Immunomodulating properties of protein hydrolysates for application in cow’s milk allergy

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Abstract
Cow’s milk proteins cause allergic symptoms in 2-3 % of all infants. In these individuals the tolerogenic state of the intestinal immune system is broken, which can lead to sensitization against antigens and eventually to allergic responses. Although a true treatment for food allergy is not available, symptoms can be avoided by providing the infants with hydrolyzed proteins. Hydrolyzed proteins are proteins that are enzymatically degraded. They lack typical allergenic IgE-binding epitopes but are also thought to play a pertinent role in other mechanisms inducing hypoallergenic effects. This review discusses the mechanisms and evidence for immunomodulating properties of cow’s milk hydrolysates. Hydrolysates are found to strengthen the epithelial barrier, modulate T cell differentiation, and decrease inflammation. Some studies suggest a role for hydrolysates in manipulating pathogen recognition receptors signaling as underlying mechanism. Peptides from hydrolysates have been shown to bind to TLR2 and TLR4 and influence cytokine production in epithelial cells and macrophages. Current insight suggests that hydrolysates may actively participate in modulating the immune responses in subjects with cow’s milk allergy and those at risk to develop cow’s milk allergy. However more research is required in order to design effective and reproducible means to develop targeting strategies to modulate the immune response.
Introduction

Awareness of food allergy as a serious health issue is increasing in the Western world. Its burden on morbidity, quality of life, and health care costs is more and more recognized [1, 2]. Despite this, there is a lack of treatment options. The only reliable therapy up to now is avoidance of allergens. In order to design more effective treatments, more insight is required in the mechanisms associated with food allergy.

A type of food allergy that has been subject of many studies is cow’s milk allergy in infants. Infant formula, which is often cow’s milk based, is the only approved alternative for breast feeding. However, cow’s milk proteins cause allergic symptoms in 2-3 % of the infants [3]. These allergic symptoms can be avoided by providing the infants with so-called hydrolysates of cow’s milk protein instead of the intact proteins [4]. Peptides in hydrolysates possess different immunological properties which prevent allergy [5].

The mechanisms by which hydrolysates modulate allergic responses are still subject of debate. One of the mechanisms is that after being taken up by intestinal epithelial cells and presentation of the peptides to the gastrointestinal immune system, the response is different and more tolerogenic than the response against the larger proteins [6]. The gastrointestinal immune system therefore seems to play an essential role. The barrier is composed of an epithelial layer and a variety of immune cells [7]. It is covered with mucus. The proteins and peptides are taken up by the epithelial cells and presented to the mucosal immune system. Here they interact with the gastrointestinal immune system (figure 1) to either induce tolerance or an immune response. In this review, we will give an overview on current knowledge on immunomodulatory properties of cow’s milk hydrolysates and its interaction with the mucosal immune system. In order to do this we will first discuss the immunological processes involved in maintaining oral tolerance and the onset of an allergic reaction.
Figure 1. Layers of the intestinal immune barrier. The barrier consists of a protective layer of epithelial cells covered with mucus and a range of immune cells. The main cell type in the epithelial layer are gate-keeping epithelial cells, but other specialized cells are also present. The crypts of the villi contain both mucus producing goblet cells and anti-bacterial compound secreting Paneth cells. The lamina propria under this epithelial layer contains many immune cells including dendritic cells, macrophages and lymphocytes. After antigen passed the epithelial barrier it is taken up by dendritic cells. The antigen presenting dendritic cells migrate to the mesenteric lymph nodes and presented their MHC-antigen complex to T cells inducing T cell activation. In the small intestine Peyer’s patches are present. In these organized lymphoid nodules antigen presenting dendritic cells also interact with T cells. T cells then regulate the immune response activating other immune cells, for example B cells.

Large proteins can cause allergy in infants

Newborns have specific dietary needs. To achieve optimal growth, development and health in the first months of life, breast milk is required. When breastfeeding is not possible, infant formula is the only approved infant nutrition. However, this is not without consequences. Infants are not yet able to digest all the novel enteral nutrients. The immature infant’s digestive system is not producing enough enzymes essential for protein digestion, such as gastric pepsin [8]. Also, the relatively high pH in the infant’s stomach does not accommodate optimal digestion of proteins [9]. This results in partly or undigested proteins in the gastrointestinal tract of the infant during a vulnerable period for the development of food allergy. As a result, more and larger proteins may pass the gastrointestinal barrier and induce, in the presence or absence of a pathogen, an aberrant Th2-skewed immune response [10]. This aberrant immune response is the first step towards allergic sensitization.
Furthermore, an important characteristic of the infant’s intestine is the relatively high permeability of the epithelial layer separating the intestinal lumen from the mucosal immune system [11], which is associated with allergic reactions [11]. This can also contribute to a higher uptake of larger proteins, although it is more related to the later allergic reactions than to the sensitization phase.

