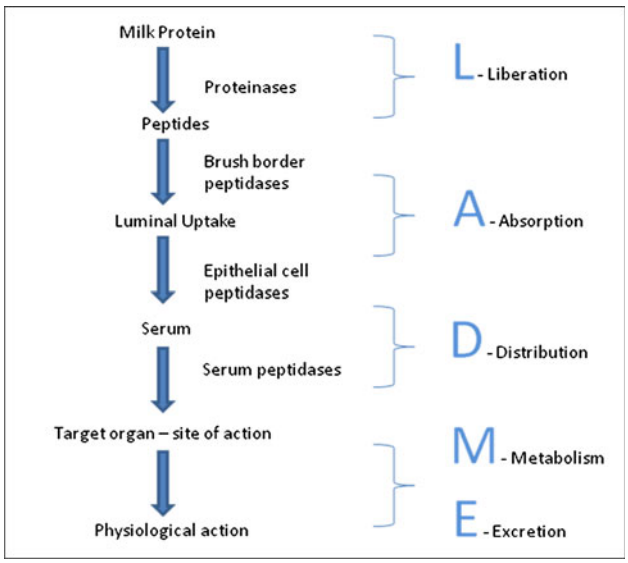

Fig. 3 Physiological route for bioactive peptide from intact protein to target site and the corresponding nutrikinetic parameter

Spontaneously hypertensive rats (SHR), an accepted model for oxidative stress, were fed (0.5 g/kg/day) a hen egg white hydrolysate (EWH) over 4 months and the AO status (plasma ORAC and MDA) and lipid profile were evaluated (Manso et al. 2008). Consumption of the EWH significantly increased plasma ORAC (~55 %), decreased aortic MDA levels (~40 %), plasma triglycerides (~17 %) and cholesterol (~13 %), while there was no change in high density lipoprotein. These data suggest that EWH may be a useful means to prevent oxidative stress and inhibit lipid peroxidation.

The AO activity of douchi (a fermented soy bean product) was evaluated in vitro and in vivo (Wang et al., 2008). Douchi extracts had AO activity in vitro (DPPH, ABTS and ferrous chelation). The in vitro AO activity was also seen in vivo in rats fed douchi for 4 weeks (2–4 % w/w). In vivo AO activity was evaluated by changes in AO enzymes (GPx, SOD, CAT) and TBARS in liver and kidney. Douchi extracts had tissue-specific effects on the enzymes. SOD activity was significantly increased in liver (~40–70 % relative to control) and kidney (~45 %), CAT activity increased in liver (~7–11 %), GPx activity increased in kidney (~20 %) and TBARS decreased in both liver and kidney. Whey protein supplementation (100 mg/kg for 15 day pre and post wounding) has been found to significantly shorten wound healing (closure) in diabetic rats (Ebaid et al. 2011). Whey protein supplementation restored inflammatory cytokines (IL-1β, TNF-α), required to initiate early stages of healing, to levels similar to that of the control animals. Diabetic rats also have increased oxidative stress; whey protein supplementation reduced oxidative stress in these animals as it decreased tissue MDA, ROS, NO and increased tissue GSH levels.

Bioavailability of antioxidant peptides

The biological functionality of an AO peptide is dependent on its bioavailability. Peptides must be resistant to gastrointestinal, brush border and serum peptidases, furthermore, these peptides must escape hepatic metabolism while making their way to the target organ or site of action. The basic pharmacokinetic principle (liberation, absorption, distribution and metabolism, LADME) can be used to illustrate how the body may process a bioactive. However, food bioavailability is more complex than for a pharmaceutical compound; therefore, a new concept of

Table 3 Summary of different mechanisms of intact peptide absorption

Peptide property	Transport mechanism	Details	References
Large water soluble peptide	Paracellular route	Peptides pass between cells by energy-independent passive diffusion through the tight junctions	Gardner (1988)
Hydrophobic peptides	Transcellular route	Energy-dependent diffusion through the brush border membrane of the muscosal cells via facilitated diffusion, carrier mediated or phagocytotic mechanisms	Ziv and Bendayan (2000)
Lipophillic peptides. Too large to be absorbed into portal circulation	Lymphatic system	Absorption of peptides from the interstitial space into the lymphatic system	Rubas and Grass (1991)
Hydrolysis resistant peptides	Peptide transporters	Peptides are transported from the enterocyte into the portal circulation via transporters located on the intestinal basolateral membrane. Example: PepT1	Gardner and Michael (1984)

nutrikinetics (Berger et al. 2011) has been developed from the traditional pharmacokinetic model (Fig. 3).

