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Chapter 1

CASEIN HYDROLYSATES AND PEPTIDES: PROPERTIES, FUNCTIONS AND HEALTH IMPLICATIONS

Yan Zhong, Sarmauli Manurung, Eric A. F. van Tol and Tim T. Lambers*
Mead Johnson Pediatric Nutrition Institute,
Nijmegen, The Netherlands

ABSTRACT

Casein, one of the major proteins found in milk sources, can be associated with many health benefits. Moreover, bovine casein-derived hydrolysates or specific casein-derived bioactive peptides have been demonstrated to affect a diverse range of physiological processes. Casein peptides can be naturally present in human or bovine milk, formed during gastrointestinal digestion or generated through the processes of protein hydrolysis for specific nutritional applications. Both casein hydrolysates and peptides thereof have been shown to influence multiple processes in our body and support a diverse range of physiological benefits under healthy or disease conditions.

One of the primary fields of nutritional applications of casein hydrolysates is infant nutrition, where casein hydrolysates are applied for the dietary management of cow's milk allergy and/or management of

* Corresponding Author: Tim.Lambers@mjn.com.

gastrointestinal symptoms. Extensive bovine casein hydrolysates are devoid of intact milk protein allergens but specific sequence from these hydrolysates can modulate the immune system and thereby have been demonstrated to accelerate the acquisition of tolerance to milk proteins in infants with cow's milk allergy. In addition to immune modulation, other health benefits of casein hydrolysates that have been reported in different areas of application include; antimicrobial activity and effects on gut microbiota; increased energy expenditure and appetite regulation; blood pressure regulation through angiotensin converting enzyme (ACE) inhibition; regulation of mineral absorption; modulation of muscle glycogenesis and protein synthesis; anti-oxidant activity; and modulation of gut function, including improving gut barrier function and modulation of epithelial signaling.

The functionality of specific casein hydrolysates is related to the degree of hydrolysis and associated peptide profiles. In this respect, new insights from the field of peptidomics has revealed that peptide profiles of different hydrolysates provide a distinct and descriptive signature which could explain observed differences in overall functionality.

Applications in human nutrition require a thorough mechanistic understanding, through *in vitro*, animal and or biomarker studies and consistent results from carefully controlled and sufficiently powered clinical intervention studies. This chapter reviews the scientific evidence around health promoting effects of casein hydrolysates and (bioactive) peptides thereof from an infant nutrition perspective.

INTRODUCTION

Within the field of bioactive peptides and hydrolysates, casein is among the best studied sources. Casein peptides can be naturally present in human or bovine milk, formed during gastrointestinal digestion or generated through the processes of protein hydrolysis for specific nutritional applications. In nutrition, bioactive peptides mostly refer to specific sequences. Although studied in isolation *in vitro*, *in vivo* mostly whole protein hydrolysates are studied as production of specific peptide sequences is laborious and industrially challenging. Some degree of purity can however be obtained industrially by applying hydrolysation procedures to purified/enriched preparations of the targeted parent protein or combinations of (selective) hydrolysis and further purification by fractionation (mostly selective precipitation and/or membrane filtration).

Protein hydrolysate preparations are typically classified by protein source and their degree of hydrolysis, while a more detailed description can be given

by chromatographic mass distribution analyses [1]. The distinctive capacity of mass distribution is however rather poor and therefore more sensitive technologies based upon automated Edman degradation may be better suitable to define hydrolysate composition [2]. These methods however do not include information about peptide sequences which, particularly in the field bioactive peptide/hydrolysate functionality, may be essential. In this respect, peptidomics are nowadays applied providing a more detailed description about peptide sequence composition and have, amongst others, provided novel insights in the field of bioactive peptide/hydrolysate research [3, 4]. Peptidomics methodology excel as one of the most informative methods for peptidome analyses as it enables identification of multiple peptides simultaneously with high sensitivity [5].

To date several peptidomics studies have provided insights in the human milk peptidome [6]. Although breast milk sampling, lactation stages, study populations etc. may have been different, all studies describe that the endogenous human milk peptidome is dominated by sequence that originate from β-casein. This may be explained by the fact that β-casein resides in casein micelles and hydrolysis may occur at the micelle interface by micelle-associated proteases [7]. Moreover, it is well known that the serum fraction of milk contains many protease inhibitors that may protect whey proteins from hydrolysis [8]. Interestingly, most of the identified sequences in human milk are derived from specific locations within their parent proteins suggesting that protein hydrolyses in the mammary glands are specific rather than random events [7]. Speculative, the latter may also suggest that the human milk peptidome may contribute to overall human milk functionality. Although to date functional work with human milk-derived sequences is scarce and their overall role remains elusive, homology with known bioactive sequences from bovine or endogenous proteins may provide insights, at least to a certain extend. In this respect, human milk peptides with homology to known bioactive peptides may contribute to activities previously associated with human milk such as innate protection (including antimicrobial activity) and immune modulation [7].