In order to understand where and how food allergy against proteins can develop, we first need to discuss the comprehensive regulatory system that is involved in tolerance induction.

**The gastrointestinal epithelial cells as gatekeeper**

The first line of defence against intruders are, as outlined above, the epithelial cells separating the intestinal lumen from the mucosal immune system. The epithelial cells are highly organized and connected by tight junctions on both the apical and basolateral site. In adults, the connections make the barrier impermeable for molecules larger than 3,5 kDa [12]. For infants, the exact permeability for macromolecules is not known, and depends on birth weight, gestational and postnatal age [13]. Epithelial cells carry the polymeric Ig receptors on the basolateral side which continuously transport neutralizing IgA antibodies into the lumen, which bind to harmful antigens that are then expelled by the peristaltic process [12].

The permeability of the epithelial layer is variable over time. Binding of food components to immune-related receptors can actively increase and decrease permeability to sample antigens from the lumen [14]. Such receptors are for instance pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) [15]. Binding of a luminal antigen to PRRs can lead to release of serine-proteases such as zonulin by epithelial cells [16]. These serine-proteases digest the tight junctions between epithelial cells [17], which leads to an increase in permeability and consequently entry of luminal antigens in the lamina propria (figure 2). How often this occurs and how it quantitatively contributes to tolerance for specific food antigens is unknown.

Another, more in detail-studied process, is antigen sampling by dendritic-like cells that are located in between the epithelial cells. These dendritic cells, often referred to as the CD11b+CX3CR1+ cells create protrusions into the lumen of the gastrointestinal tract to sample its contents [18]. The major part of luminal antigen sampling, however, is not occurring by the epithelial or dendritic cells but in the gut-associated lymphoid tissues (GALT). In the small intestine these are called the Peyer’s patches. Here, specialized epithelial cells called M-cells transport luminal antigens to the underlying dendritic cell-rich area [19]. Here the antigens are taken up by antigen presenting cells (APC) (mainly dendritic cells) and processed for presentation to T cells. These dendritic cells are referred to as the CD103+CX3CR1-. They are found in high quantities in the lamina propria and take up food components, including proteins, that are sampled by the epithelial cells or CD11b+CX3CR1+ dendritic cells. Currently, the CD103+CX3CR1- dendritic cells are considered to be the only dendritic cell population that is able to migrate to mesenteric lymph nodes [20], to induce tolerogenic responses. In order to do so the cells need appropriate tolerogenic signals. An important source of tolerogenic factors are the epithelial cells [21]. When the epithelial cells secrete factors such as IL-10, TGF-β, and TSLP [21], CD103+CX3CR1- dendritic cells differentiate into tolerogenic dendritic cells, before presenting their antigens to T cells in the mesenteric lymph nodes.
Figure 2. The intestinal epithelium separates the lumen of the intestine from the lamina propria and the immune system. It keeps pathogens and antigen from entering the body. This layer of epithelial cells is covered by a thick layer of mucus. The mucus layer is a reservoir for IgA, which agglutinates peptides. This stimulates expelling of the peptides. Epithelial cells are joint together by tight junctions. These structures, consisting mainly of the proteins ZO-1, occludin and claudin, make the paracellular spaces impenetrable for most proteins. However, the permeability of the epithelial layer is not always that low. Epithelial cells express receptors including MHC class II and pattern recognition receptors. Binding of food derived molecules to these receptors is able to regulate the permeability.

