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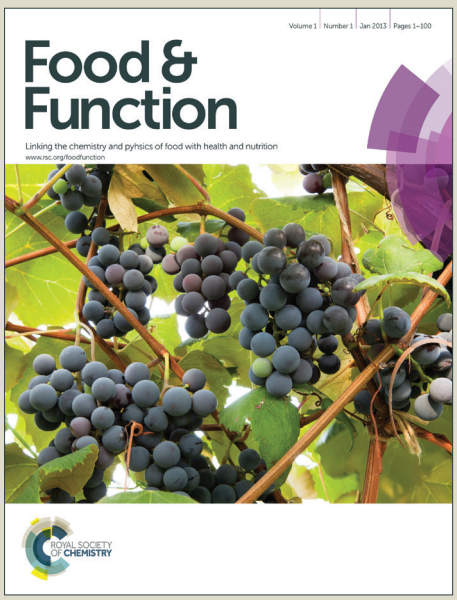

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ARTICLE TYPE

Milk proteins as a source of tryptophan-containing bioactive peptides

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Tryptophan (W) is an essential amino acid which is primarily required for protein synthesis. It also acts as a precursor of key biomolecules for human health (serotonin, melatonin, tryptamine, niacin, nicotinamide adenine dinucleotide (NAD), phosphorylated NAD (NADP), quinolinic acid, kynureric acid, etc.). Among dietary proteins, milk proteins are particularly rich in W. W residues within milk proteins may be released by proteolytic/peptidolytic enzymes either as a free amino acid or as part of peptide sequences. Different W-containing peptides originating from milk proteins have been shown *in vitro* to display a wide range of bioactivities such as angiotensin converting enzyme (ACE) inhibition along with antioxidant, antidiabetic and satiating related properties. Free W has been shown in certain instances to have an effect on cognition and the aforementioned bioactive properties. However, a higher bioactive potency has generally been observed with specific W-containing peptides compared to free W. Since W is thermolabile, the impact of processing on the stability of W-containing peptides needs to be considered. Milk protein-derived W-containing peptides may have significant potential as natural health promoting agents in humans.

1. Introduction

The name "tryptophan" (W, molecular formula: $C_{11}H_{12}N_2O_2$, molecular mass: 204.23 Da) originates from the time when this amino acid was first isolated from a casein (CN) tryptic digest by Hopkins and Cole [1]. Of the twenty conventional amino acids, W is the only one which possesses an indole structure. W is classified within the category of hydrophobic amino acids. It is formed through the condensation of serine (S) with an indole group. This reaction is catalysed by W synthase in lower organisms, including microorganisms. As an essential amino acid, it cannot be synthesised by humans and animals because they are lacking in W synthase. Therefore, W has to be supplied to the body by exogenous sources, notably through the diet [2,3]. A requirement of 4 mg kg^{-1} day^{-1} for adults has been set by the WHO for healthy metabolism [4]. W has been reported to be the amino acid with the lowest concentration within the human body [5,6]. W deficiencies can have several consequences for human health including the onset of diseases such as pellagra (caused by niacin deficiency), as well as psychological, cognitive and behavioral disorders such as dementia and depression [5,6].

In the human body, the primary function of W is protein synthesis, with the L isomer being the only one which can be incorporated in proteins [5,6]. W is also a precursor for the synthesis of serotonin, melatonin, tryptamine, niacin, nicotinamide adenine dinucleotide (NAD), NAD phosphorylated (NADP), quinolinic acid and kynureric acid (Fig. 1) [2,5,6]. Kynurenine acts as a precursor of kynurenic and quinolinic acid which are antagonists and agonists of the glutamate receptor, respectively. Kynurenine has also been shown to retard cataract formation due to its role as a UV filter in the retina [5]. W is the sole precursor of serotonin, which has been reported to have an effect on psychological status and satiety in humans. This effect may be modulated by tryptamine, which results from the decarboxylation of W [5]. A link between W and the immune response has also been suggested. It is thought that W breakdown can reduce T cell proliferation on the one hand. On the other hand, the metabolites generated within the kynurenine pathway (taking place in the immune system) can modulate various inflammatory responses [2].

W is found within a wide range of dietary proteins (See USDA National Nutrient Database for W [7]). However, certain protein sources are richer in W than others. Several dietary proteins have been described as good sources of W (> 0.3% (w/w)), these include egg, soy, beans, seafood, poultry and milk proteins [7]. The major milk proteins contain between 1 (β- and κ-CN) and 13 (lactoferrin – LF, Table 1) W residues per molecule. In general, whey proteins possess more W residues than CNs. In particular, α-lactalbumin (α-La), which possesses 4 W residues per molecule, shows the highest W content (5.3% (w/w); Table 1) of milk proteins.

Following milk protein digestion, free amino acids and peptides are released in the gastrointestinal tract (GIT). These free amino acids and peptides may have different beneficial effects as health promoting agents [8]. Numerous studies have shown that milk proteins comprise specific peptide sequences, known as bioactive peptides (BAPs), which are able to modulate different key health biomarkers [9,10]. Various antihypertensive, antioxidant, mineral binding, antimicrobial and anticancer activities have been demonstrated *in vitro* [11-15]. A number of human intervention studies have concluded in positive health outcomes following the consumption of dairy products and milk

protein-derived BAPs [16-24].