Within the field of nutrition, infant formulae are among the primary fields of applications for casein hydrolysates, where casein hydrolysates are applied for the dietary management of cow's milk allergy and/or management of gastrointestinal symptoms. Other areas of applications for biological active casein peptides/hydrolysates are sports nutrition, dietary strategies for blood pressure regulation and nutrition for the aging population. Herewith, biological activities such as immune modulation, antimicrobial activity, effects on gut

microbial composition and metabolism, appetite regulation, blood pressure regulation through angiotensin converting enzyme (ACE) inhibition; regulation of mineral absorption; modulation of muscle glycogenesis and protein synthesis; anti-oxidant activity; and modulation of gut function, including improving gut barrier function and modulating epithelial signaling are suggested to underlie overall functionality of the peptides/hydrolysates. In this review we provide an overview of health promoting effects of casein hydrolysates and (bioactive) peptides thereof with a particular focus on relevance to infant nutrition.

IMMUNE MODULATION; CASEIN HYDROLYSATES AND DIETARY MANAGEMENT OF ALLERGY AND AUTOIMMUNITY

Infant nutrition is one of the primary fields of application of milk protein hydrolysates and several health benefits have been associated with casein hydrolysates/peptides in this area. Best described benefits are associated with allergy, where hydrolyzed milk proteins are applied for the dietary management of cow's milk allergy (CMA).

ORAL TOLERANCE

Oral tolerance induction refers to the feeding of proteins or peptides in order to induce mucosal and systemic tolerance upon re-challenge with the native protein through dietary intake. In case of a defective or deregulated tolerogenic response this may lead to widespread inflammation, tissue damage and loss of intestinal protective and absorptive functions. In addition to the mucosal immune compartment and intestinal barrier function the development of oral tolerance also involves microbial composition and metabolism [9]. Disruption of epithelial tight junctions along the intestinal tract may compromise barrier function thus allowing increased influx of and exposure to potential food allergens. The exposure to tolerogenic protein sequences should not be looked at in isolation. Tolerizing peptide antigens are present in breast milk and thus key in the prevention of allergic sensitization [10, 11]. In fact, both food and aerosolic allergens are processed and transferred through human milk to the neonate driving tolerance induction [10, 12]. Moreover, the

efficacy of conveying tolerance to the infant through human milk depends in part on the presence of other factors like growth factors and immunoglobulins [10, 13] that further stimulate the immune system to develop a tolerogenic response.

From an immunological perspective there are multiple mechanisms of tolerance induction that include anergy of the allergen specific T cell population, deletion of a specific allergen reactive T cell population, or the generation of specific tolerogenic or regulatory T cells. Route of exposure, antigenic properties and dosing, individual genetic background, as well as microbiota composition and immune status, are all key elements in tolerance induction. The process and mechanisms of mucosal tolerance induction and the role of nutrition and microbial composition/metabolism in food allergy have been reviewed in detail [9, 14-18].

COW'S MILK ALLERGY

CMA is one of the most prevalent (2-5%) food allergies that develop early in life [19], with quite strong demographic differences in disease manifestation. The natural history and etiopathogenesis of CMA has been evolving over the last decades as reflected by increased disease persistence and severity until later ages [20]. Although allergy to cow's milk proteins may be transient and resolve over the first years in life [21], it is well accepted that early allergic sensitization may be underlying the increased susceptibility for allergic manifestations later in life, also referred to as the "atopic march" [22]. Furthermore environmental factors, lifestyle changes that may involve altered dietary patterns and microbial and toxin exposure. Apart from life style, socio-economic status and demographics it is also well established that genetic factors greatly contribute to the development of CMA. Thus there has been considerable interest in the role of nutrition on genetic susceptibility for allergy development. Studies in the area of epigenetics have shown environmental factors (including bacteria and food components) to be able to modulate gene function in allergy [23, 24]. And in a study by Canani et al. tolerance acquisition induced with an extensive casein hydrolysate formula was associated with specific alterations of relevant cytokine genes [23].