Upon ingestion of a protein hydrolysate, the availability of peptides and amino acids is influenced by the rate of gastric transit, intestinal motility, luminal hydrolysis and mucosal absorption. Gastric transit is three times slower than transmucosal transfer making it the rate limiting step in the bioavailability of peptides. The differing physicochemical properties of milk proteins result in contrasting gastric kinetics (Biorie et al. 1997). It has been suggested that peptide hydrolysates have marginally faster gastric emptying and intestinal absorption than intact proteins (Moughan et al. 1991). However, other studies have reported no significant differences in gastric emptying between intact proteins and their hydrolysates (Calbet and Holst 2004; Power et al. 2009). Small differences in absorption may be attributed to the fact hydrolysates can simultaneously use two transport systems, the amino acid and peptide transport systems (Silk et al. 1982). This may result in reduced competition for specific transporters, thus increasing the rate of delivery across the small intestine (Webb 1990). An AO peptide delivered as a protein hydrolysate could confer a kinetic advantage over intact protein during gastrointestinal transit and potentially maximise any physiological effect.

Peptides can be absorbed and transported by a variety of mechanisms. The molecular size and structural properties such as peptide hydrophobicity will determine the mechanism of transport. Short peptides, typically 2–6 amino acids, can be absorbed by peptide-specific transporters (Grimble 1994). Peptides can be absorbed by a number of mechanisms including the paracellular and transcellular routes, the lymphatic system and via basolateral transporters (Table 3). PepT1 is a proton-dependent transporter that uses an electrochemical proton gradient to move small hydrolysis resistant peptides from the enterocyte to the bloodstream (Segura-Campos et al. 2011). The AO dipeptide, carnosine, has been shown to be transported via PepT1 in CaCo-2 cells (Shimizu 2004). These transport mechanisms are not mutually exclusive and may occur simultaneously. Peptide transporters can also become saturated limiting the amount of bioactive peptide that can reach the peripheral circulation (Gardner and Michael 1984).

The antioxidative potency of a protein hydrolysate has been linked to the increase in concentration of specific amino acid residues (Sect. 6). Therefore, it is important to consider the amino acid transport system and how it may influence the biological availability of regulatory amino acids. Amino acids are transported via group specific transporters and the individual amino acids have differing affinity for these transporters (Keohane et al. 1985). This can result in competitive inhibition of some amino acids by those having a higher affinity for a shared transport system. The amino acids transporters are located along the surface of the brush border and basolateral membrane of the small intestine with the greatest abundance in the jejunum. They are classified based on kinetic parameters and substrate preference. They can use a variety of transport mechanisms including simple diffusion, facilitated diffusion and active transport. Active transport moves amino acids against a concentration gradient. Some are sodium dependent and use the energy potential of the Na^+ gradient to drive the process. Others are sodium independent and do not require metabolic energy to transport the amino acids against the concentration gradient. Absorption studies have shown that the essential amino acids are absorbed in greater quantities than the non-essential amino acids. In addition, $\sim$20 % of the amino acids absorbed do not appear in the portal circulation which can be accounted for by intestinal and hepatic metabolism of amino acids (Webb 1990).

The in vivo potency of an AO peptide could be greater than that predicted from the in vitro response. Potential synergy with other components within the food matrix may increase bioactivity. If a peptide AO and non-peptide AO are consumed together, they may elicit their AO activity by

different mechanisms, exploiting different pathways to produce a synergistic increase in the AO activity such as the combined action of WPC and spirulina outlined in Sect. 8. Peptides must have sufficient stability to survive the gastrointestinal tract and reach their target site intact. Human blood contains a large number of serum peptidases, which can further hydrolyse the bioactive peptide and reduce activity. For example, the β-lactoglobulin-derived ACE-inhibitory peptide (f142–148; Ala–Leu–Pro–Met–His–Ile–Arg) was shown to be degraded in vitro by gastrointestinal and serum proteinases destroying its potential bioactive properties (Walsh et al. 2004).