The detection of peptides within complex food protein hydrolysates and biological fluids has been made easier with the enhanced precision of liquid chromatography mass-spectrometry (LC-MS) analysers [25]. Furthermore, bioinformatic tools have allowed the establishment of structure-function relationships between BAPs and their *in vitro* bioactivity [26,27]. In addition, peptide alignment strategies have made it possible to discriminate peptide motifs which correlate with specific bioactive properties [28,29]. Establishment of structure-function relationships has highlighted the importance of specific amino acid residues within BAPs. For example, Y, L, P and W residues appear to play a specific role in opioid, antioxidant, insulinotropic, along with angiotensin converting enzyme (ACE) and dipeptidyl peptidase IV (DPP-IV) inhibitory peptides [8,28,30,31]. This review assesses the contribution of W and W-containing peptides to the bioactive properties of milk protein hydrolysates. The targeted release of free W and W-containing peptides during the digestion of milk proteins will be outlined. Furthermore, the literature on the stability of W and W-containing peptides following different processing conditions, which are relevant to food manufacture, will be assessed.

Table 1: Percentage (% (w/w)) of tryptophan (W) residues found within the major milk proteins.

Protein*	Molecular mass (Da)	Number of W residues per molecule	Percentage of W in the protein (% w/w)
α-lactalbumin (P00711)	14186	4	5.3
β-lactoglobulin (P02754)	18281	2	2.0
bovine serum albumin (P02769)	67206	2	0.6
lactoferrin (P24627)	76145	13	3.2
α_{s1}-casein (P02662)	20374	2	1.8
α_{s2}-casein (P02663)	24349	2	1.5
β-casein (P02666)	23584	1	0.8
κ-casein (P02668)	18975	1	1.0

*The accession number for the protein is given in brackets

2. Release of free W and W-containing peptides during the digestion of milk proteins

A limited number of studies have investigated the release of peptides from milk proteins during gastrointestinal (GI) digestion in humans [32-36]. Free W and/or W-containing peptides originating from milk proteins may be released in the GIT following the digestion of milk proteins [32,36,37]. Furthermore, using *in silico* approaches, the release of free W and W-containing peptides by GI enzymes can be predicted (Table 2). Chymotrypsin, in particular, is able to cleave milk proteins at the C-terminus of W residues [38]. *In silico* digestion of the major milk proteins with GI enzymes has shown that free W may be released by chymotrypsin from α_{s1}-CN, α-La and bovine serum albumin (BSA). In addition, W-containing peptides with 2-71 amino acid residues may also be released from all major milk proteins (α_{s1}-, α_{s2}-, β- and κ-CN, α-La, β-Lg, LF and BSA, Table 2).

However, to date, only a limited number of W-containing peptides have been identified in biological samples obtained from humans (Table 3), including the jejunal contents [32] and the sera [24]. Peptide cutter programs generally only allow prediction of peptides which may be released by one enzyme activity, while digestion in humans involves the hydrolytic activity of several enzymes (proteinases and peptidases). In general, the peptides which were predicted *in silico* to be released by GI enzymes (Table 2) have not been identified in the GIT (Table 3). However, larger β-CN peptides predicted to be released *in silico* (Table 2) may act as precursors of the five β-CN derived W-containing peptides which has been identified in the jejunal contents of humans [32]. These peptide precursors may be further cleaved by carboxy- and aminopeptidases present in the GIT of humans [39,40].

In the late 1980's, contaminated batches of W supplements caused several cases of eosinophilia myalgia syndrome, which was fatal in certain instances. However, it has been demonstrated that W is a non-toxic amino acid [41]. Its median lethal dose (LD_{50}) was determined as being as high as 1.6 g kg^{-1} in rats [42], corresponding to ~ 1.4 g kg^{-1} in humans [6]. It is well accepted that free amino acids and peptides are more easily absorbed than unhydrolysed proteins [40,43,44]. W is found in the circulation mainly in a bound form with BSA [6]. However, W is thought to be able to cross the blood brain barrier (BBB) in a free form as it has a higher affinity for BBB transporters than for BSA [5]. The transfer of W to the brain is thought to be reduced by the presence of large neutral amino acids (LNAA) which compete for the same transporter [6]. In this context, W supplements, which are more concentrated than dietary sources, allow for its increased bioavailability in the brain [41]. However, because the incorporation of W into proteins depends on the presence of other amino acids, it has been suggested that intake of W from supplements causes an imbalance which may not be consistent with efficient protein synthesis. Therefore, intake of dietary W appears more efficient for protein synthesis than W supplements [6].

Foltz, et al. [26] studied the intestinal stability of dipeptides *in silico* and *in vitro*. Most W-containing dipeptides found within the milk proteins which were evaluated, were not stable to *in vitro* intestinal digestion, with the exception of GW (LF (f124-125) and LF (f466-467)). There are a limited number of reports of W-containing peptides being identified in the circulation of humans. LW, an ACE inhibitory peptide which is predicted to be released from α_{s1}-CN by pepsin (Table 3), was identified in the serum of human subjects who consumed a yoghurt beverage enriched with the lactotripeptides IPP and VPP [24].

Table 2: Free tryptophan (W) and W-containing peptides released by gastrointestinal enzymes following the *in silico* digestion of major milk proteins. *In silico* digestion was carried out with an in-house algorithm using the peptide cutter from Matlab (version 2014b, The MathWorks Inc., Natick, MA, USA).