The protein compartment in bovine milk consists predominantly of caseins (~80%) and with a smaller proportion of whey proteins (~20). And although there is a large variation in individual reactivity against these proteins

in CMA patients, reactivity against αS1-casein and β-lactoglobulin seem to be most prevalent [25].

CASEIN HYDROLYSATES; COMPOSITION

Hypoallergenic casein hydrolysate formulations are recommended for the dietary management of CMA in infants, whereas for the less common but persisting and severe cases an amino acid based formula devoid of any protein or peptide sequences is recommended. Hypoallergenic formulae are produced through enzymatic hydrolyses of the different bovine milk protein fractions, casein or whey or a mixture of both. Depending on the degree of hydrolyses and thus the peptide weight/length distribution in the product it is either referred to as a partial or an extensive hydrolysate [3, 26]. The hydrolysis process reduces the protein and molecular weight, and thus minimizes the allergenicity of the product. However, there is accumulating evidence that peptides generated through such a hydrolysis process are still harboring a variety of biological activities that may contribute to the clinical efficacy of the products. Modification or mutation of protein allergens may affect the binding to IgE and the mast cell effector arm of the allergic sensitization, whereas it may not affect specific T cell responses [27]. Typical extensive casein hydrolysate formulae may have a peptide distribution of 85-95% <1000 Da. Residual allergenicity can be evaluated with high sensitivity *in vitro* using specific antibody binding assays, immunoblotting, as well as *in vitro* cellular response in peripheral blood cells isolated from both healthy and CMA individuals [28, 29]. *In vitro* systems that could mimic human gastrointestinal digestion processes further contribute to our understanding of reduction of protein allergenicity, as well as generation of further tolerogenic peptide sequences along the gastrointestinal tract [30]. Specific peptide motifs that are highly active in immune cell activation and or IgE binding can be identified *in vitro* using overlapping synthetic casein peptide sequences. Emerging research suggests that despite the hypoallergenic nature of extensive casein hydrolysate formulae these products do contain smaller peptide sequences that may display bioactivity. These smaller peptides could still be epitopes for immune cells stimulation or may affect other relevant biological functions that could support health and reduce the risk of allergy development [3]. Certain bioactive peptides from hydrolysates can modulate the immune response by interacting with specific receptors involved in bacterial signaling, epithelial barrier function as well as modulation of specific regulatory T-cell reactivity thus

potentially supporting tolerance induction [31]. Immunological tolerance is critical to allow the immune system to properly respond to potential pathogens while maintaining appropriate regulation or irresponsiveness to harmless food proteins or microbial antigens. Moreover, with the rapidly developing proteomics technologies the protein and peptide composition of human milk can be further elucidated with special reference to protein/peptide composition and the association with health and disease risk.

CASEIN HYDROLYSATES: CLINICAL USE

Obviously prevention of the development of cow's milk protein allergy may be achieved by complete elimination of allergic protein containing formulations or foods. Prenatal and postnatal allergen avoidance in children at risk because of family history, in combination with the use of extensive casein hydrolysates and to a lesser extent also partial hydrolysate, was also shown to be highly efficient in reducing food sensitization and allergy especially in the first year of life [32]. Note that this study was geared towards allergen avoidance and no evidence for active tolerance induction was provided. A prospective 4 year study tassessed prevention effects of feeding a casein hydrolysate formula to infants with a family history of allergy [33]. The study protocol included 4 months feeding of an extensive casein hydrolysate formula versus routine cow's milk formula. There was a clear long term protective effect of the casein hydrolysate on allergic manifestations (atopic eczema, asthma, plasma IgE) after 4 years.

There remain differences between partial whey hydrolysates and extensive casein hydrolysates in the clinical efficacy. This was demonstrated in a study by Oldaeus et al. where the preventive effect of extensively and partially hydrolyzed formulae was compared in a group of infants with a family history of allergy [34]. There was lesser wheezing and atopic dermatitis symptoms developed in the extensively hydrolyzed formula as compared with partially hydrolyzed formula or routine formula control group. It was suggested that partially hydrolyzed formula would still contain allergic determinants that could have caused immunological reactivity. In a similar study by Halken et al. the preventive effect of partially hydrolyzed formula and extensively hydrolyzed casein formula was evaluated in infants with high risk of allergy [35]. Again in this study design, with equal distribution of breast feeding between groups and recommended 4 month formula intake, the extensive

casein hydrolysate formulation was found to be performing better than the partial whey hydrolysate.

