General discussion

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Hydrolyzed proteins, called hydrolysates, have been developed as a protein source in infant formulas for food allergic infants and infants at risk for allergy [1,2], since the destruction of allergenic epitopes during hydrolyzation was assumed to protect against allergy development [3]. Recent *in vitro* findings show that hydrolysates also possess many immunomodulatory properties [4,5], which means that hydrolysates might actively contribute to the prevention of food allergy or a decrease of allergic reactions [6,7]. However, to optimally use immune modulating hydrolysates in the development of infant formulas, more knowledge about differences between hydrolysates, and the mechanisms involved in immune effects of hydrolysates is needed. Due to ethical restraints, mechanistic studies can not be done by invasive studies in neonates.

Therefore, the aim of the studies in this thesis was to investigate *in vitro* how different hydrolysates of cow’s milk, soy and wheat proteins modulate the immune system by affecting epithelial and immune cells. A second goal was to identify fractions or actual proteins and peptides that are involved in this immunomodulation. Our studies started by applying a technology platform in which we first selected hydrolysates that were interacting with Pathogen Recognition Receptors (PRRs), which are central immune receptors responsible for immunomodulation. This approach allowed us to start with high numbers of hydrolysates and end up with a few bioactive candidates that could be mechanistically explored in immune cells, which is laborious and could only be done with a limited number of hydrolysates. In hydrolysates with confirmed immunomodulatory effects, we investigated which protein fractions of the hydrolysates were responsible for the immune effects observed.

**Protein aggregates in hydrolysates of different protein sources induce TLR signaling**

We started with a high throughput screening of hydrolysates for TLR modulating effects, which has not been done before for hydrolysates. Many immune effects of single hydrolysates have been described in literature, but they are difficult to compare due to the different production methods and immune assays that were used. Our data demonstrate that screening and comparing a range of different hydrolysates contributed to our understanding of the observed immune effects and its underlying mechanisms. Therefore, it should be applied more often to understand product differences.

In chapter 2, we demonstrate that a range of cow’s milk hydrolysates, both from whey and casein, were able to modulate TLR signaling. The proteins or peptides responsible for the signaling were found to be formed during the hydrolysis process, since effects of hydrolysates were different than those of intact proteins. Effects depended on multiple characteristics of the hydrolysates. The effects were protein type dependent, since whey hydrolysates were found to exclusively induce TLR activation, while casein hydrolysates only induced inhibition of ligand activated TLR signaling. Furthermore, the actual production method of the hydrolysates is important, since TLR activation by whey hydrolysates depended on the degree of hydrolysis. TLR activation was highest in mildly hydrolyzed proteins, which are known to still contain larger proteins. When we studied TLR modulating effects of soy and wheat hydrolysates in the same manner (chapter 3), we also found both TLR activating and inhibiting effects. Again, hydrolysates containing the highest amount of larger proteins were the ones with most TLR activation effects. Therefore, hydrolysates from different protein sources were found to induce similar TLR activation effects, which seems to require the presence of larger proteins.
This observation is further studied in chapter 6 to identify the actual proteins structures responsible for TLR signaling. Here we fractionated a soy and whey hydrolysate with significant TLR activating effects, and tested the TLR activating properties of the obtained fractions separately. In this way we showed that, for both hydrolysates, a fraction containing proteins larger than 1000 kD was responsible for the TLR activating effects of hydrolysates. This fraction is thought to contain aggregated proteins, as no single intact whey or soy protein is this large [8,9]. During the hydrolysis process the proteins are heated, which is known to trigger denaturation and aggregation of whey and soy [10,11], despite structural differences between proteins. We demonstrate that these aggregated protein structures are responsible for the TLR activation that was observed for both soy and whey hydrolysates.

The identification of TLR modulating protein aggregates in hydrolysates is an interesting finding since research on immune modulating effects of hydrolysates mainly focusses on bioactive peptides present in hydrolysates. Standard hydrolysate analysis procedures are not taking these large structures into account, while our data clearly indicate that the aggregate content of hydrolysates contributes to the overall immune effects of hydrolysates. Therefore, investigating the aggregate formation during the hydrolyzation process should be taken into account in order to develop hydrolysates with optimized immune effects. TLR activation, as stimulated by the aggregates, is an effective way to prevent allergy [12].