Protein*	Enzyme	Free W and W-containing peptides
α_{s1}-CN (P02662)	CH-LO	W
	PEPS	LW
	PEPS2	DAYPSGAWYYVP
	PEPS2	LW
	TRYPS	QFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEK
	TRYPS	TTMPLW
	ELAS	WY
	CH-HI	QLDAYPSGAW
	CH-HI	SDIPNPIGSENSEKTTMPLW
	CH-LO	DAYPSGAW
α_{s2}-CN (P02663)	PEPS	WDQVKRNAVPITPTL
	PEPS	WIQPKTKVIP
	PEPS2	LNPWDQVKRNAVPITPTL
	PEPS2	KTVYQHQKAMKPWIQPKTKVIPYVRYL
	TRYPS	FPQYLQYLYQGPIVLNPWDQVK
	TRYPS	AMKPWIQPK
	ELAS	NPWDQV
	ELAS	MKPWIQPKTKV
	CH-HI	QGPIVLNPW
	CH-HI	QHQKAMKPW
	CH-LO	NPW
	CH-LO	KPW
β-CN (P02666)	PEPS2	QSWMHQPHQPLPPTVMFPPQSV
	TRYPS	YPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK
	ELAS	WMHQPHQPL
	CH-HI	TESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAF
	CH-LO	QSWM
κ-CN (P02668)	PEPS2	QWQV
	TRYPS	SPAQILQWQVLSNTVPAK
	ELAS	QWQV
	CH-HI	AKPAAVRSPAQILQW
	CH-LO	QW
α-La (P00711)	CH-LO	W
	PEPS	PEW
	PEPS	WCKDDQNPHSSNICNISCDK
	PEPS2	PEWVCTT
	PEPS2	QINNKIWCKDDQNPHSSNICNISCDK
	PEPS2	TDDIMCVKKILDKVGINYW
	PEPS2	DQW
	TRYPS	GYGGVSLPEWVCTTFHTSGYDTQAIVQNNDSTEYGLFQINNK
	TRYPS	IWCK
	TRYPS	VGINYWLAHK
	TRYPS	LDQWLCEK
	ELAS	PEWV
	ELAS	WCKDDQNPHSS
	ELAS	WL
	ELAS	DQWL
	CH-HI	GGVSLPEW
	CH-HI	QINNKIW
	CH-HI	LAHKALCSEKLDQW
	CH-LO	GGVSLPEW
	CH-LO	QINNKIW
	CH-LO	DQW
β-Lg (P02754)	PEPS2	LDIQKVAGTWYS
	PEPS2	QKWENGECAQKKIIAEKTKIPAV
	TRYPS	VAGTWYSLAMAASDISLLDAQSAPLR
	TRYPS	WENGECAQK
	ELAS	GTWY
	ELAS	QKWENGECA
	CH-HI	LIVTQTMKGLDIQKVAGTW
	CH-HI	VEELKPTPEGDLEILLQKW
	CH-LO	DIQKVAGTW
	CH-LO	QKW
BSA (P02769)	CH-LO	W
	PEPS	FWGK
	PEPS	WSVARL
	PEPS2	FWGKY
	PEPS2	LKAWSVARL

	TRYPS	FWGK
	TRYPS	AWSVAR
	ELAS	DEKKFWGKY
	ELAS	WS
	CH-HI	GERALKAW
	CH-LO	KAW
LF (P24627)	PEPS	APRKNVRW
	PEPS	CTISQPEW
	PEPS	KCRRWQW
	PEPS	TRVVW
	PEPS2	APRKNVRWCTISQPEW
	PEPS2	KCRRWQWRMKK
	PEPS2	GRSAGWIIPMGI
	PEPS2	SWTES
	PEPS2	IWK
	PEPS2	LRETAEEVKARYTRVVWCAVGPEEQKKCQQWSQQSGQNVTCATASTTDDCIV
	PEPS2	TWNS
	PEPS2	KDKKSCHTAVDRTAGWNIPMG
	PEPS2	VKNDTVWENTNGESTADWAKN
	TRYPS	WCTISQPEWFK
	TRYPS	WQWR
	TRYPS	SAGWIIPMGILRPYLSWTESLEPLQGAVAK
	TRYPS	EDLIWK
	TRYPS	VVWCAVGPEEQK
	TRYPS	CQQWSQQSGQNVTCATASTTDDCIVLVLK
	TRYPS	ANEGLTWNSLK
	TRYPS	TAGWNIPMGLIVNQTGSCAFDEFFSQSCAPGADPK
	TRYPS	NDTVWENTNGESTADWAK
	ELAS	RWCTI
	ELAS	QPEWFKCRRWQWRMKKL
	ELAS	GWI
	ELAS	WTES
	ELAS	WKL
	ELAS	WCA
	ELAS	GPEEQKKCQQWS
	ELAS	TWNS
	ELAS	GWNI
	ELAS	WENTNGES
	ELAS	DWA
	CH-HI	APRKNVRW
	CH-HI	CTISQPEW
	CH-HI	KCRRW
	CH-HI	QW
	CH-HI	QLDQLQGRKSCHTGLGRSAGW
	CH-HI	LSW
	CH-HI	KECHLAQVPSHAVVARSVDGKEDLIW
	CH-HI	TRVVW
	CH-HI	CAVGPEEQKKCQQW
	CH-HI	LAVAVVKKANEGLTW
	CH-HI	NSLKDKKSCHTAVDRTAGW
	CH-HI	VKNDTVW
	CH-HI	ENTNGESTADW
	CH-LO	APRKNVRW
	CH-LO	CTISQPEW
	CH-LO	KCRRW
	CH-LO	QW
	CH-LO	GRSAGW
	CH-LO	SW
	CH-LO	IW
	CH-LO	TRVVW
	CH-LO	CAVGPEEQKKCQQW
	CH-LO	TW
	CH-LO	TAVDRTAGW
	CH-LO	VKNDTVW
	CH-LO	ENTNGESTADW

*The accession number for the protein is given in brackets

CN: casein; α-La: α-lactalbumin; β-Lg: β-Lactoglobulin; BSA: bovine serum albumin; LF: lactoferrin

Enzymes used in the peptide cutter were: pepsin pH 1.3 (PEPS) and > pH 2 (PEPS2), pancreatic elastase (ELAS), trypsin (TRYPS), chymotrypsin high (CH-HI) and low (CH-LO) specificity

5

Table 3: Milk protein-derived tryptophan (W)-containing peptides identified in human biological samples.