Nevertheless, with emerging science that helps to understand hypoallergenicity and peptide functionality of extensive casein hydrolysates, the use of these formulations may be broadened from dietary management to more preventive strategies. In a study by Juvonen et al. infants were fed either breast milk, a cow's milk formula or a casein hydrolysate formula for 3 days immediately after birth, and then switched to breast milk feeding. Serology at various time points during the first year revealed antibodies to cow's milk protein to be lowest in the casein hydrolysate group [36], even after such short early life intervention. Primary prevention of food allergy in children at higher risk because of a family history has been described in the German Infant Nutritional Intervention (GINI) Study. Early intervention for 4 months with a partially or extensively hydrolyzed casein or whey formulation revealed long-term effects on the incidence of allergic manifestations [37]. This was most pronounced for the extensive casein hydrolysate showing a reduced incidence of atopic dermatitis over the first years of life. On the long term, the prevalence of asthma between 11 and 15 years was significantly reduced in the extensively hydrolyzed casein formula group compared to intact cow's milk formula. Moreover, the cumulative incidence of eczema was found to be reduced for both extensively hydrolyzed casein formula and to a lesser extend partial hydrolyzed whey formula [38]. Again underpinning the potential role of dietary peptides in actively supporting tolerance induction.

It seems that intestinal microbial composition, diversity as well as specific metagenomic profiles of the microbial community, i.e., presence of butyrate producing species, may be associated with lower incidence of atopic disease development [17, 39]. Thus, the potential role of casein hydrolysates and peptides thereof, in combination with other factors from the diet, on microbial composition and metabolism deserves further study. Indicative of such peptide functionality is the observation from a clinical study in which extensive casein hydrolysate intervention accelerated tolerance acquisition in CMA infants [40]. The rate of oral tolerance acquisition after 12 months was significantly higher with extensive casein hydrolysate formulation compared to other formulae and this was associated with changes in microbial composition and metabolism [41].

EXPERIMENTAL ANIMAL MODELS FOR COW'S MILK ALLERGY AND CASEIN SENSITIZATION

To date, studies with casein hydrolysates in animal models of cow's milk protein allergy are limited. It is known though that mice can be sensitized to oral casein exposure when given in the context of a co-adjuvant such as cholera toxin [42]. This resulted in symptoms that are in large part similar to human food allergy, i.e., acute allergic reactivity in the skin (ear swelling), casein specific serum IgE elevation, increased mast cell mediator MCP-1 serum levels, colonic motility and water reabsorption changes suggestive of diarrhea [42] as well as Ig free light chain elevation [43]. Mouse immunization with cow's milk protein preparations resulted in specific allergic sensitization patterns against casein (IgE, splenocyte allergic cytokines responses, fecal histamine levels), more so then against whey protein that were also present in the immunizing cow's milk protein preparation [44]. Further establishment of such models will allow to study mechanism of action of casein-derived peptides that seem to comprise relevant biological activity that may contribute to observed clinical effects of casein hydrolysates.

CASEIN HYDROLYSATE AND AUTOIMMUNE DISEASE

Reactivity to cow's milk protein has also been associated with the development of autoimmune disease like type I diabetes [45, 46]. Infants from diabetic mothers indeed have high levels of casein specific circulating antibodies which can be dramatically reduced by providing a casein hydrolysates based formula [47]. In the TRIGR study, that included infants with a high risk for developing type 1 diabetes, the early intervention with a casein hydrolysate seemed to have a beneficial effect in reducing the levels of relevant autoantibodies known to be associated with the development of type 1 diabetes [48]. In a much larger, and likely phenotypically more diverse population, this protective effect could however not be reproduced [49]. The potential protective effect of extensive casein hydrolysates was shown in rodent models of type 1 diabetes [50, 51]. The studies also included an amino acid formulation comparison and revealed that the protective effect of the casein hydrolysate could not solely be explained by the avoidance of whole proteins as protective effects were not observed in the amino acid group. Distinct effects of the peptides in the extensive hydrolysate were reported on

immune cell activity and microbial composition and were suggested to underlie the observed beneficial clinical effects [50, 51].