Protection of epithelial barrier function by a soy hydrolysate
Epithelial cells are lining the entire lumen of the intestine, and come into close contact with dietary molecules in the lumen of the gut. Knowing that the epithelial barrier is more permeable in infants, and that too high permeability increases the risk of food allergy [13], we investigated in chapter 4 the effects of a range of soy hydrolysates on the epithelial barrier function in vitro. Preincubation with one of the tested soy hydrolysates was found to protect an epithelial cell layer from increased permeability induced by calcium ionophore A23187 but like with TLR signaling this was hydrolysate dependent. The protective effect was associated with increased claudin-1 and decreased claudin-2 expression by the soy hydrolysate. When we further investigated the underlying mechanism of this effect by comparing the protective effect of the soy hydrolysate on increased permeability induced by different disruptors, we concluded that the effect is mediated via PKC, and not via the MAPK pathway.

PKC is known to be involved in the regulation of tight junction expression, and therefore epithelial barrier permeability by dietary molecules and probiotics [14-16]. In addition to our data, this indicated that it is likely that the soy hydrolysate also works via this pathway, but we feel that a good follow up on the performed experiment would be the use of PKC inhibitors. This is a good strategy to confirm our results, since previous studies have successfully proven PKC mediated epithelial barrier effects in this way [16,17]. Another advantage of this method is that PKC inhibitors specific for certain PKC isoforms are available. Since different PKC isoforms can have opposite effects on epithelial barrier function [18], knowing which isoforms are involved in the observed effects is essential for a detailed understanding of the mechanism behind the protective effect of the soy hydrolysate in vitro.
Another aspect of the working mechanism of the soy hydrolysate that needs to be elucidated is the possible receptor(s) involved. So far, only TLR2 is known to protect epithelial barrier integrity in a direct way, via PKC [19]. However, the T84 cells in which the protective effect was observed, are relatively unresponsive to TLR2 ligands [20]. Therefore, other receptors are possibly involved. A wide screening of other receptors that could be involved in protective effects on the epithelial barrier integrity is needed. Another possible mechanism is via IL-10, which also has protective effects on the barrier function [21]. The only soy hydrolysate with barrier protective effect was also found to be the only IL-10 inducing soy hydrolysate (chapter 3).

A layer of epithelial cells attenuates hydrolysate aggregate activated dendritic cells (DCs)
To further study the effects of a TLR activating whey and soy hydrolysates on immune cells, we first quantified effects on cytokine production in hydrolysate stimulated DCs and epithelial cells separately (chapter 5). In DCs, cytokine production of IL-6, IL-12, and IL-10 and chemokines IL-8, MIP-1α, and MCP-1 was increased, indicating maturation and activation of the stimulated DCs. These effects were thought to be induced via TLR activation by aggregates in the hydrolysates, since in chapter 6 we observed that for both hydrolysates the fraction containing proteins larger than 1000 kD not only showed TLR activation in reporter cells used, but was also responsible for the induced effects in DCs in an NFκB dependent way. Epithelial cells only produced increased levels of IL-8 and MCP-1 after soy hydrolysate stimulation, but not after stimulation with whey hydrolysate.

In order to investigate the effect of the hydrolysates on the interaction between dendritic and epithelial cells, DCs, and epithelial cells were cocultured in a transwell system mimicking the situation in the intestine. A layer of confluent intestinal epithelial cells covered DCs cultured in the lower chamber of a transwell system, and hydrolysates were applied on the apical side of the epithelial layer. We found no induced cytokine production in this setup after hydrolysate stimulation. We concluded that the presence of the epithelial layer physically prevents the DCs to come into contact with the hydrolysates and thereby prevents the pronounced cytokine production by hydrolysate stimulated DCs. Since we concluded before that the hydrolysate effects in DCs are caused by aggregates (chapter 6), the fact that we did not see an increased cytokine production seems to be logical since these large structures are not able to pass the intact epithelial barrier. This has indeed been described for whey proteins [22]. The fact that soy hydrolysates even actively strengthen the epithelial barrier (chapter 4), would implicate that even less aggregates can pass the epithelial barrier.