Parent protein	Fragment	Peptide sequence	Location	Reference
β-CN (P02666)	140-148	LQSWMHQPH	Intestine	32
	142-148	SWMHQPH		32
	143-148	WMHQPH		32
	143-154	WMHQPHQPLPPT		32
	143-155	WMHQPHQPLPPTV		32
CNs/whey	diverse	LW	Serum	24

The accession number of β-CN is given in brackets

CN: casein

3. Effect of W and W-containing peptides on psychological and cognitive function

W and W-containing peptides have been shown to play a key role in various bioactive properties which may be relevant to the management of diseases in humans. Their role in psychological/cognitive function and as antihypertensive, antioxidant, antidiabetic and satiating agents will be outlined in the following sections.

Intake of W has been linked with an increase in serotonin level in the brain of animal [45] and human subjects [41]. Increased serotonin levels in the central nervous system may beneficially modulate stress-related and cognitive function [41,46-49]. A significant increase in plasma W:LNAA ratio was seen, which induced anxiolytic-like effects in rats fed with α-La enriched whey proteins (190 g kg^{-1}). The anxiolytic effects may be related to an increase in serotonin synthesis and release [45]. Consumption of α-La, a W rich protein, induced a 48% increase in plasma W:LNAA ratio compared to a CN diet [50]. This increase was thought to be followed by a higher serotonin concentration in the hypothalamus of human subjects [50,51]. A positive effect on depressive mood and a better ability to cope with stress (reduction of cortisol-related stress) was seen in stress-vulnerable human subjects [50]. Evening intake of α-La induced a relatively high increase in plasma W:LNAA ratio (+ 130%, 2 h after intake) followed by improved morning alertness (reduced sleepiness) and vigilance performance which were explained by a better sleep quality [52]. Similarly in another study, free W intake was shown to improve cognitive function (significantly shorter reaction time in a memory scanning test) in highly stressed human subjects [51]. However, this positive effect was not seen in low stress vulnerable subjects. Increased serotonin level in highly stressed subjects may have caused a reduction of stress levels which was followed by better cognitive performances [51]. Other studies have also reported an increase in the plasma W:LNAA ratio in healthy females following α-La intake (40 g). However, this was not followed by beneficial modifications in subjective ratings of mood or anxiety [53].

It has been proposed that the plasma W:LNAA ratio may be further increased by the intake of hydrolysed vs. intact α-La [46]. To date, it appears that most studies involving dietary protein hydrolysate effects on plasma W and psychological outcomes have been conducted with the proprietary egg protein hydrolysate, LumiVida™ (DSM, Delft, The Netherlands) [54-56].

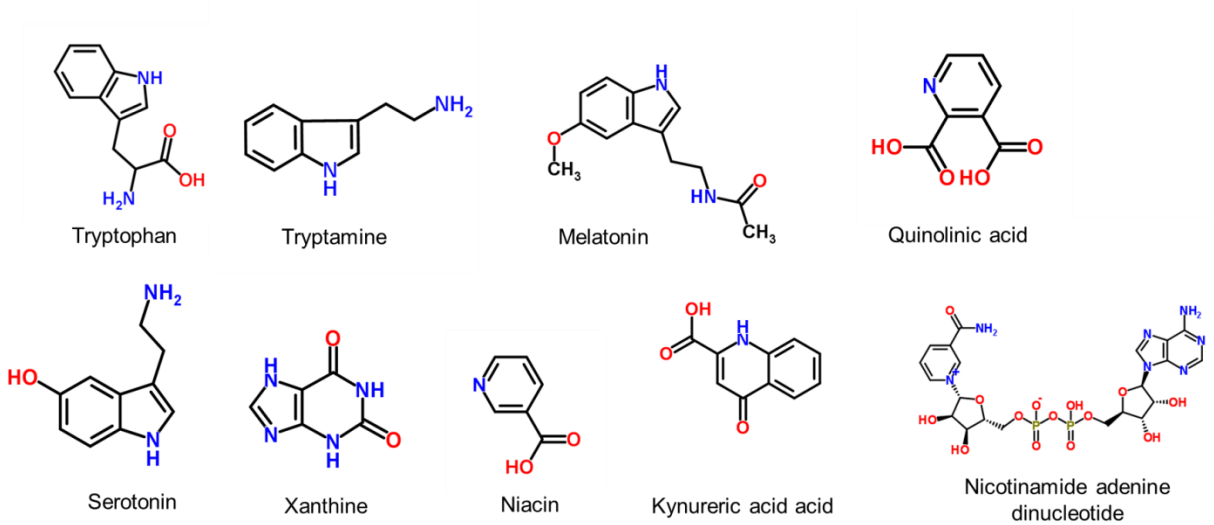

Fig. 1: Structure of tryptophan (W) and biomolecules with W-like structures. The structures were taken from www.chemspider.com.

4. ACE inhibitory properties of W and W-containing peptides

ACE is a metabolic enzyme which is involved in the cleavage of the potent vasodilator bradykinin, resulting in a loss in its activity [30]. ACE inhibition has therefore been suggested as a means to reduce blood pressure. Numerous studies have focused on food protein-derived peptides with ACE inhibitory properties. A large number of human intervention studies have been conducted with milk protein-derived ACE inhibitory peptides and more particularly with the CN-derived lactotripeptides IPP and VPP [57,58].