REGULATION OF ENERGY METABOLISM, BODY WEIGHT AND COMPOSITION

Obesity is becoming a major health issue world-wide, and further understanding of the dietary impact on energy metabolism in infants is crucial for the modulation of energy balance and stimulation of a healthy weight development. Accumulating evidence shows that protein can induce satiety, increase secretion of gastrointestinal hormones, and increase diet-induced thermogenesis [52]. Diets with a higher proportion of energy from protein are therefore suggested to contribute to weight loss and, particular for casein, additional benefits may be associated with specific peptide sequences or hydrolysates.

HYDROLYZED CASEIN AND BODY WEIGHT/COMPOSITION

Overall, data on the long-term effects of hydrolyzed casein in the area of body weight development in infants is still limited. One clinical study with infants with a family history of atopic disease was performed in this area [53]. It was found that, compared to hydrolyzed whey protein or cow's milk-based formulae, infants that consumed hydrolyzed casein-based formulae had an overall decreased BMI due to a lower body weight gain during the first year of life. Importantly, other factors including overall length did not differ illustrating that the observed effects in the hydrolyzed casein group is a consequence of lower weight gain and not a result of overall impaired growth development. In another study with healthy infants, comparing whole protein formulae with hydrolyzed formulae, cow's milk formula were found to accelerate growth [54] which is in line with previous studies in this area. Interestingly though, weight development in the hydrolyzed casein formula fed group was normative. No differences in length-for-age z scores and linear-growth velocity were observed, further supporting that growth differences between groups were specifically associated with weight across the study period [54].

Further insights related to differences between intact and hydrolyzed casein came from animal studies [21, 55]. The authors describe that mice fed Western-style diets containing hydrolyzed casein gained less adipose tissue mass than mice fed the same diet with intact casein, particularly evident in inguinal white adipose tissue [55]. Moreover, relative to intact casein, hydrolyzed casein tended to induce a lower respiratory quotient than intact casein, indicating lower utilization of carbohydrates as energy substrate. The latter was further supported by lower plasma concentrations of glucose and lactate in the hydrolyzed casein group. Furthermore, in inguinal white adipose tissue, the mice fed hydrolyzed casein had greater non-CO2 β-oxidation capacity along with induced expression of genes involved in mitochondrial fatty acid oxidation and mitochondrial uncoupling. Clausen et al. further demonstrated that the reduced body fat accretion in the hydrolyzed casein group might be related with enhanced liver lipid metabolism in C57BL/6J mice [21].

Although the underlying mechanisms are not yet fully elucidated, overall these studies suggest that consumption of hydrolyzed casein may contribute to an optimal weight development and may, at least in part, contribute to prevention of obesity early in live. Further studies would be required to confirm the observed effects.

DIGESTION/ABSORPTION KINETICS AND METABOLIC RESPONSES

Metabolic responses to protein from different sources may differ and will, amongst others, depend on their intrinsic properties, such as amino acid profile, overall bioavailability and digestion/absorption kinetics [56, 57]. Particularly for casein, both human and animal studies have demonstrated that postprandial metabolism is different after consumption of intact or hydrolyzed casein [58-61]. Although, in general, amino acid composition will not vary between intact or hydrolyzed casein, these observations can be explained by other factors. First, specific free amino acids and/or peptide sequences in hydrolyzed casein may stimulate sensory receptors in the oral cavity and/or gastrointestinal tract, which in turn, may serve as key signals for food intake regulation and satiation [62]. Second, intact casein coagulates in the acidic environment of the stomach, which delays its digestion and gastric passage and overall induces a slower postprandial increase in plasma amino acids [56].

Hydrolyzed casein does not coagulate under gastric conditions [63] and consequently gastric emptying is increased, coinciding with higher postprandial amino acid absorption rates, and elevated plasma glucose-dependent insulinotropic polypeptides [58].

A recent crossover design study compared the postprandial digestion between intact and hydrolyzed casein in elderly men [60]. Compared to the same amounts of intact casein, a single bolus of 35 g hydrolyzed casein induced accelerated protein digestion and absorption from the gut and a transient more rapid increase in plasma amino acid concentrations. Consequently, a tendency of increased incorporation rates of amino acids into skeletal muscle protein relative to ingestion of intact casein was observed. Thus, the ingestion of hydrolyzed casein, compared to intact casein, may result in a different nitrogen utilization in humans. As an overall result, ingestion of hydrolyzed casein with an associated more rapid amino acid delivery may induce a lower net energy accretion in the postprandial period, especially because soluble dairy proteins with a high intestinal absorption rate increases the deamination of the ingested amino acids, leading to a higher urinary urea excretion compared with micellar casein or total milk proteins [64].