In addition, a bioactive peptide may not need to be absorbed in order to elicit the AO function. Binding to an intestinal receptor may be sufficient to trigger the bioactive (i.e. antioxidant) response. AO peptides may also elicit a beneficial biological effect by reducing oxidative damage within the gastrointestinal tract (Xiong 2010).

Therefore, simultaneous evaluation of the in vitro and in vivo AO capacity is the most effective means to establish the exact relationship between the in vitro and in vivo potency of milk-derived bioactive peptides. However, to date, no such evaluation appears to have been systematically performed.

In vivo AO capacity

Establishing in vivo efficacy of a potential AO peptide is essential and reliable biomarkers are central to this process. Many of the assays described herein have also been used to measure the AO capacity in biological fluids including serum, plasma, urine and semen. There has been some debate as to which biological matrix (plasma or serum) is most appropriate for determination. When both matrices were evaluated in the ORAC assay, they had a coefficient of variation of $\leq$12.5 % (Fernandez-Pachon et al. 2005). The absolute values for plasma were significantly higher than those for serum. Release of ROS during platelet aggregation may have contributed to the lower serum values (Ghiselli et al. 2000) making plasma the preferred matrix for AO capacity evaluation in vivo.

Plasma antioxidant capacity (PAC) is derived from exogenous dietary AOs (e.g. ascorbate, α-tocopherol) and endogenous compounds (e.g. uric acid, GSH, albumin). Therefore, quantitative determination of the PAC evaluates the free radical scavenging capacity of all the radical scavenging AO's present in plasma having been absorbed through the gut or excreted by tissues. PAC has been proposed as a marker of food-derived AO bioavailability (Fernandez-Panchon et al. 2008). Physiologically, AO compounds are intrinsically linked through cooperative and synergistic action between the different compounds, within the aqueous phase of plasma GSH regenerates oxidised ascorbate, which in turn regenerates α-tocopherol (Packer et al. 1979). However, measurement of each individual AO compound is difficult, time consuming and may not reflect the in vivo oxidative balance (Fernandez-Panchon et al. 2008). PAC is a non-specific measure of AO status; however, it does show some correlation with endogenous AO compounds. Regression analysis has shown high correlation between baseline PAC (FRAP) and uric acid ($R^2 = 0.8$) and PAC (ORAC) and albumin ($R^2 = 0.81$) (Fernandez-Pachon et al. 2005).

To date, only a small number of studies have evaluated the efficacy of AO peptides in human trials. Intact WPC has shown some promise as an anticancer agent. Both anti-carcinogenesis and anti-tumoral effects have been shown in animal and in some human studies (Bounous 2000). The anti-carcinogenesis effect of WPC was attributed to the ability of the WPC to boost tissue GSH levels and enhance the removal or detoxification of free radicals. Anti-tumoral effects may be related to the ability of GSH to stimulate the immune system. It has been suggested that high pressure treatment of WPI can increase its bioavailability and Cys content. Two weeks of supplementation with (15–45 g/day) pressurised WPI increased lymphocyte GSH concentration by up to 24 % (Zavorsky et al. 2007). This response was dose dependent at the higher doses and the 30 and 45 g/day groups showed increases above baseline.