In this coculture setup a confluent layer of intestinal epithelial cells (T84) was used to study effects of hydrolysates on immune cells. However, the epithelial barrier in newborn infants shows an increased permeability [13]. Therefore, an interesting option for further research would be to develop a coculture model in which the epithelial layer resembles the infant epithelial barrier more, and repeat the experiment. This could for example be done by using non-confluent intestinal epithelial cells, or by using a fetal intestinal epithelial cell line. With a more permeable epithelial barrier, more immune effects on cells in the lamina propria, for example DCs, might be induced.
Although the epithelial barrier does not provide uptake of aggregates of hydrolysates, Peyer’s patches located in the jejunum and ileum are known to take up protein aggregates [23], after which they can be sampled by DCs. Furthermore, some DCs extrude into the lumen of the intestine, mainly in the jejunum [24]. We hypothesize that by this sampling TLRs on DCs might get activated in a controlled way, leading to maturation and activation of these DCs. Matured, activated DCs are known to stimulate T cell differentiation [25], which could contribute to an hypoallergenic effect of hydrolysates when this dampens Th2 responses. In these studies we stimulated T cells with conditioned medium from hydrolysate stimulated DCs or cocultured DCs and IECs. However, this did not lead to skewing of T cell differentiation. We believe that physical contact between DCs and T cells might be needed to induce effects. In future studies this should be tested by incubating T cells with DCs which have been preincubated with hydrolysates. The effects of hydrolysates on T cell differentiation remains an important issue that should be studied in order to get a complete understanding of adaptive effects of hydrolysates.

Differences between soy and whey hydrolysates in resistance to digestive conditions
For the above described immune effects to occur, protein aggregates of the soy and whey hydrolysates should be present intact and in sufficient amounts in the intestine. The jejunum is thought to be an important site, since DCs extruding in the lumen and Peyer’s patches, which are immune organs in the intestinal wall taking up antigen, are present here [24,26]. Therefore, in chapter 6 we studied the digestion of soy and whey hydrolysates in the stomach and duodenum in an in vitro infant digestion model. Soy aggregates were found to resist both stomach and duodenum digestion, resulting in a preserved TLR activation capacity in the intestine. Whey aggregates were digested in the duodenum to such an extent that whey hydrolysates did not activate TLRs in the reporter cells anymore after digestion. Therefore, the above described TLR induced immune effects are, according to this digestion model, likely to occur after soy hydrolysate consumption, but not after consuming whey hydrolysate.

Soy protein has indeed a lower digestibility compared to whey [27], which is due to the different structures of whey and soy proteins. Legume proteins, including soy proteins, contain a relatively high number of β-sheets and cysteine, making them particularly resistant to proteolytic digestion [10]. However, the difference in degree of hydrolysis might also contribute to the difference in digestion between soy and whey hydrolysate. Since infant formulas containing soy hydrolysates are more and more used [28], knowledge about the soy hydrolysates, including their immune effects, is important.

From our in vitro data, it is clear that soy aggregates are able to resist digestion in the intestine, while aggregates in the whey hydrolysate completely disappeared in the intestine. However, since some studies described the presence of whey aggregates in the intestine of mice [23], it is doubted whether whey aggregates are always completely digested in vivo, as our data suggests. Whether these differences are due to differences in aggregate structure or digestion conditions remains to be elucidated.
Immune effects by a partially whey hydrolysate in a cow’s milk allergy mouse model

The performed *in vitro* studies were essential for unraveling effects of hydrolysates at a cellular level. To understand the effects of hydrolysates on the development of allergic reactions and underlying immune effects, *in vivo* studies are indispensable. However, although food allergy mouse models have been described in literature, effects of hydrolysates on immune cell populations involved in allergic reactions have hardly been studied [29]. In *chapter 7* we performed a study in which we, as we believe for the first time, show a stepwise analysis of immune cell populations in different immune organs in a cow’s milk allergy mouse model. Mice were orally sensitized either with a partial whey hydrolysate or with intact whey (positive control) using cholera toxin in order to break tolerance, and challenged intradermally to induce clinical symptoms, followed by an oral challenge, after which the animals were terminated.