GUT HORMONES RELEASE/SIGNALING

Relative to intact casein, hydrolyzed casein is absorbed and metabolized in a way that promotes greater satiation, possibly because of altered gut nutrient-sensing and/or more rapid absorption [65]. It is hypothesized that the digestion of proteins gives rise to certain peptides that possess the capacity to induce several satiety signals in the gut [22]. For example, particular peptides in hydrolyzed casein stimulate cholecystokinin (CCK) release, which in turn contributes to food intake suppression and satiation [66]. Additionally, hydrolyzed casein may provide satiety signals through opioid receptors (also see section ‘Casein-derived opioid receptor ligands’ of this review). Although speculative, the induced satiation signals and stimulated earlier meal termination could potentially contribute to the observed overall slower weight gain over time in infant who consume hydrolyzed casein formula [54].

Insulin signaling regulation by diet intake is important in the development of obesity and obesity associated diseases such as type II diabetes [23]. The increased insulin concentrations after feeding may promote anabolic processes in the body such as protein and glycogen synthesis [67]. Although the data about the insulinotropic effects of different protein sources in healthy human

subjects are sparse, studies have demonstrated that the response of insulin concentration is associated with the availability of specific plasma amino acids [68], with rapidly absorbed proteins inducing a greater response of insulin concentration than slowly absorbed proteins [44]. Therefore, a higher insulin response was observed after ingestion of hydrolyzed casein than after ingestion of intact casein, especially during the initial postprandial hours when plasma amino acids were higher [59].

Overall, the ingestion of hydrolyzed casein will initiate a multiplicity of satiety signals from the gut depending on the peptide released from the digestion, which will stimulate satiation and suppress food intake.

PROTEIN SYNTHESIS

After ingestion, hydrolyzed casein may induce a stronger muscle protein synthetic response then intact casein, mainly because of their fast systemic availability and concurrent stronger metabolic responses [60]. Besides, presumably due to the early hyperinsulinemia and possibly hyperleucinemia, there might be a preferential utilization of dietary nitrogen for splanchnic protein syntheses from hydrolyzed casein, which will contributes to muscle protein anabolism, especially after resistance exercise. This may particularly be important for individuals, such as elderly to prevent sarcopenia [69]. A randomized, prospective 12-week study was performed comparing the changes in body composition after consuming diet containing hydrolyzed casein, hydrolyzed whey or control diet in individuals who followed resistance exercise training [70]. The results showed that the hydrolyzed casein group had the highest lean mass gain and highest increase in strength likely due to improved nitrogen retention and overall anabolic effects caused by the peptide components of the hydrolyzed casein in combination with resistance exercise training. Similarly, whole-body protein synthesis stimulating effects of hydrolyzed casein were observed in chronic obstructive pulmonary disease patients with nutritional depletion [70].

Although not fully understood in infants, the described intrinsic differences in metabolic responses after consumption of hydrolyzed or intact casein may, at least in part, contribute to the observed growth difference of infant fed either intact milk or hydrolyzed casein formula.

OTHER HEALTH BENEFITS ASSOCIATED WITH CASEIN HYDROLYSATES

Although overall of lesser relevance for infant nutrition, milk protein hydrolysates/peptides, in particular casein-derived, have been demonstrated to exert other activities such as improved mineral absorption, ACE inhibition, antioxidant activity, opioid receptor activation and analgesic activity.

BLOOD PRESSURE REGULATION

Among the different bioactivities of casein-derived peptides, angiotensin converting enzyme (ACE)-inhibitory peptides are receiving special attention due to an increased prevalence of hypertension in the Western population. Both animal and human studies have studied effects of casein peptides/hydrolysates over the past two decades. Lactotripeptides (LTP) valine-proline-proline (VPP) and isoleucine-proline-proline (IPP), which are released from β-casein and ƙ-casein, are amongst to best studied sequences and nowadays have found application in specialized nutrition for blood pressure regulation [71-73].

Gastrointestinal digestion of casein releases ACE inhibitory peptides. In addition, bacterial or fungal proteinases are also capable to hydrolyse casein and release ACE inhibitory peptides [74]. For example, the potent casokinins, IPP and VPP were found in skim milk fermented with *Lactobacillus helveticus* CP790 and *Saccharomyces cerveisiae* [75]. While *in vitro* models provide valuable insights related to ACE inhibitory potency, testing in human subjects would be required to confirm overall effects on blood pressure and eventual commercial application.