Adaptive tolerogenic T cell responses to food antigens

Depending on the amount of antigen, three distinct immunological mechanisms can contribute to immunological tolerance to food antigens: i.e. induction of regulatory T cells (Tregs), clonal anergy of T cells, and deletion of T cells [21]. Tregs are mainly generated by CD103+CX3CR1-dendritic cells in the mesenteric lymph nodes. Here the tolerogenic CD103+CX3CR1- dendritic cells present the food antigens to CD4+ T cells. Tolerogenic CD103+CX3CR1- dendritic cells, but also macrophages, mostly express IL-10, and TGF-β, which induce Treg formation [22]. Upon interaction of dendritic cells with the Treg subtype Th3, a cascade of Treg subtype induction and maintenance is initiated, while the secreted TGF-β at the same time inhibits Th1 and Th2 subtype T cells [21]. This type of tolerance induction is only initiated when the food antigen is present in lower doses. This mechanism is considered to be a central mechanism of tolerance induction for food antigens.
At higher doses tolerance is mainly caused by clonal deletion and clonal anergy. PRR mediated activation, and especially TLR signaling, has also been found to be essential in these processes. TLRs have been shown to function as co-stimulatory molecule in the interaction between T cells and dendritic cells [23]. By this, TLRs influence the T cell response, as peptide-MHC-II-complex presentation together with a lack of co-stimulatory molecules on dendritic cells during high antigen exposure is thought to cause T cell clonal anergy [24]. Clonal deletion of T cells is the other process that occurs at high antigen exposure. This usually occurs in the Peyer’s patches. It is thought to be caused by natural killer T cells, possibly via Fas-FasL interaction with T cells that leads to apoptosis of food antigen recognizing T cells [25] (figure 3).

Figure 3. Mechanisms of oral tolerance induction. In a normal situation immunity is induced by the interaction between an antigen presenting dendritic cell and a naïve T cell. Due to the binding of the TCR (T cell receptor) with the MHC-peptide complex and the costimulatory molecules the T cell gets activated. However, the default state in the gut is tolerance against dietary antigens. Different mechanisms are involved in inducing tolerance. When a high dose of antigen is present, the main mechanisms involved are anergy and deletion of T cells. Anergy occurs when costimulatory molecules are lacking during the interaction between dendritic cells and T cells. This leads to an unresponsive T cell. Deletion also occurs at high-antigen levels. Antigen specific T cells go into Fas-Fasligand induced apoptosis after interacting with for example a natural killer T cell. The main mechanism inducing tolerance at a low dose of antigen is the induction of regulatory T cells. Tolerogenic factors produced by epithelial cells result in a more tolerogenic dendritic cell. When this cell interacts with a T helper 3 cell, the T cell produces TGF-β, which leads to differentiation towards a regulatory T cell and the inhibition of Th1 and Th2 cells.
**Food allergy through broken tolerance**

Sensitization to a food antigen may occur, when one of the above mentioned processes fails. The antigen, such as a food protein, then causes an inappropriate Th2 skewed immune response. In the sensitization phase antigens are processed by APCs and presented to T cells. These T cells become activated Th2 cells, which stimulate class switching to IgE and B cell differentiation into B plasma cells by secreting a mix of cytokines, including IL-4, IL-5, and IL-6 [26]. The antigen-specific IgE antibodies bind to the high-affinity IgE receptor FcεRI on mast cells and basophils. Re-exposure to the same antigen results in cross-linking of the IgE antibodies bound to FcεRI, inducing degranulation of the mast cells, which causes an immediate hypersensitivity reaction [27]. Mast cells release several inflammatory mediators including histamine, which contributes to clinical symptoms like nausea, vomiting, and abdominal pain [27] (figure 4). Upon activation, mast cells also secrete chymases, and various cytokines, such as TNFα, IL-13, and IL-8 [28], that can break open the tight junctions between epithelial cells, which allows more dietary antigens to cross the epithelial barrier. These changes in barrier function after antigen exposure lead to changes in osmotic water pressure over the epithelial barrier with diarrhea as a consequence [29]. When the barrier cannot be closed, the result will be severe inflammation which subsequently leads to more epithelial breakdown.

The immediate hypersensitivity reaction can be followed by a T cell mediated late phase response, which occurs 12-48 hours after antigen exposure [30] and is caused by the continuous presence of antigen [31]. During this late phase reaction, Th2 cells and intestinal epithelial cells keep producing Th2 related cytokines such as IL-4, IL-5, IL-9, and IL-13 [28]. This leads to maintenance of high IgE levels, elevated mucus production and the infiltration of basophils, eosinophils and lymphocytes [32].
Figure 4. Overview of the events during sensitization and an allergic reaction. When antigen passed through the epithelial barrier it is taken up and processed by antigen presenting cells (APCs). These cells present the antigen in a MHC class II molecule together with costimulatory molecules to antigen specific naive T cells, which get activated and develop a Th2 phenotype. Activated T cells stimulate B cell differentiation into plasma cells, which start to produce antigen specific antibodies (IgE). These antibodies bind to the high-affinity receptor FcεRI on mast cells. Upon re-exposure of the same antigen the antibodies cross-link and degranulation of the mast cell is induced. The release of a range of compounds results in allergic symptoms.