Seacure, a commercial supplement made from fermented pacific hake reduced non-steroidal, anti-inflammatory drug (indomethacin) damage to the small intestine in a human clinical trial (Marchbank et al. 2008). Subjects consumed 1 g of the FPH or placebo three times daily for 7 days and for the final 5 days they also received 50 mg of indomethacin with each dose. It was proposed that the AO activity was related to glutamine (Gln) content of the FPH and to the stimulation of GSH production. A peptide extracted from a Chinese medicinal mushroom (Ling Zhi) had AO activity in vitro (Sun et al. 2004). Acute ingestion of a Lingzhi supplement significantly increased PAC. This response was dose dependent and only the higher dose, 3.3 g per subject, resulted in a statistically significant 3 % increase in PAC 90 min post ingestion, (ΔC_{max} FRAP 23 µmol/l; Wachtel-Galor et al. 2004a). This acute benefit of a Lingzhi supplement was not reproduced in short-term (10 days) or long-term (4 weeks) supplementation. In the 10-day study, there were increases (although not statistically significant) in ascorbic acid, uric acid, α-tocopherol and erythrocyte SOD and GPx. However, there was no statistically significant increase in any biomarker of AO status (Wachtel-Galor et al. 2004a, b). The acute benefits did was not give rise to any demonstrable cumulative effect on plasma AO status.

As already outlined, milk protein hydrolysates display AO activity in vitro. The relationship between in vitro AO activity of milk proteins/peptides and in vivo PAC following ingestion has yet to be fully elucidated. A number of studies have demonstrated that foods with high in vitro antioxidant capacity (AOC) can produce an AO response in vivo. Consumption of 240 g of strawberries increased PAC (ORAC) by 14 % (Prior et al. 2007). This response was related to the in vitro AOC of the fruit but it was not directly proportional and was also influenced by the type of AO present (phenolics) and potential differences in absorption and metabolism of these AO compounds. Ingestion of red wine (300 ml) produced a 20 % increase in PAC (chemiluminesence) of which 60 % was accounted for by changes in plasma urate (Whitehead et al. 1995). Acute increases in PAC post ingestion while beneficial need to be translated into demonstrable long term changes if they are to be a useful strategy to combat oxidative stress.

Health benefits to the aging population

Life expectancy is increasing and it is estimated that by 2030, ~18 % of the world's population will be older than 65 (Kinsella and Velkoff 2001). Aging is defined as the progressive accumulation of deleterious changes within the cells and tissues of the body (Harman 2001). It is characterised by a decrease in biological function and resistance to stress, an increase in fat mass, inflammation and an increased susceptibility to disease (Kregel and Zhang 2007). The aging process is poorly understood because it is inherently complex but oxidative stress has also been linked to the aetiology of aging (Harman 1956). This hypothesis states that as we age there is a gradual increase in ROS along with a decrease in AO production. Oxidative damage to proteins during aging can lead to an accumulation of protein carbonyls and can contribute to the aging process (Berlett and Stadtman 1997). There appears to be an age-dependent decrease in PAC (FRAP). When plasma FRAP was plotted as a function of age there was a negative linear correlation, i.e., between the age of 20 and 80 years there was ~60 % decrease in plasma FRAP (Rizvi et al. 2006). The reduced efficiency of nutrient absorption during aging and the lower intake of dietary AO by elderly may have contributed to this observation. A cross-sectional study of healthy, older adults showed an association between dietary consumption of AO-rich foods and serum AO status, suggesting that consumption of AO-rich foods may contribute to healthy aging (Khalil et al. 2011). While many people have a genetic predisposition to develop age-related diseases, environmental and lifestyle factors are also major regulators.

Age-related sarcopenia is a multi-factorial phenomenon linked to insufficient dietary protein and low levels of physical activity. Sarcopenia manifests itself as a loss of skeletal muscle mass which can reduce muscular function, decrease physical activity and ultimately compromise independence in the older adult (Doherty 2003). Therefore, as the body ages, there is also an increased requirement for dietary protein in order to prevent sarcopenia (Paddon-Jones et al. 2008). This age-related loss of muscle mass can be delayed or prevented by an effective combination of dietary protein and exercise (Boirie 2009). Muscle wasting is also associated with an increase in oxidative stress. Both animal and human biopsy studies have shown that age-related increases in oxidative stress can damage proteins, lipids and DNA within muscle (Semba et al. 2007). ROS induced muscle damage can up-regulate pro-inflammatory cytokines (TNF-α, IL-6) and compromise the already weakened muscle. Sarcopenic individuals tend to have a lower intake of protein and AO micronutrients (Chaput et al. 2007). The combined action of Leu and AOs (vitamins A and E, selenium and zinc) had a beneficial effect on muscle metabolism of aging rats (Marzani et al. 2008). Following 7 weeks of supplementation with AOs, the older rats supplemented with AOs had an increase in Leu stimulate protein synthesis, reduced inflammatory (α-macroglobulin) and oxidative stress (heart TBARS) markers. This suggests that a protein which provides a rich source of essential amino acids and AO peptides could protect the aging muscle from oxidative damage and provide multi-functional nutritional support for healthy aging.