Numerous human studies involving IPP and VPP were reported and a meta-analysis restricted to 1306 European subjects involved in 14 trials confirms that this peptide combination can contribute to maintaining a normal blood pressure [76]. The mean pooled effect for decrease in systolic blood pressure with IPP/VPP was 1.28 mm Hg (95% CI, –2.09 to –0.48, $P = 0.0017$) without heterogeneity among the analyzed studies. However, the authors pointed out potential confounding effects such as subject age and blood pressure status among subjects as factors influencing the outcome. For example, four trials on subjects with a mean age below 50 years, all of which were hypertensive subjects, showed a large effect of systolic blood pressure

(up to -10.3 mmHg) whereas three trials on subjects with a mean age above 50 years, all with high blood pressure, showed no beneficial effects. Overall, these studies suggest IPP/VPP as a potential lifestyle intervention for middle-aged adult Europeans with high blood pressure. Focusing on a different population, a recent meta-analysis restricted to Japanese subjects reported even a stronger effect of IPP/VPP combination [72]. The analysis that includes 18 trials with a total of 1194 subjects reported a mean pool effect of systolic blood pressure reduction at -5.63 mm Hg (95% CI, -6.87 to -4.39, $P<0.0001$). This report reaffirms previous findings that the Japanese population are better responders to IPP/VPP than Europeans.

Mechanisms responsible for antihypertensive effect of IPP/VPP are however not completely understood. The inhibition of ACE, production of vasoactive mediators and effects on the sympathetic nervous system were among the proposed mechanisms [77].

CASEIN-DERIVED OPIOID RECEPTOR LIGANDS

The existence of opioid ligands in milk have been proposed since 1979 when Brantl and colleagues identified β-casomorphins in milk as part of experiments to prove a hypothesis that opioids in the blood of mammals may permeate into mammary tissue [78]. Most of β-casomorphins are agonists selectively towards μ-opioid receptors, however, specific sequences may also have affinity for δ- or ƙ receptors. Opioid receptors (μOR, δOR, and ƙOR) are widely distributed in the human body and play crucial roles in numerous physiological processes. This include pain signaling in the central and peripheral nervous system, reproduction, satiety, growth, respiration, and immunological responses [79].

β-casomorphin 7, BCM7 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile), was one of the first peptides out of the β-casomorphin group detected and isolated from bovine casein digests [80]. A shorter analogue peptide, BMC5 (Tyr-Pro-Phe-Pro-Gly), showed a stronger opioid receptor activation than BCM7 [78]. A longer β-casomorphin fragment (1-11) Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu was isolated from the gastrointestinal tract of minipigs fed with β-casein. This peptide later showed to also behave as an opioid ligand in various *in vitro* and *in vivo* experiments [80]. Besides for β-casein-derived peptides, opioid activities have also been shown for α-casein and ƙ-casein–derived casomorphins [81].

An early report on the fate of the ingested β-casomorphins in young and adult animals showed that β-casomorphins were absorbed readily into blood but no transfer into lymph nor appearance in the cardiovascular system was detected, possibly due to rapid post absorptive degradation [82]. Absorption of β-casomorphins across gut epithelial cells may allow contact with extrinsic sensory neurons which endings are extended to submucosa of the gut. Activations of μOR expressed on these neurons reduce neurotransmitters release thereby mediating less neuronal excitations transmitted to the central processing [79, 83]. Many extrinsic sensory neurons that innervate the gut are silent nociceptors, meaning mechanically insensitive under basal/healthy conditions. However, under inflamed conditions they are readily activated by normally innocuous mechanical and chemical forces and remain excitable and overly sensitive after inflammation has diminished [84]. It has been suggested that peripheral opioid ligands are involved in reversing this phenomena resulting in reduced visceral pain [85]. Besides affinity towards opioid receptors expressed on enteric neurons, BCM5 and its derivatives are ligands to central opioid receptors reported to be involved in antinociception effect shown in rat neonates [86]. Prove that casein -derived opioid receptor ligands may mediate such activities was given in a recent clinical study in healthy adults where the authors reported that β-casomorphins were continuously released within the lumen of subjects who ingested casein. The amount released in jejunum 6 h postprandial were sufficient to elicit opioid ligand effects as discussed above [87].