Beyond Th1 and Th2 cells

Although food allergy was classically thought to be a Th2 type response, recent studies have shown that the Th1/Th2 paradigm is an oversimplified view of the real situation. Besides Th1 and Th2 T cells, other T cell subtypes, such as Th17 and Th22, have been identified and found to be important in gut homeostasis [33]. In healthy individuals, low numbers of Th17 cells are present, mainly in the lamina propria [34]. During infection IL-17 induces the recruitment of neutrophils, and the epithelial increase of chemokine CCL20 attracts more Th17 cells [35]. Current insight suggests Th17 cells also play a role in cow’s milk allergy as will be outlined below.

Th22 cells produce IL-22. IL-22 has been found to be essential in the defence against Gram-negative bacteria in the intestine [36], showing its important role in the regulation of host defence and homeostasis. IL-22 elicits these protective effects by inducing for example the expression of antimicrobial peptides [36].

The above suggest that both Th17 and Th22 cells are important in maintaining homeostasis in the intestine and thus protect the state of tolerance. Although it is well established that these cells play a role in other allergic diseases, such as allergic airway inflammation [37], their exact role in cow’s milk allergy needs more attention of researchers. Earlier, a trend towards a negative correlation between whole blood IL-17 levels and sensitization to some food antigens was described [38], which is in line with the study of Duhan and d’Hennezel who found a lower production of IL-17 in CD4+ T cells in children with food allergies compared to healthy controls. In vitro, CD4+ T cells from these allergic children showed impaired IL-17 production after antigen administration [39].

Possibility to prevent/treat allergy with hydrolyzed proteins

As outlined before allergic reactions against cow’s milk protein in cow’s milk allergic infants can be prevented or avoided by administration of hydrolysates of cow’s milk. During hydrolysis of the original protein, mixtures of smaller peptides with different properties can be produced. The formulas available differ by the degree of hydrolysis. There are extensively or partially hydrolyzed formulas available, made from whey or casein. Extensively hydrolyzed formulas are intended to avoid allergic reactions in already cow’s milk allergic infants. This hypoallergenic effect has longer been known to be due to the destruction of the epitopes on the proteins, which are responsible for IgE binding [41]. Partially hydrolyzed formulas are used for infants at risk for developing food allergy [40], and are (together with extensively hydrolysates) now more and more considered to be hypoallergenic by modulating the immune response and therewith preventing sensitization. Some peptides in hydrolysates (but also proteins in cow’s milk) have indeed been described
to actively influence the immune system and modulate the allergic response [42]. When encountering the epithelial cells and CD11b+CX3CR1+ dendritic cells in the intestine peptides can bind to specialized PRRs involved in inducing tolerogenic responses [43]. By binding to the receptors, which are able to recognize a variety of molecules, hydrolysates may be able to affect the epithelial barrier or the dendritic cells.

Jaziri et al. already proved in 1992 the concept of peptides binding to specific receptors [44]. They showed that the immunostimulating peptides Gly-Leu-Phe (GLF) and Val-Glu-Pro-Ile-Pro-Tyr (VEPIPY), which were isolated from casein, bound to specific sites on human phagocytic blood cells. Although the binding sites were not further characterized it was thought that the two investigated peptides were recognized by two different receptors. GLF bound specifically to monocytes and polymorphonuclear leukocytes, while VEPIPY only bound to monocytes and macrophages. By using different analogues, the great selectivity of the binding sites was demonstrated. Iskander et al. [45] recently observed a decline in the LPS-induced IL-8 production in respiratory epithelial cells after the administration of whey protein hydrolysates. When they studied the mechanism involved, they found that the hydrolysate did not suppress the IL-1β and TNFα but induced IL-8 production. Therefore TLR4, which binds LPS, was suggested to be involved. It was demonstrated that the hydrolysate did neither neutralize LPS nor change the expression of the TLR4 receptor. Therefore, it was concluded that the effect of the hydrolysate was probably due to a direct binding of the hydrolysate to the TLR4 receptor, thereby preventing the binding of LPS. However, hydrolysates may not only hinder the binding of inflammatory stimuli to TLR receptors, they may also directly activate TLR signaling [46]. Inhibition of either TLR2 or TLR4 was shown to abolish the increased production of IL-6 in intestinal epithelial cells treated with a yellow pea protein hydrolysate [46].