Since most allergenic epitopes have been destroyed during partial hydrolysis of whey proteins, no sensitization against intact whey by the hydrolysate was expected. However, sensitization clearly occurred, as the animals showed increased whey-specific IgE levels, which were in some animals higher than whey-specific IgE levels in intact whey sensitized animals. We believe that this unexpected result can be explained by the presence of protein aggregates in the hydrolysate. Roth-Walter et al. describe that aggregated whey proteins are not passing the epithelial barrier in mice, which is corresponding to our *in vitro* data, but are more likely to be taken up into the Peyer’s patches compared to individual intact whey proteins [23]. This alternative route of uptake results in an increased immunogenicity of aggregated proteins, leading to increased IgE levels.

Although protein aggregation increases sensitization in animals, individual intact whey proteins were more capable of inducing allergic reactions compared to aggregates [23]. However, when hydrolysate and intact whey sensitized animals were challenged with the same amount of intact whey, no clinical symptoms were induced in hydrolysate sensitized animals, while whey sensitized animals showed clear anaphylactic shock symptoms. This made us question whether the hydrolysate could have some immunomodulatory effects resulting in a dampening of the clinical symptoms. Indeed, in the spleens of hydrolysate sensitized animals many differences in T cell and B cell subsets were observed between hydrolysate sensitized and whey sensitized animals, suggesting that the hydrolysate indeed modulated the immune system in a specific way. Increased Treg and Breg percentages in the spleen of hydrolysate sensitized animals were of particular interest due to their regulatory function, and this would be an interesting subject for further research. Whether the observed immune effects are also induced by the protein aggregates or by bioactive peptides in the hydrolysate also remains to be investigated.

In this study, we investigated the adaptive immune response, as we believed this is a good starting point in investigating immune effects of hydrolysates *in vivo*. However, the adaptive immune system is orchestrated by innate antigen presenting cells, like DCs. Therefore, and since we showed in *chapter 5* that hydrolysates can influence DC functions, it is expected that differences in DC functioning or distribution in hydrolysate sensitized mice are at the basis of the differences in the adaptive immune system. Therefore, it would contribute to our understanding of *in vivo* immune effects by hydrolysates to also investigate the amounts and activation markers of DC subsets in the animal model.
Application of a TLR2 inhibiting wheat hydrolysate in clinical nutrition

Next, we showed that immune effects of hydrolysates also have potential to be beneficial in nutritional products targeting adults with immune related problems. When screening the TLR modulating effects of hydrolysates from different origins, we observed a strong TLR2 inhibiting effect induced by a wheat hydrolysate used in medical nutrition. Since TLR2 signaling is known to be involved in intestinal inflammation, for example induced by chemotherapeutic drugs applied in cancer patients, we hypothesized that this hydrolysate could contribute to a dampening of the inflammation. As a first step, we further investigated the TLR2 inhibiting effects. The wheat hydrolysate was found to inhibit both TLR2/1 and TLR2/6, leading to the inhibition of HKLM induced IL-6 production in DCs, which is a cytokine known to be important in the intestinal inflammatory response. Further analysis of identified bioactive fractions of the hydrolysates lead to the identification of peptides which could possibly be responsible for the TLR2 inhibition. To get a complete understanding of the TLR2 inhibiting mechanism of the hydrolysate, the identified peptide candidates should be synthesized and tested for TLR2 inhibitory effects separately.

The next step would be testing the TLR2 inhibitory effect in vivo, in order to confirm whether the observed effects are indeed capable of reducing the mucositis and ileitis symptoms. TLR2 signaling is specifically important in doxorubicin induced mucositis [30]. Therefore, a mouse model could be used in which intestinal inflammation is induced by doxorubicin administration, as performed by Kaczmarek et al [30]. They already showed that a TLR2 specific inhibitor was able to reduce inflammatory symptoms, which makes this a good model.