Several *in silico* approaches including quantitative structure activity relationship (QSAR) and molecular docking have been utilised in an attempt to predict the ACE inhibitory activity of peptides. These studies have allowed a better understanding of the structural features of peptides displaying ACE inhibitory activity (Fig. 2). These analyses indicated that the presence of branched chain amino acids (L and I) or C and G at the N-terminus and R, W, P, F or Y at the C terminus are associated with potent ACE inhibitory activity [24,27,30,59].

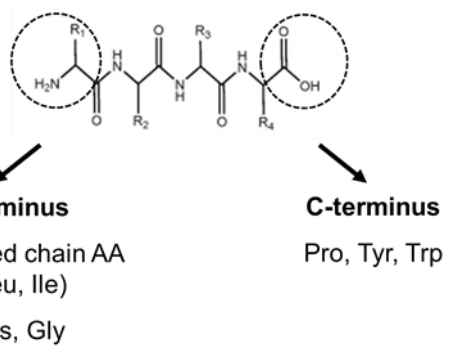

Fig. 2: Structural characteristics of potent angiotensin converting enzyme (ACE) inhibitory peptides [24,27,30]

Several W-containing peptides displaying low ACE half maximum inhibitory concentration (IC_{50}) values have been reported in the literature, some of which are listed in Table 4. These peptides have been identified within all the major milk proteins with the exception of β-CN. Their IC_{50} values vary between 0.7 (KGYGGVSLPEW, α-La (f16–26)) to 372 µM (VGINYWLAHK, α-La (f99-108)). Virtual screening approaches such as molecular docking have been used to predict the sequences of peptides with potent ACE inhibitory properties [60,61]. Selected W-containing dipeptides (AW (α_{s1}-CN (f163-164)), IW (α-La (f 59-60) and LF (f 267-268)) and VW (LF (f 346-347) and LF (f 548-549)) were shown to be competitive inhibitors of human ACE [62,63]. The α-La-derived peptides, IW and WL (α-La (f 104-105) and α-La (f 118-119)) were identified in milk protein-based infant formulae [64] and in a whey protein hydrolysate generated with a combination of chymotrypsin and thermolysin [62]. The yield for the release of IW was estimated between 4-19% in the infant formulae [64] and at 50% (w/w) in a whey protein hydrolysate [62]. While that of WL was 4% (w/w) in a whey protein hydrolysate [62]. Simulated gastrointestinal digestion (SGID) revealed that AW and IW, when present in an hydrolysate, were stable to the hydrolytic action of pepsin but were significantly degraded (59 and 40% (w/w), respectively) on incubation with pancreatic enzyme activities [62].

Localisation of the W residue at the C-terminal position of dipeptides (RW and VW) has been correlated with higher ACE inhibitory properties as compared to the reverse peptides (WR and WV). These differences were explained by the fact that higher hydrophobic interactions at the S2' hydrophobic sub-site of the C-domain of the ACE active site could take place when W was located at the C-terminal side of dipeptides [63].

LW (α_{s1}-CN (f198-199)) and IW were administered to spontaneous hypertensive rats (SHR) at 10 and 60 mg kg^{-1}, by intravenous injection and oral administration, respectively [65]. LW caused a decrease in systolic blood pressure (SBP) of -45 and -22 mmHg after intravenous injection and oral administration, respectively. While IW reduced SBP by -55 and -22 mmHg after intravenous injection and oral administration, respectively [65].

5. Antioxidant activity of W and W-containing peptides

Free amino acids and milk protein-derived peptides may act as antioxidant compounds. The mechanisms involved in the antioxidant activity comprise radical scavenging of oxidative species and the modulation of enzymes involved in the *in vivo* oxidative system [12]. Milk protein-derived peptides which are able to scavenge antioxidant species are generally short (< 11 amino acid residues) peptides containing hydrophobic (P, H, Y and W) and/or sulphur (C and M) residues [9,11,66-71]. A study on the antioxidant activity of 18 free amino acids (V, I, D, T, L, E, A, M, Y, H, F, W, C, N, K, G, Q and P) using the ORAC assay has shown that W displayed the highest activity [72]. Another study has assessed the antioxidant activity of free amino acids using the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ABTS assay. Four amino acids displayed a scavenging activity of the ABTS radical in the following order C > W > Y > H [73]. Similarly, the importance of W has been demonstrated in the scavenging of reactive oxygen and nitrogen species [3] as well as in the scavenging of peroxyl radical and the inhibition of linoleic acid autoxidation [74]. It has been suggested that phenol and indole amino acids within peptides may act as hydrogen donors, resulting in the formation of stable radicals and a reduction in the rate of oxidation propagation [11,69,72].

Table 4: Half maximal inhibitory concentration (IC_{50}) of tryptophan (W)-containing peptides from milk proteins with angiotensin converting enzyme (ACE) inhibitory properties.

