Immunomodulatory properties of protein hydrolysates
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Identification of an immunomodulating protein fraction in soy and whey hydrolysates and its resistance to digestion in an infant gastrointestinal model system

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Abstract
Hydrolysates have been found to possess immune modulating effects. For an optimal utilization of these products in for example hypoallergenic infant formulas, the working mechanisms and responsible proteins underlying the observed effects should be elucidated. In this study, the immunomodulating activity of a whey and soy hydrolysate was studied by quantifying TLR activation and assessing cytokine production of hydrolysate stimulated dendritic cells. The responsible bioactive fraction was identified and further characterized by gel electrophoresis. To determine whether the immune modulating effects are maintained under gastrointestinal conditions, hydrolysates were digested in an in vitro infant digestion assay, after which the protein content and TLR activating capacity of the digests were analyzed. Both in soy and whey hydrolysates TLR activation and cytokine production in dendritic cells was found to be induced by a fraction of proteins which contain protein aggregates larger than 1000 kD, which were formed by electrostatic interactions and disulfide bonds. Both aggregates of soy and whey proteins resisted stomach digestion, while only soy aggregates stayed intact during duodenal digestion, and maintained TLR activating capacity. This knowledge is important for a better understanding of the effects of hydrolysates, which could lead to a more tailored use of hydrolysates in infant formulas, for example for allergy prone infants.
Introduction

Cow’s milk allergy is the most common food allergy in newborns, with a prevalence of 2-3% [1], but allergies against alternative proteins such as soy can also occur [2]. Hydrolyzation of the cow’s milk and soy proteins is often used to prevent the onset of allergic symptoms [3]. The hypoallergenic properties of hydrolyzed proteins are caused by destruction of allergenic epitopes during the hydrolysis process [4], but also by creation of novel proteins and peptides with immune regulating properties [5,6,7]. How these proteins and peptides modulate the immune system and whether these properties are maintained during digestion in the infants gastrointestinal track is still largely unknown.

Our group and others recently showed using in vitro model systems that hydrolysates induce immunomodulatory effects via Toll-like receptors (TLRs) [8,9]. TLRs are a family of pathogen recognition receptors which are expressed on many cell types, including dendritic cells, lymphocytes and epithelial cells [10-12]. TLRs sample the lumen of the intestine, which has been found to be important in the mucosal immune regulation [13-15]. Due to the complex protein composition of hydrolysates, it remains to be identified which proteins and/or peptides are sampled by TLRs. This identification of responsible proteins or peptides could contribute to a better understanding of the working mechanisms of hydrolysates and are instrumental in the design of hydrolysates with predictable and reproducible health effects [16].

Most studies addressing interactions of food components with intestinal immune cells apply ingredients without pre-exposure to the hazardous stomach circumstances [17,18]. It is therefore unknown whether bioactive food such as hydrolysates maintain their immunomodulatory properties after digestion in the stomach. In order to be able to interact with immune cells, for example with small-intestine located TLR expressing dendritic cells [19], the proteins and peptides in hydrolysates should be able to resist the low pH and digestive enzymes in the stomach and the beginning of the small intestine. It is recognized that digestion in the infant’s stomach is suboptimal, because of the lower enzymatic activity and higher pH in the infant stomach compared to adults [20,21]. Therefore, digestion models mimicking these specific conditions should be used when studying infant digestion.

In the present study, we investigated the immunomodulating effects of a whey and soy hydrolysate by quantifying TLR activation. The fraction responsible for the TLR activation was identified by size based fractionation of the hydrolysates. This fraction was further characterized using Native and SDS-PAGE. To study whether the protein fraction stayed bioactive during digestion, hydrolysates were digested in an in vitro infant digestion assay mimicking the stomach and duodenum, after which the protein content and TLR activating capacity of the digests were analyzed.

Materials and methods

Tested materials

Soy and whey protein hydrolysates and their source materials (soy isolate and whey protein concentrate) were kindly provided by FrieslandCampina (Amersfoort, the Netherlands). The hydrolysates were produced by a two-step digestion of the source materials. The peptide composition of the hydrolysates was characterized with RP-UHPLC [22]. Separations were
performed on a Hypersil GOLD C18 analytical column. Elution was performed at a flow rate of 0.8 mL/min. The gradient elution was carried out with a mixture of 0.1% trifluoroacetic acid (TFA) and 1% acetonitrile (ACN) in H₂O and 0.1% TFA and 90% ACN in H₂O. All samples were tested for endotoxins by using the Limulus amebocyte lysate assay (LAL) according to the manufacturer’s instructions (ThermoFisher Scientific, Waltham, US). Endotoxin concentrations in all samples had no significant activating effects on the applied cells.