Hydrolysates affect the intestinal epithelial barrier

Up to now there are a few studies dedicated to modulation of the barrier function by hydrolysates. Visser et al. [47, 48] fed diabetes prone rats casein hydrolysates from the moment of weaning until an age of 140 and 150 days and compared the barrier function of these animals with animals fed regular chow with the same macronutrient composition [47, 48]. The in vivo barrier function improved as demonstrated by a decrease in the lactulose:mannitol ratio in both studies [47, 48]. Also in vitro the intestinal barrier was increased by hydrolysates, as demonstrated by measuring the transepithelial electrical resistance (TEER) of a sample from the ileum [48]. Looking further into the mechanisms involved in the improvement of the epithelial barrier, it was found that the mRNA expression of genes encoding the tight junction proteins myosin IXb, claudin-1, and claudin-2 in diabetic rats on casein hydrolysates were normalized in the ileum after the casein hydrolysate diet compared to healthy rats, together with an upregulation of the regulatory cytokine IL-10 [48]. These studies demonstrate that hydrolysis not only deletes allergic epitopes but that hydrolysates also stimulate immune barrier function. Interestingly, one of these studies compared the hydrolysate formulation with a formulation containing only single amino acids [47], and found that the hydrolysate formulation was superior in epithelial barrier protection. This underscores the important role of peptides present in the hydrolysate mixture.

Hydrolysates may also influence the ‘inflammatory’ status of the epithelial cells, and since
inflammation is associated with cell damage and consequently damage to the epithelial barrier, hydrolysates can affect the epithelial barrier via this way. This was shown by Nielsen et al. who studied the inflammatory state of intestinal epithelial cells *in vitro* by using intestinal epithelial cells treated with the inflammation inducing drug indomethacin [49]. Casein treated with the enzymes pepsin and corolase was observed to decrease the transcription of several inflammation markers, including cyclo-oxygenase 2 (COX-2) and nuclear factor kappa beta (NF-κB), compared to casein treated with pepsin only, suggesting an anti-inflammatory effect of this hydrolysate [49]. Hydrolysates from other sources can also cause anti-inflammatory effects. For instance, a hydrolysate obtained from pea protein was observed to decrease the production of IL-8, which is a proinflammatory cytokine, in Caco-2 cells compared to an unhydrolyzed pea protein extract [50]. However, this hydrolysate also inhibited epithelial cell division, and thereby hindered normal epithelial cell renewal and decreased epithelial integrity.

Hydrolysates thus appear to be able to stimulate the epithelial barrier. Because an increased permeability of the intestines is associated with intolerance and food allergy [51], an improved epithelial barrier is beneficial in at-risk or already allergic infants. Therefore, peptides that improve the epithelial barrier are expected to have a hypoallergenic effect. It is therefore important that further (mechanistical) studies into the effect of hydrolysates on the intestinal epithelial barrier are performed.

**Hydrolysates show anti-inflammatory effects in innate immune cells, which can influence macrophage differentiation and the allergic reaction against dietary proteins.**

Hydrolysates have also been shown to inhibit inflammatory responses and even stimulate tolerogenic responses in antigen presenting cells. Oseguera-Toledo [52] used a hydrolysate mix from the common bean (*Phaseolus vulgaris* L.) obtained from hydrolysis using the enzymes alcalase and pepsin-pancretin. After administrating the hydrolysate, LPS activated RAW 264.7 macrophages showed a decrease of inflammation markers, such as COX2 expression and related prostaglandin E2 production, inducible NO synthase (iNOS) expression and related NO production. The decrease of these inflammation markers was associated with a decrease of NF-kB, due to a decreased translocation of its subunits p50 and p65 [53]. However, these results were not compared to the intact protein extract from the common bean. Similar results for p50 and p65 were found when using a peptide called lunasin or a lunasin-like peptide, which was obtained from soy [54]. Interestingly, this is one of the few peptides to be studied as individual protein giving unique insight in structure- function relationships. Lunasin was found to inhibit NO, prostaglandin E2, iNOS, and COX2 as well. Furthermore, the proinflammatory cytokines IL-6 and IL-1β, which are also under the control of NF-kB, were also found to be reduced.

Combined, the foregoing studies suggest that in addition to improving barrier function, peptides in hydrolysates may contribute to preventing allergy by inducing a more tolerogenic response in antigen presenting cells. *In vitro* studies suggest that a decreased overall amount of NF-kB and in particular the p50 subunit regulate the polarization of macrophages away from a pro-inflammatory (M1) towards an alternative (M2) state, which are directed towards a regulatory and tissue repair function [55] (figure 5). Therefore, the anti-inflammatory peptides could also help to control inflammation in the late phase of the allergic reaction [56].
Performing paw oedema tests in mice fed with hydrolysates indeed showed that different whey hydrolysates have anti-inflammatory effects in vivo, which is promising in a setting of cow milk allergy [57].