Peptide sequence *	Protein fragment	ACE IC_{50} (μM)	Reference
KGYGGVSLPEW	α-La (f16–26)	0.7	75
VW	LF (f346-347), LF (f548-549)	1.6	59
IW	α-La (f59-60), LF (f267-268)	2	59
FW	BSA (f133-134)	5.9	27
AW	α_{s1}-CN (f163–164)	10	59
RW	LF (f7-8), LF (f21-22)	16	59
DKVGINYW	α-La (f97–104)	25.4	75
GW	LF (f124-125), LF (f466-467)	30	59
LQKW	β-Lg (f58–61)	34.7	76
YW	α-La (f103–104)	43.7	27
TTMPLW	α_{s1}-CN (f194–199)	51	77
WLAHK	α-La (f104–108)	77	78
DAYPSGAW	α_{s1}-CN (f157–164)	98	77
WI	α_{s2}-CN (f193-194), LF (f125-126)	100	24
WL	α-La (f104-105), α-La (f118-119)	105	24
WY	α_{s1}-CN (f164-165), α-La (f19-20)	113	24
LW	α_{s1}-CN (f198–199)	144	24
VGINYWLAHK	α-La (f99–108)	372	78

* Peptide sequences are ordered by increasing ACE inhibitory IC_{50} value

The inhibition and activation of metabolic enzymes involved in the oxidative system have also been demonstrated in a few studies with W-containing peptides. The role of W in xanthine oxidase (XO) inhibition was hypothesised based on its structural similarities with its natural substrate, xanthine (Fig. 1), and a drug inhibitor of XO, Allopurinol [79]. This was confirmed by the fact that out of the 20 amino acids tested, W was the only one to display inhibitory properties against XO [80]. The inhibition of xanthine oxidase (XO) *in vitro* by free W, RW, IW, KW, WV and VW has been reported [79,80]. Similarly, LF hydrolysates were also able to inhibit XO. This was attributed to the W-containing peptides within the hydrolysates as shown following further enrichment of the hydrolysates using activated carbon [79]. Pro-oxidant activity of free W has been reported in Caco-2 cells [81]. Interestingly, an adaptive response through the nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) pathway was demonstrated, which resulted in an increase in glutathione peroxidase transcription level.

A compendium of antioxidant activity of W and W-containing peptides, as determined by different assays, is presented in Table 5. Free W displays a lower anti-oxidant capacity than peptides, e.g., with 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging. However, in other instances, free W antioxidant activity was as high as that of W-containing peptides such as with the oxygen radical absorbance capacity (ORAC) assay and with XO inhibition (Table 5). In addition, the peptide sequence also affected the antioxidant activity as demonstrated in studies conducted with reverse peptides [79,82,83] or when substituting amino acids in peptides [74]. It is interesting to note that certain peptides yielded good antioxidant properties across different assays (Table 5). This was the case for WV and VW which were able to scavenge DPPH and superoxide (SO) radicals and inhibit XO; for WY which displayed ORAC activity and scavenged DPPH and for KW which scavenged DPPH and inhibited XO.

6. Antidiabetic potential of W and W-containing peptides

The consumption of intact and hydrolysed milk proteins has been linked, in certain instances, with an increase in insulin secretion in the post-prandial phase. This insulinotropic effect may be followed by a reduction in serum glucose both in animal models [84-86] and in humans [16,18,87-90]. The insulinotropic effect of milk proteins has been linked with an increase in plasma amino acid level, in particular branched chain amino acids (BCAA), following the consumption of milk proteins. A human study has reported on the insulinotropic and serum glucose lowering effect of free W. It was shown that intravenous infusion of W (amount infused: 2.5-22.5 g) increased plasma insulin, displaying a similar potency as L [91]. However, the role of W in insulin secretion has not been extensively studied. This may arise from limitations in the analytical methodologies used to quantify free amino acids which often lead to a heat-induced destruction of W, thus making it challenging to quantify [92]. However, different strategies for W residue protection during acid hydrolysis involving the utilisation of antioxidants (e.g. thioglycolic acid), reducing agents (e.g. pyridine borane) and organic acids (e.g. p-toluene sulfonic acid) have been suggested. A lower level of W degradation is generally observed during alkaline hydrolysis in the absence of oxygen [93].

Table 5: Tryptophan (W)-containing peptides from milk proteins with antioxidant activity.

Peptide sequence	Protein fragment	SO EC_{50} (mM)	DPPH EC_{50} (mM)	ORAC activity (T.E./µmol)	XO IC_{50} (µM)
W	diverse	na	358.5 [94]	4.65 [72]	1259.8 [79]
WV	α-La (f 26-27)	131.02 [94]	242.0 [94]	na	1195.7 [79]
VW	LF (f 346-347), LF (f 548-549)	114.52 [94]	654.2 [94]	na	1301.7 [79]
RW	LF (f 7-8), LF (f 21-22)	na	na	na	1136.9 [80]
KW	β-Lg (f 60–61)	na	4.14 [83]	na	1450.9 [80]
IW	α-La (f 59-60), LF (f 267-268)	na	na	na	1046.6 [80]
WC	α-La (f 60-61), LF (f 8-9), LF (f 348-348)	na	0.26 [83]	na	na
WY	α_{s1}-CN (f 164-165), α-La (f 19-20)	na	1.28 [83]	7.67 [95]	na
WpY*	α_{s1}-CN (f 164-165), α-La (f 19-20)	na	2.43 [83]	na	na
WL	α-La (f 104-105), α-La (f 118-119)	na	2.71 [83]	na	na
WQ	κ-CN (f 76-77), LF (f 22-23)	na	3.92 [83]	na	na
WF	LF (f16-17)	na	4.20 [83]	na	na
WR	LF (f24-25)	na	4.43 [83]	na	na
WT	LF (f138-139)	na	4.92 [83]	na	na
WN	LF (f448-449), LF (f467-468)	na	4.92 [83]	na	na
YW	α-La (f 103-104)	na	0.57 [83]	na	na
LW	LF (f198-199)	na	1.71 [83]	na	na
FW	BSA (f133-134)	na	3.04 [83]	na	na
GW	LF (f124-125), LF (f466-467)	na	3.68 [83]	na	na
SW	β-CN (f 157-158), LF (f137-138)	na	3.97 [83]	na	na
EW	α-La (f 25-26), LF (f15-16)	na	3.07 [83]	na	na
WIQP	α_{s2}-CN (f 193-196)	na	2.1 [96]	na	na
VAGTWY	β-Lg (f 15–20)	na	na	5.63 [97]	na
WYS	β-Lg (f 19–21)	na	na	4.45 [95]	na
WYSL	β-Lg (f 19–22)	na	na	4.51 [95]	na
WYSLA	β-Lg (f 19–23)	na	na	4.59 [95]	na
WYSLAMAASDI	β-Lg (f 19–29)	na	na	2.62 [72]	na