Six months of supplementation with AOs was an effective means to boost the AOC in vivo (Wouters-Wesseling et al. 2003). The AO status of an elderly cohort was evaluated before and after supplementation with a vitamin and mineral enriched drink containing a Cys-rich whey protein. The 250 ml daily dose contained; 9 g protein, 22 g carbohydrate, 9 g fat and a range of micronutrients 30–150 % of the recommended daily allowance. After 6 months the supplemented group had significantly elevated levels of vitamin C (97 %), E (59 %), plasma Cys (8 %) and PAC (TEAC; 7 %) compared to the placebo group.

In addition to sarcopenia, there is an increase in fat mass with aging, Approximately, 60 % of those older than 65 are classified as overweight (body mass index, BMI > 25 kg/m^2; Chapman 2010). This increase in fat mass is multifactorial and linked to a decrease in physical activity, decrease in metabolic rate and decrease in production of growth hormones. Obesity can exacerbate the metabolic changes that occur during aging and ultimately reduce lifespan. Oxidative stress is linked to the aetiology of age-associated weight gain. The accumulation of fat mass has been shown to correlate with markers of systemic oxidative

stress in humans (Furukawa et al. 2004). Within the adipose tissue there is an increased production of ROS and a reduced expression of AO enzymes in the body (Mendoza-Núñez et al. 2007).

Obesity is major risk factor for the development of type 2 diabetes, increasing the susceptibility to this disease within the aging population. There is a linear relationship between BMI and the risk of type 2 diabetes making obese individuals 10 times more likely to develop diabetes than those with normal weight (Stein and Colditz 2004). Diabetes is associated with an increase in oxidative stress. Diabetic hyperglycaemia results in disproportionate production of free radicals through glucose auto-oxidation. Other factors include cellular oxidation–reduction imbalances, decreased production of AO and elevated levels of pro-oxidants in diabetic patients (Maritim et al. 2003). Therefore, it has been suggested that an increased intake of AO may help reduce the incidence of diabetes in the aging population. The ATTICA study found that a higher total dietary AO intake was associated with improved glycaemic biomarkers (Psaltopoulou et al. 2011). Peptides derived from whey proteins have been used to stimulate insulin secretion and regulate blood glucose in type 2 diabetic subjects (Frid et al. 2005). Therefore, an insulinotropic peptide that also has AO activity potentially offers a strategy for the treatment of type 2 diabetes in the aging population.

These studies highlight the potential health effects of AO peptides for the aging population. However, further studies are required to elucidate the mechanism of action of AO peptides and their long-term protective effects in the active aging population.

Regulatory requirements for functional foods

If food-derived bioactive components are to have a real impact on human health, they must be accepted by the consumer, have a demonstrable health effect, have sufficient scientific evidence to substantiate health claims and the food industry must be interested and capable of commercialising the final food product (Tapsell et al. 2005). Bioactive peptides offer a new frontier in health promotion and disease prevention. However, there is no universally accepted definition of a functional food with different definitions put forward by industry, government and academia. Simply stated they can be described as any food which provides health benefits beyond basic nutrition (Goldberg 1994). More comprehensively as a natural food or food component to which something has been added or removed or in which the bioavailability of one or more components has been modified and thus demonstrates a beneficial effect on health or reduces the risk of disease (Roberfroid 2000).