Figure 5. Immunomodulating peptides might be able to stimulate M2 differentiation in macrophages by regulating the expression of the NF-κB subunits p50 and p65. Specific peptides are able to decrease the overall NF-κB activity in the cell, which leads to a more M2 type macrophage. TLR2 activation for example results in autophagy of both p50 and p65. Administration of peptides that change the expression of a particular subunit can also change the phenotype of the macrophages. This results in a different amount of regulatory NF-κB complexes containing a p50 homodimer. This complex stimulates the expression of genes related to an M2 phenotype and inhibits the expression of multiple M1 genes.

Specific hydrolysates skew the T cell differentiation from a Th2 subtype towards Th1 or Treg, which is beneficial in food allergy. Peptides from hydrolysates may also encounter lymphocytes in the lamina propria when passing the barrier. Especially in infants where the epithelial barrier is rather permeable [11] this is likely. Many studies have shown that hydrolysates from different sources can have a direct stimulatory effect on the proliferation and activation of lymphocytes. For example, hydrolysates obtained from soy and whey (or more specifically the proteins β-lactoglobulin or lactoferrin) were shown to enhance proliferation in murine spleen lymphocytes [58]. Also intact whey proteins stimulate proliferation in murine spleen lymphocytes, however, they were less potent than the whey hydrolysates [59].
Although the above-mentioned results show an effect of hydrolysates on number of lymphocytes, it is not the number of lymphocytes that may prevent or inhibit an allergic response, but the skewing of Th2 responses towards Th1 responses [60]. These T cell responses can be induced without causing an allergic response, depending on the size of the peptides in the hydrolysate. For IgE crosslinking the peptides need a minimal length of 30 amino acids. Smaller peptides are not able to induce mast cell degranulation, but can still be recognized by T cells and therefore skew T cell differentiation [61].

Only a few studies have addressed this issue of T cell differentiation skewed by peptides at the moment. One study investigated the effect of a yak milk casein hydrolysate on the Th1/Th2 balance by measuring mRNA levels of Th1 cytokines (IL-2 and IFN-γ) and the Th2 cytokine IL-4 in murine spleen lymphocytes [62]. They found that this hydrolysate increased the Th1 cytokines, but it did not alter IL-4 levels. Therefore, this specific hydrolysate skewed the differentiation of T cells towards a Th1 subtype. Furthermore, Wu et al. mentioned that the IFN-γ/IL-4 ratio increased in spleen T cells from mice fed with chitosan hydrolysate, suggesting a change towards a more Th1-like phenotype [63]. On the contrary, specific hydrolysates derived from whey did not show individual effects on specific cytokines [61].

Because IL-10 producing Treg cells can inhibit Th2 cells, another way to dampen the Th2 response is to promote the differentiation of Treg cells by administration of specific peptides [31]. Various studies have shown an effect of hydrolysates on Treg formation by showing an IL-10 upregulation after treatment of lymphocytes with a hydrolysate obtained from the seaweed Porphyra columbina [64] or from casein hydrolysate [65]. This upregulation of IL-10 production was also observed in splenocytes obtained from mice treated with β-lactoglobulin trypsin hydrolysates, while intact β-lactoglobulin on the contrary downregulated IL-10 [66]. Ndiaye et al. also found an increased amount of IL-10 producing cells in the small intestine lamina propria of mice after oral administration of yellow pea protein hydrolysate [46].

Hydrolysates are not only thought to elicit effects on the epithelial barrier by binding to TLRs, but the observed effects on lymphocytes described above could also be induced via TLRs expressed on dendritic cells and T cells. TLR activation not only induces tolerogenic dendritic cells, but in general, activation of TLRs on antigen-presenting cells also prevents a Th2 driven allergic response by skewing a more Th1 like response [67]. Multiple animal studies have indeed shown that stimulation of a range of TLRs ameliorates the allergic response, including TLR2, TLR3, TLR4, TLR5, TLR7, and TLR9 [68]. Some clinical studies using non-pathogenic probiotics as TLR ligand showed a decrease in the incidence of atopic dermatitis, although the number of sensitized individuals was the same in the placebo and the experimental group [69]. However, although effects of hydrolysates on TLR signaling in epithelial cells [45] and effects of hydrolysates on dendritic cell and T cell activation and proliferation have been shown, more research is needed to show the direct interaction of hydrolysate peptides and TLRs on dendritic cells and T cells.