Although to a large extend yet to be fully confirmed some of additional suggested functions of casein-derived opioid receptor ligands include: stimulation of mucus production [88]; central nervous system regulations [89] and anti-inflammatory activity through activation of μOR expressed by various immune cells in the lamina propria [79].

Their biological functions in infants are still largely unknown, however one particular non-clinical experiment with rat pups found that β-casomorphins was associated with reduced visceral sensitivity towards thermal stimuli [86]. Simulated gastro-intestinal digestion of infant formulae samples showed variable amount of released BCM7 (2.25 to 337 nmol/L), depending on the casein to whey ratio of the infant formulae and pH value under which the simulated hydrolyses were performed [90].

MINERAL ABSORPTION

Several studies have been performed in the last three decades on the functionality of casein peptides as carriers for different minerals [91]. The ability of casein to maintain calcium and phosphate ions in a soluble and bioavailable state is attributed to the tryptic multi- phosphorylated peptides of caseins, known as casein phosphopeptides (CPP). Since CPPs bind and solubilize minerals, they have been linked to overall physiological benefits including the prevention of osteoporosis, dental caries, hypertension and anemia [92].

CPP were detected in human ileostomy fluid, confirming their ability to survive gastrointestinal digestion [93] and facilitate the passive diffusion of minerals *in situ*. The benefit of CPP to improve iron absorption and availability has been suggested. However, trials reported conflicting outcomes. Partly this could be explained by additional *ex vivo* experiments where the authors reported that only β-casein and its CPP enhanced Fe absorption, whereas α-casein–rich fractions or their CPP reduced Fe absorption [91].

ANTIOXIDANT ACTIVITY

Oxidative species such as reactive nitrogen and oxygen species (ROS) are naturally found within humans. The ingestion of milk proteins has been suggested to reduce oxidative stress [94]. Partly, this effect is attributed to radical scavenging activity, inhibition of lipid peroxidation and metal ion chelation properties of peptides mostly studied *in vitro* [95]. In one particular study, a specific casein-derived peptide sequence Tyr-Phe-Tyr-Pro-Glu-Leu (YFYPEL) showed potency to scavenge 1,1-diphenyl-2-picrylhydrazyl radicals *in vitro* and further characterization suggested that the Glu-Leu sequence is most important for the activity [95].

Fermentation of casein with *Bifidobacterium longum* KACC91563 for 24 h release hydrolysate fractions with antioxidant activity as shown *in vitro* [96]. Further characterization and isolation of peptides within the most potent fraction (< 3kDa) identify 2 peptides containing the fragment VLPVPQ.

A trial in healthy adults reported the bioavailability of casein-derived peptide displaying antioxidant properties [87]. The peptide (Val-Leu-Pro-Val-Pro-Gln-Lys, β-CN (f 170-176)), were found in the jejunum of subjects as digestion products after consumption of milk casein proteins. However, the

overall physiological effects of this and other antioxidant peptides in humans are still to be elucidated.

FUTURE PERSPECTIVES

Applications in human nutrition require a thorough mechanistic understanding, through *in vitro*, animal and or biomarker studies and consistent results from carefully controlled and sufficiently powered clinical intervention studies. To date, some of the activities around bioactive peptides are mainly studied *in vitro* making the overall application and translation to nutritional outcomes challenging.

In this respect, one understudied feature in the field of bioactive peptide/hydrolysate research is bioavailability and systemic distribution. Several activities associated with casein-derived sequence may occur along the gastrointestinal tract, or within the proximity of the gut lumen interface where systemic availability is less of a factor, e.g., for immunological mechanisms such as tolerance induction specific macrophages have been demonstrated to sample antigens from the lumen and transfer antigenic sequences to other immune cells to induce tolerance [97]. Other suggested benefits of casein peptides will however depend on systemic availability. For example, *in vitro* assessment of ACE inhibition activity has long been a struggle given the lack of insight in systemic availability of milk-derived peptides. However since the mid-1990s significant progress has been made and nowadays it is clear that specific dairy-derived peptides may become available which coincided with a decreased blood pressure, both systolic and diastolic [72, 98]. With respect to longer casein-derived sequences data is more limited. Some studies reveal that a small proportion of specific sequences may also become available although overall bioavailability was low and half-live was short [99]. Thus more research in this area may be warranted to understand the mechanisms of overall peptide and hydrolysate functionality within nutrition.

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