The concept of functional food is not new. Research in this area began in Japan in the early 1980's. In 1991, the Japanese Ministry for Health, Labour and Welfare established labeling regulations for Foods for Specific Health Use (FOSHU) making Japan the first country to introduce legislation. The FOSHU system grants approval to products based on evidence of safety, efficacy and having a positive impact on a physiological function. There are three FOSHU classifications, i.e., qualified, standardized and disease risk reduction FOSHU (Shimizu 2003). FOSHU approval is issued for the final product and not the food ingredient. In Japan, the term AO does not appear on the list of approved FOSHU health related claims. However, there are some FOSHU ingredients including green tea and soy beans which are known to have AO properties (Ministry of Agriculture Fisheries and Food 2011). The food industry places less emphasis on the AO activity and more on the health related benefits of these products. Research output in the area of functional foods has increased rapidly and by April 2011, 955 Japanese products have been approved to carry the FOSHU mark on their product label (Japanese Consumer Affairs Agency 2011).

Within the US, there are a number of pieces of legislation that govern health claims for food products. The Nutrition Labeling and Education Act (NLEA) of 1990 gave the US Food and Drug Administration (FDA), the authority to regulate health claims on food labels (Anon 1990). These claims establish a link between a food and a disease or health condition. Under the NLEA an FDA panel of experts will conduct a systematic review of the scientific evidence. If the panel agrees that sufficient evidence has been provided a qualified health claim will be issued along with a letter of enforcement. If there is some scientific agreement a qualified health claim can also be issued under enforcement discretion. That is, the company is permitted to make a link between a nutrient and reduction of the risk of disease once they qualify that the claim is not endorsed by the FDA due to limited or inconclusive evidence. For example, AO vitamin supplements, vitamin C and E, can claim to reduce the risk of certain cancers provided the qualification is also made (Food and Drug Administration 2003). The FDA Modernization Act of 1997 allows food manufacturers to use health claims based on authoritative statements by a scientific body of the US government, such as the National Institutes of Health (Anon 1997). These claims relate to a food or food component that reduces the risk of disease. The Dietary Supplements and Health Education Act 1994 (DSHEA) allows structure function claims to be made. They describe the role of a nutrient or dietary ingredient that affects a bodily function or describes the mechanism of action.

In Europe, applications for nutrition and health claims are submitted to the European Food Safety Authority

(EFSA) under Regulation 1924/2006 and are evaluated by the Dietetic Products, Nutrition and Allergies (NDA) panel of scientific experts (Anon 2006). There are two categories within the legislation defining nutritional claims and health claims. Nutritional claims are as those relating to food composition and defines food with a beneficial nutritional composition and specifies limits for specific nutrients (e.g. low fat, <3 g fat per 100 g solid) whereas health claims suggest or establish a relationship between food and health (Lalor et al. 2010). The primary objective of the regulation is to protect consumers by ensuring that any claim made on a food label in the European Union (EU) is clear and substantiated by scientific evidence. The opinion of the panel of experts is presented to the European Commission which, in turn, votes on whether the claim should be accepted or rejected.

There are three categories of health claims as defined by EU legislation. Article 14 claims are those relating to disease risk reduction or related to children's health. Article 13.1 claims are general function claims and refer to the role of a nutrient or substance in physiological or psychological function e.g. weight management, reduction in energy intake, feeling of satiety. Article 13.5 claims are defined as new function or emerging science claims. Due to the proprietary nature of some of this data, claims applications are not published. EFSA has a responsibility to verify the scientific evidence presented to them. A positive outcome is centred on three questions; has the food or food constituent been sufficiently characterised, is the proposed claim beneficial to human health and has a cause and effect relationship been established.