The use of the obtained knowledge in nutritional product application and development

The observed immune and intestinal barrier effects of hydrolysates can have many beneficial effects, but might also have a negative impact when applied in the wrong situations. The knowledge gained in this thesis is to our opinion important for its application. The presence and identification of immune stimulating aggregates in the hydrolysates has crucial consequences in this. Since aggregation of proteins might increase the sensitization capacity, it is advisable to avoid aggregate containing formulas in infants at high risk of food allergy. It would be better to either apply extensively hydrolyzed proteins, which do not contain aggregates, or hydrolysates of which the aggregates are filtered out. The fact that a clinical study by Boyle et al. described that an unfiltered hydrolysate (which was applied in combination with a prebiotic) did not prevent eczema in high-risk children, although some regulatory immune effects were induced [31], might also be an indication that the presence of aggregates can promote sensitization.

In infants who have just developed an allergic reaction, aggregate containing hydrolysates could be beneficial. Allergic symptoms might then be reduced by dampening the allergic response via TLR signaling induced by protein aggregates. One point of attention in this is that the aggregate containing hydrolysate given to an allergic infant shouldn’t contain other proteins that can trigger an allergic response. This means that in the case of a sensitization against cow’s milk allergy, which is most frequent, we believe a soy hydrolysate containing aggregates should be provided, and is preferred above an extensively hydrolyzed cow’s milk hydrolysate which does not contain aggregates. Furthermore, this soy hydrolysate may have the additional benefit of decreasing the epithelial barrier permeability, which is also associated with reduced allergic
symptoms. However, before the suggested application strategy, which is based on our in vitro data, can be used in allergic infants, in vivo studies should be performed to confirm our findings. Our data suggest that hydrolysates could also be applied at a broader scale in adults. Humans with an increased epithelial permeability such as patients with IBS, some forms of IBD and TLR dependent intestinal disorders could benefit from consuming hydrolysates with protective effects on intestinal barrier function, since an impaired barrier function negatively affects many health aspects, including metabolic and mental functions [32,33]. This is often observed in children and elderly, so they might form specific treatment groups [34]. In people with more severe intestinal disorders, like inflammation due to TLR2 signaling, wheat hydrolysates might help dampen this response. Overall our data suggest that hydrolysates have many beneficial properties that have not been used to their full extend. Applying our technology platform with reporter cell lines, complex culture systems in the presence and absence of simulated digestion systems allows identification of hydrolysates with specific immunomodulatory properties. This should now be followed up by a rational design for testing in specific target groups and could lead to more efficacious treatment of intestinal disorders and allergies.

Conclusion and future perspective
In this thesis, we determined the relation between hydrolysis level, protein source and immune effects by screening immune effects of a range of hydrolysates. Soy, whey and wheat hydrolysates had an effect on dendritic and epithelial cell functioning via TLR modulation, but all in a hydrolysate specific way. A whey hydrolysate was also found to modulate adaptive immune responses in vivo. For all hydrolysates, the responsible fractions in the peptide composition were identified.

For an optimal use of immune modulating hydrolysates in nutritional products, a standardized, high throughput method is needed to identify which hydrolysates could have positive immune effects in specific target groups. Based on this thesis, we propose a cell- based assay to do this. In this way, although additional studies in animals and ultimately humans are needed, hydrolysates can be identified for the use in tailored nutritional products with targeted immunomodulatory effects, for example hypoallergenic infant formulas or anti- inflammatory clinical nutrition. Ultimately, identifying and pinpointing specific beneficial effects of hydrolysates to biomedical phenomena such as barrier function improvement or stimulation of specific immune processes, will allow tailoring or selection of novel effective hydrolysate formulations with a high value for innovative hydrolysates. Furthermore, it will allow industry to develop new products to provide solutions for intestinal disease and allergy.
References