*pY: phosphorylated tyrosine

CN: casein; α-La: alpha-lactalbumin; LF: lactoferrin; BSA: bovine serum albumin.

EC_{50}: half maximal effective concentration; IC_{50}: half maximal inhibitory concentration.

DPPH: 2,2-diphenyl-1-picrylhydrazyl scavenging activity; na: not available; ORAC: Oxygen radical absorbance capacity; SO: superoxide scavenging activity; T.E.: Trolox equivalent; XO: xanthine oxidase.

DPP-IV is a metabolic enzyme which is responsible for the cleavage and subsequent inactivation of the incretin hormones glucagon like peptide 1 (GLP-1) and glucose inhibitory polypeptide (GIP) [98]. These incretins, which have been shown to have an insulinotropic activity during the post-prandial phase, contribute to the regulation of serum glucose levels in humans. Therefore, inhibition of DPP-IV is a strategy which is being employed, notably with pharmaceutical drugs (gliptins) to help in the management of type 2 diabetes [99]. Several food protein hydrolysates have been shown to display DPP-IV inhibitory properties *in vitro* [100]. Out of the 20 conventional amino acids, it was shown that M, L and W were able to inhibit DPP-IV [80]. However, their IC_{50}'s were relatively high (> 2000 µM). In certain instances specific peptide sequences have been linked with DPP-IV inhibition [94,101-105]. Milk protein-derived DPP-IV inhibitory peptides generally have an IC_{50} value in the µM range [28], which is several orders of magnitude higher than that of gliptins (nM) [106]. To date, four *in vivo* studies have demonstrated that food protein hydrolysates or peptides with *in vitro* DPP-IV inhibitory properties were able to induce an increased insulin secretion and a reduction in plasma glucose following an oral glucose tolerance test in rodents. This was the case with a tryptic digest of β-Lg administered (300 mg kg^{-1} body weight) to mice [107], the peptide LPQNIPPL (β-CN (f70-77)) isolated from Gouda-type cheese, administered (300 mg kg^{-1} body weight) to rats [108] and porcine and Atlantic salmon skin gelatin hydrolysates administered (300 mg day^{-1} for 42 days) to streptozotocin-induced diabetic rats [109,110]. The insulinotropic effects observed were only directly linked with DPP-IV inhibition *in vivo* in two instances where plasma DPP-IV activity was shown to be reduced following the ingestion of a DPP-IV inhibitory porcine [110] or Atlantic salmon skin gelatin hydrolysate [109].

A limited number of potent DPP-IV inhibitory peptide sequences have been identified to date. While several DPP-IV inhibitory peptides possess between 2-8 amino acids [111,112], longer peptide sequences with > 13 amino acid residues have also been identified [113]. DPP-IV IC_{50} values of W and W-containing peptides inhibitors of DPP-IV are listed in Table 6. Their IC_{50} values vary between 37.8 (WR) to 4280.4 µM (W). The peptides originate from all the major milk proteins with the exception of β-CN.

Table 6: Half maximal inhibitory concentration (IC_{50}) of tryptophan (W)-containing peptides from milk proteins with dipeptidyl peptidase IV (DPP-IV) inhibitory properties.

Peptide sequence *	DPP-IV IC_{50} (μM)	Protein fragment	Reference
WR	37.8	LF (f24-25)	83
WK	40.6	LF (f268-269)	83
WL	43.6	α-La (f104-105), α-La (f118-119)	83
WV	65.7	α-La (f26-27)	94
TW	84	β-Lg (f18-19), LF (f447-448)	114
WA	92.6	LF (f560-561)	83
WQ	120.3	κ-CN (f76-77), LF (f22-23)	83
WI	138.7	α_{s2}-CN (f193-194), LF (f125-126)	83
WLAHKALCSEKLDQ	141	α-La (f104-117)	102
WN	148.5	LF (f448-449), LF (f467-468)	83
VAGTWY	174	β-Lg (f15-20)	115
WIQP	237.3	α_{s2}-CN (f193-196)	96
WM	243.1	β-CN(f158-159)	83
WY	281	α_{s1}-CN (f164-165), β-Lg (f19-20)	83
WLAHKAL	286	α-La (f104-110)	102
WC	420	α-La (f60-6), LF (f8-9), LF (f347-348)	83
WT	482.1	LF (f138-139)	83
WS	643.5	LF (f361-362), BSA (f213-214)	83
WRM	673	LF (f24-26)	116
LW	993.4	α_{s1}-CN (f198-199)	83
W	4280.4	all	80