**Conclusion and future perspectives**

In order to prevent immune reactions against the numerous antigens present in the lumen of the gut, the different arms of the intestinal immune system contribute to maintaining a tolerogenic
state. When this state of tolerance is broken, this can lead to the onset of an allergic reaction. The present review discusses the evidence that although dietary proteins may serve as allergen-antigens, some bioactive peptides from hydrolysates have the ability to modulate the immune response in a hypoallergenic or other beneficial way [70].

An important part of these beneficial effects is attributed to the immunomodulatory properties of the hydrolysates [71]. There is increasing in vitro evidence that hydrolysates contain specific immunomodulating peptides, which, possibly by binding to TLR, have been found to improve the epithelial barrier, modulate the Th1/Th2 balance and the amount of Tregs towards a less Th2 skewed response and decrease inflammation. The limited in vivo studies confirm these findings (table 1). However, to get a better understanding of the immunomodulatory effects of hydrolysates especially human studies are needed, since many different effects of peptides, of both a pro- or anti-inflammatory nature, were found, which makes it hard to predict the final outcome in humans.

Another complicating factor is the diversity and a lack of documentation of the chemical properties of the hydrolysates tested, making side-by-side comparison of studies complicated. Every hydrolysate composition, and therefore its effects, is unique due to the use of different raw materials and hydrolysis production methods. Therefore, general statements about the hypoallergenic and immunomodulating effects of hydrolysates are not possible. Our recommendation is to document as many details as possible about the hydrolysates, including their degree of hydrolyzation, the source, lot-number and way of processing. It is also important to isolate individual peptides from hydrolysates and study characteristics of these individual peptides separately, in order to elucidate the exact interaction between a specific peptide and e.g. a receptor.

Hydrolyzed proteins are already used in infant formula for infants with cow’s milk allergy or infants at high risk to become allergic. However, up to now the suitability of these peptides in cow’s milk allergy is based on observations of absence of symptoms in the infants treated, while the underlying mechanisms are not well understood. Elucidating the exact effects and working mechanisms of specific peptides together with a better understanding of tolerance induction could provide mandatory information for a more efficient application of specific immunomodulating peptides in order to induce a hypoallergenic effect in infants. Therefore, more knowledge about the hypoallergenic effects of these hydrolysates will contribute to a more efficient treatment of this vulnerable group of newborns, and could ultimately also be beneficial for other individuals with a higher risk of allergy.
Table 1. Overview recent studies investigating immunomodulating effects of hydrolysates

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<th>Reference</th>
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<td>46</td>
<td>Ndiaye et al.</td>
<td>2012</td>
<td>Increased amount of IL-10+ cells in the small intestine lamina propria after oral administration of yellow pea protein hydrolysate</td>
<td>In vivo</td>
<td>mice</td>
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<td>47</td>
<td>Visser et al.</td>
<td>2012</td>
<td>Decreased lactulose:mannitol ratio (also compared to AA diet) after casein hydrolysate diet</td>
<td>In vivo</td>
<td>rats</td>
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<td>48</td>
<td>Visser et al.</td>
<td>2010</td>
<td>After casein hydrolysate diet decreased lactulose:mannitol ratio, increased transepithelial electrical resistance in an ileum sample (ex vivo), mRNA expression tight junction genes were normalized, and IL-10 upregulation</td>
<td>In vivo</td>
<td>rats</td>
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<td>57</td>
<td>Tavares et al.</td>
<td>2013</td>
<td>Anti-inflammatory effects from a whey hydrolysate were observed using a paw oedema test</td>
<td>In vivo</td>
<td>mice</td>
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<td>63</td>
<td>Wu et al.</td>
<td>2006</td>
<td>IFN-γ/IL-4 ratio increased in spleen T cells from mice fed with chitosan</td>
<td>In vivo</td>
<td>mice</td>
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Rational and outline of the thesis

As outlined in the preceding section, hydrolysis of intact proteins is a promising method for the generation of bioactive proteins and peptides with effects on different layers of the immune system. However, a thorough and broad screening of immune effects of different hydrolysates is still lacking. Furthermore, the underlying mechanisms of the immune effects are not known yet. Therefore, the aim of the studies described in this thesis is to investigate how a range of hydrolysates of different sources affect epithelial and immune cells involved in the allergic reaction. We tested hydrolysates from cow’s milk proteins, which is the main source of proteins used in infant formulas, as well as hydrolysates from soy and wheat, which are used as alternative plant protein sources. Hydrolysates with different degrees of hydrolysis were compared. The data obtained in these studies contribute to better insights in the effects of hydrolysates on food allergy and will ultimately lead to the design of better and more tailored hypo-allergenic infant formulas.