There is still a lot of confusion within the food industry as to what evidence is required to substantiate a claim within the EU. In response, the NDA panel has issued opinion documents on the substantiation of claims related to various health areas including one on premature aging and AO activity (EFSA Panel on Dietetic Products, Nutrition and Allergies 2010). In this document the panel define an Article 13 claim permissible in this area as having "AO activity" or "AO properties" beneficial to the general population. Proposed wording may include, "AO containing foods support healthy aging," however, wording such as "protect cells from premature aging" were deemed not to comply with the criteria set out in the regulations. The panel also stated that a cause and effect relationship should be established between consumption of an AO containing food and a beneficial physiological effect. Beneficial physiological effects may include protection of molecules such as DNA, proteins and lipids from oxidative damage. The food component needs to be sufficiently characterised using in vitro test systems such as ORAC, FRAP, TEAC and ferrous oxidation–xylenol orange assays. However, in vitro assays and animal models are not sufficient to substantiate biological functionality in humans. The panel concluded that there was no evidence that a food having the capacity to scavenge free radicals or act as a reducing agent will result in beneficial physiological effect. Therefore, human studies to investigate the effects of the food or food constituent on reliable markers of oxidative damage to body cells or to molecules such as DNA, proteins and lipids are essential. To date no Article 13.5 AO peptide dossier has received a positive opinion from the NDA panel. Positive opinions have been issued for polyphenols in olive oil (EFSA Panel on Dietetic Products, Nutrition and Allergies 2011b) and general function claims for vitamin A, C, E and selenium (EFSA Panel on Dietetic Products, Nutrition and Allergies 2011a). Since its inception, the regulatory process has to date reviewed 2,758 article 13 claims and only 222 have gained approval (European Food Safety Authority 2011).

Despite this new product, development in the area of AOs has been increasing rapidly. In 2009, Mintel's Global New Product Database recorded 2,057 AO-labelled products on the market that is approximately four times more than the number recorded in 2005 (Mintel 2009). However, there are no milk protein-derived AO peptides with regulatory approval and the majority of AO peptides are still at the laboratory research stage. There are only a limited number of commercial peptide products that are reported to have AO activity. Dutch company, DMV, produce a Cys-rich peptide from a whey protein hydrolysate. The hydrolysate is further purified to enrich the Cys content between 7 and 20 %. This ingredient has been patent protected and is reported to boost GSH production, improving sleep and boosting energy (Mallee et al. 2003). Another commercial, patented (Gold et al. 1993) product, ImmunoPro®, produced in the US contains a proprietary whey protein along with lactoferrin, immunoglobulin and dipeptides. This product claims to enhance GSH production, immune function, cellular repair and muscle growth although this has not been evaluated by FDA. Other patent protected AO peptides include a peptide derived from a hydrolysate of lactoferrin (Tominta and Schimamura 1998), an AO peptide derived from hydrolysis of CN that is further purified by a phase separation and an enrichment strategy (Han et al. 2002) and Cys-rich peptides reported to improve thiol homeostasis (Sprong et al. 2008).

Conclusion

Bioactive peptides offer a new means to promote health and can provide health benefits beyond their basic nutritional role. Food protein-derived AO peptides are emerging as a natural alternative to chemical-based AO and complementary to traditional dietary AO. Whey and CN-

derived AO peptides have been reported, but the mechanism of action is yet to be fully elucidated. Some of these peptides display multi-functional properties (e.g. AO, antimicrobial, anti-inflammatory) and synergetic actions with other foods (e.g. WPC and spirulina) which may in turn increase their physiological effectiveness. In some cases, peptides derived from milk proteins have in vitro activity similar to or greater than chemical AO's (BHT, BHA). However, there is no standardised methodology to assess in vitro AO activity and the diversity of methodologies in use hinders direct comparison of the data.

The relationship between the composition of a peptide and its AO activity is yet to be full established. With improved understanding of the structure–function relationship, we may be able to design targeted enzyme hydrolysis strategies to release these peptides. Greater understanding of the biological fate of peptides and the site of action will allow delivery of an effective dose and formulation of the peptides to ensure that they reach their target sites. Confirmation of the efficacy of AO peptides in vivo is still in its infancy. Some food protein-derived peptides and intact whey proteins have shown a beneficial effect in vitro and in acute human intervention studies. However, in some cases, acute benefits did not translate to longitudinal effects. Therefore, we need to gain a better understanding of the relationship between these in vitro activities, the long-term health benefits in humans and establish appropriate biomarkers of biological efficacy. AO peptide interventions could be most appropriate for specific *compromised* populations such as the aging. As the body ages, there is an increased risk of sarcopenia and oxidative stress. Therefore, nutrient solutions that provide a rich source of essential amino acids and AO peptides could be an efficient strategy to meet the nutritional needs of this population and provide a novel approach to promote healthy aging.

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