*Peptide sequences are ordered by increasing DPP-IV inhibitory IC_{50} value

An attempt to link *in vitro* DPP-IV inhibitory properties to the physicochemical characteristics (molecular mass, hydrophobicity hydrophobic moment (μH), isoelectric point (pI) and charge) of the peptides has been conducted [117]. However, no obvious relationship between the physicochemical characteristics of the peptides and their bioactivity could be determined. In contrast, other specific features in DPP-IV inhibitory peptide sequences have been shown to correlate with their *in vitro* activity. It was reported that hydrophobic peptides with Xaa-Pro, Pro-Xaa or Xaa-Ala (with Xaa, an amino acid residue) sequences may be relatively potent DPP-IV inhibitors [118]. Different structural features have been elucidated from relatively potent DPP-IV inhibitory peptides (with IC_{50} values < 200 μM) using a peptide alignment approach. This analysis revealed that these peptides generally contained a W at the N-terminus and/or a Pro at position 2 [28]. These characteristic features of DPP-IV inhibitory peptides were further confirmed in a more recent study where a similar approach was taken [29]. Similarly, analysis of a dipeptide library revealed that the most potent DPP-IV inhibitory peptides generally contained a W residue at the N-terminal position [114]. Interestingly, the specific location of W at the N-terminus of peptides is crucial for DPP-IV inhibitory activity. It was shown with dipeptide isomers that DPP-IV inhibitory activity was significantly reduced or lost when the W residue was located at the C-terminal position of the peptide [83].

Several W-containing peptides were shown to be non-competitive, linear mixed- or parabolic mixed-type inhibitors of DPP-IV [83,94,116,119]. This suggests that these peptides do not directly bind to the active site of DPP-IV [94,120]. Taking *in silico* approaches, it was proposed that W-containing peptides were likely to bind to a secondary binding site located in the neighborhood of the active site of DPP-IV [80]. Certain W-containing peptides appear to act through different mechanisms which are still not fully understood and would merit more investigation since these peptides appear to be relatively potent DPP-IV inhibitors.

7. Satiating properties of W and W-containing peptides

Consumption of milk proteins has been proposed as a means to increase satiety in humans [87,121-123]. The satiating effects of milk proteins are thought to involve the kinetics of amino acid appearance in the circulation and the rheological properties of the proteins in the GIT [124]. Satiating effects have particularly been observed with whey proteins [125-127], which are digested faster than CNs [40,128]. In particular, when α-La was administered at breakfast to adults, a subsequent 25% reduction in energy intake in an *ad libitum* lunch was seen [129]. No effect was seen with CN, whey, and whey with CMP [129].

A link between serotonin activation and milk protein intake has been suggested. This has been explained by the fact that milk proteins are a source of W, the sole precursor of serotonin [130]. Consumption of α-La has been shown to increase plasma W in rats [45] and in humans [50,129], which may result in higher hypothalamic serotonin concentrations [50]. Different milk protein

(whey and CN-derived) hydrolysates have been shown to act as serotonin 5-HT_{2C} receptor agonist in embryonic kidney cell lines (HEK 293A) [131,132]. While no effect was seen *in vivo* with the 1 kDa permeate of a whey protein hydrolysate, the 1 kDa permeate of a CN hydrolysate was show to yield a significant reduction in cumulative food intake when administered intraperiteonally (500 mg kg^{-1} body weight) to mice [131].

8. Effect of processing conditions on W stability and bioactivity

The stability of W and W-bound peptides/proteins to different processing conditions has been reviewed [92]. W is relatively stable to processing conditions (steam sterilisation, industrial and home cooking) in comparison to other amino acids such as K, M and C [92,133]. However, the application of severe processing (heat in the presence of oxygen and sunlight exposure) applied to milk proteins can lead to the degradation of W and subsequently to reduced bioavailability or the formation of antinutritional and toxic/carcinogenic (carboline and Maillard) compounds [92]. The reactivity (oxidation and substitution reactions) of W to different chemicals has been attributed to its indole structure. At temperatures > 100°C, the degradation of W in acidic or basic conditions requires the presence of oxygen. It was estimated that in sterilising conditions (125°C, 30 min), only 1% of W would be lost from CNs at pH 8.0 [92].

The condensation of W with aldehydes leads to the generation of β-carboline or Maillard like products [93]. Maillard reactions occur between primary amino (ϵ- or α-NH_2) groups and reducing carbohydrates following heat treatment. The ϵ-NH_2 group from W has been shown to be able to participate in Maillard reactions [93]. In addition, W may be further degraded by oxidative species which are generated during Maillard reactions [134]. The oxidative degradation of W via Maillard reaction products is also termed glycoxidation (glycation and oxidation). W glycoxidation only appears to occur in native α-La. This may arise from the fact that W residues within heat-denatured α-La are not easily accessible to radicals due to the formation of α-La aggregates [134]. Similarly, the effect of lipid-derived oxidative products on the reduced bioavailability of W (-27.9%) from whey proteins following storage at 37°C for 4 weeks has been reported [133].

9. Conclusion

W is a central amino acid residue for human nutrition as shown by its numerous physiological functions in the body. Dietary sources of W have been reported to be more efficient than W supplements in contributing to the primary role of W in the human body, i.e., protein synthesis. In this context, dietary sources of W such as milk proteins appear to be relevant for human nutrition. In addition, milk protein-derived W-containing peptides have been shown to display a wide range of bioactive properties. Several W-containing peptides have been identified *in vitro* for their potential antihypertensive, antioxidant and antidiabetic properties. In addition, W is generally considered to be stable to conventional processing as applied during food manufacture. The detection of W and W-containing peptides still appears to be challenging namely due to the oxidation of W which may be caused by the severe conditions applied during acid and alkaline hydrolysis of proteins. These analytical limitations may have hindered the discovery of novel W-containing BAPs from milk. However, there is strong evidence in the scientific literature suggesting that W and W-containing BAPs originating from milk proteins may help to improve human health.

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