Since an extensive comparison of the effects of multiple hydrolysates is lacking, in chapter 2 we performed a detailed screening of a broad range of cow’s milk hydrolysates for their possible activating effects on immune cells. Then, we investigated whether the effects of cow’s milk hydrolysates could be due to interaction of the hydrolysates with Pathogen Recognition Receptors (PRRs). We studied the Toll-like receptor (TLR) interacting capacity of hydrolysates of two fractions of cow’s milk proteins, whey and casein, and investigated whether the degree of hydrolysis of the hydrolysates with the same protein source influenced the observed effects. In chapter 3 we did the same experiments for soy and wheat hydrolysates, to screen immune effects derived from plant-based hydrolysates and discuss basic immunomodulatory differences between animal and plant source hydrolysates.

As dietary molecules in the lumen of the intestine have been described to be able to modulate the epithelial barrier, in the next chapter, we studied effects of hydrolysates on the intestinal epithelial barrier. Therefore, in chapter 4 we studied the potential protective effects of hydrolysates on the epithelial barrier after disruption of the barrier by the disrupter A23187. After an extensive screening of epithelial barrier effects of cow’s milk, soy and wheat hydrolysates, we used soy hydrolysates, since strongest effects were observed in a hydrolysate from this protein source. The most potent soy hydrolysate was used to further investigate the effects of the soy hydrolysate on tight junctions. Furthermore, we studied the specificity and underlying mechanism of the protective effect by comparing the barrier protection after administering disruptors acting via different mechanisms.

In the previous chapters, we studied the effects of hydrolysates on immune cells and epithelial cells in monocultures. In a physiological setting, the interaction between the different cell types is crucial for the outcome. Therefore, in chapter 5, we first studied the interaction between dendritic cells and epithelial cells after hydrolysate administration in vitro by using a transwell coculturing system. In this experiment we compared the effects of a whey and soy hydrolysate which were both found to be potent TLR activating hydrolysates in chapters 2 and 3. Then, we investigated the effects of soluble factors produced by dendritic cells and epithelial cells on T cells.

During hydrolysis of intact proteins a complex mixture of many different proteins and peptides is formed. To determine effector-function relationships, it is important to identify the active
protein or active protein fraction in the hydrolysate that is responsible for the effects observed. Therefore, in chapter 6 we fractionated the mildly hydrolyzed whey and soy hydrolysates based on molecular mass, and tested the TLR activating potential of the fractions obtained. Next, the protein structures in the size defined fraction found to induce TLR activation were further characterized. Polyacrylamide gel electrophoresis (PAGE) was used to visualize the protein fraction responsible for TLR modulation, and by applying different PAGE conditions we determined the molecular forces and bonds involved in the tertiary and quaternary protein structures responsible for immune modulation. In order to maintain its immunomodulatory effect in vivo the protein fraction should withstand the harsh conditions and digestive enzymes in the stomach and intestine. We therefore also assessed the fate of hydrolysates during digestion in an in vitro infant digestion model.

In chapter 7 the immune effects of a partially hydrolyzed whey protein in a cow’s milk allergy mouse model were studied. In order to investigate the sensitizing capacity of the hydrolysate tested, animals were first sensitized with the whey hydrolysate, followed by an intradermal challenge with intact whey after which the clinical symptoms were measured. To gain more insight in the immunological effects underlying the clinical outcome of sensitizing with a partial whey hydrolysate, we studied immune cell populations both in the systemic and intestinal immune organs after an oral whey challenge. In this way, we were able to determine a possible mechanism explaining the observed clinical outcome of whey hydrolysate sensitization.

During our screening of the immune effects of a wide range of hydrolysates, we observed specific effects of a wheat hydrolysate that could have a beneficial effect on the intestinal health of adults as well and might serve specific purposes in medical nutrition. One such field is providing nutrition and suppressing inflammation in cancer patients on chemotherapeutics and suffering from mucositis or ileitis. Mucositis is inflammation of the mucosal intestinal tissue, which is initiated when PRRs are activated by danger signals released by chemically injured epithelial cells. Especially TLR2 is involved. During our screening for TLR interacting capacities of hydrolysates, we observed a strong TLR2 inhibitory effect of a wheat hydrolysate. Therefore, to take a first step in the development of wheat hydrolysates that might be effective in mucositis or ileitis, in chapter 8 we investigated the TLR2 inhibitory effects of different wheat hydrolysates and the underlying mechanism. We also identified the peptides present in the hydrolysate that could be involved in the observed effects.

Ultimately, an overall summery and discussion of the results in this thesis is available in chapter 9.
References


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Chapter 1