Fractioning of hydrolysates
The hydrolysates were fractionated based on size using 2 mL Amicon centrifugal filters with molecular weight cut-offs (MWCOs) of 10 kD, 100 kD, and 1000 kD (Merck, NJ, USA). Hydrolysates were dissolved in 5 mL sterile PBS at a concentration of 20 mg/mL by stirring the solution for 15 min at room temperature (RT). Before filtering, samples were centrifuged at 2000g for 10 min. in order to remove undissolved particles that could block the centrifugal filters. A sample of 1 mL of the supernatant was stored (and used in the cell assay as total hydrolysate), the remaining supernatant was used for filtering. Filtration was performed following the manufacturer’s instructions. Briefly, hydrolysates were added to the filter tubes, and centrifuged at 4000g for 10 min at RT. Then, the retentate on the filter was mixed by pipetting it up and down to prevent blocking of the filter, and the tubes were again centrifuged at 4000g for 5 min at RT. This last step was repeated. The permeate was collected and 2 mL of PBS was added to the retentate which was remaining on the filter of the tube. The retentate was washed by two repetitions of centrifugation of 5 min at 4000g. Then, the retentate was collected by inverting the centrifugal filter tubes, followed by short centrifugation.

Protein concentration of the hydrolysate fractions obtained were determined using Pierce BCA protein assay, following the manufacturer’s instructions (Thermoscientific, Waltham, US). When fractions were tested in THP-1 reporter cells, the protein concentrations for complete samples and fractions were equalized.

THP-1 reporter cell assay
To test the TLR activating capacity, we used a THP-1-XBlue™-MD2-CD14 (Invivogen, Toulouse, France) reporter cell assay. These cells endogenously express TLRs. To quantify TLR activation, the cell line contained a construct for Secreted Embryonic Alkaline Phosphatase (SEAP), which was coupled to the nuclear factor kB/Activating protein-1 (NF-κB/AP-1) promoter. NF-κB/AP-1 is a known downstream target of TLR receptors [23,24].

Cells were cultured as described before [23]. The cells were seeded in a flat bottom 96 wells plate at a concentration of 100,000 cells/well (100 μL/well), and stimulated with total hydrolysates or fractions for 24 hours (37 °C, 95% oxygen, 5% CO₂) or with 10 ng/mL LPS as a positive control. Hydrolysates alone were tested at a concentration of 2 mg/mL. Medium was used as a negative control. After incubation, Quanti-Blue detection medium was used to analyze the cell supernatant as described before [23]. Absorbance (650 nm) was measured using a VersaMax microplate reader (Molecular Devices GmbH, Biberach an der Riss, Germany) and SoftMax Pro Data Acquisition & Analysis Software to determine SEAP activity, which represents activation of NF-κB/AP-1.
**Stimulation of dendritic cells (DCs)**

DCs were purchased from MatTek Corporation (Ashland, MA, USA). DCs were generated from umbilical cord blood CD34+ progenitor cells (hematopoietic stem cells). DCs were thawed and cultured according to the manufacturer’s instructions.

Stimulations were performed by seeding 6x10⁴/well freshly thawed DCs in a 96 wells plate (in 200 μL). Cells were precultured for 24 hours before starting the experiment as described in the manufacturer’s instructions. Then, cells were exposed to hydrolysates or its fractions for 24 hours and supernatant was collected and stored at -80 °C for cytokine measurements.

In order to confirm NF-κB dependency, which is a known downstream target of TLR receptors, the experiment was repeated in the presence and absence of 10 μM of the NF-κB inhibitor celastrol (Invivogen, Toulouse, France). Cells were preincubated with celastrol for 30 min before samples were added.

**Assessment of cytokine expression**

The quantity of IL-1β, IL-1RA, IL-10, IL-12, IL-6, IL-8, MCP-1, MIP-1α, RANTES, TNFα, and TSLP in the DC supernatant were measured using a custom made ProcartaPlex® multiplex immunoassay (Affymetrix, CA, USA). The immunoassay was performed according to the manufacturer’s protocol. Briefly, cytokine standards were resuspended, and serial dilutions were prepared. Antibody magnetic bead mix was added to the plate. After washing, standards and samples were added (50 μL/well), the plate was sealed, and incubated while shaking (30 min at RT, overnight at 4 °C, and again 30 min at RT). After washing the plate twice, detection antibodies were added (25 μL/well) and the plate was incubated for 30 min at RT on a plate shaker. After incubation, the plate was washed twice and 50 μL/well streptavidin-phycoerythrin was added. Again, the plate was incubated at RT for 30 min while shaking. To prepare the plate for analysis, the plate was washed, and 120 μL/well of reading buffer was added. After shaking the plate for 5 min at RT fluorescence was measured using a Luminex 100 System. The data obtained were analyzed using StarStation software.

**Polyacrylamide gel electrophoresis (PAGE)**

We studied the involvement of charge interactions in the buildup of the proteins by comparing the Native PAGE result with an Sodium Dodecyl Sulfate (SDS)-PAGE gel in which a uniform charge density was established with SDS. In the other experiment, we performed an SDS-PAGE in the presence of DTT, which is known to break sulfide bonds.

Under all conditions, gel electrophoresis was performed using precast 4-20% criterion TGX stain-free gels (Biorad, CA, USA). All samples were loaded at a concentration of 1 mg/mL, after being heated for 3 min at 95 °C. After centrifugation, 10 μL of each sample was loaded on the gel. First, a voltage of 100V was applied for 10 min. Then, the voltage was increased to 150V which was applied for 50 min. Proteins in the gel were visualized by exposing the gel to UV-light and pictures were taken using a Chemidoc MP Imaging system (Biorad, CA, USA).

For the Native PAGE, samples were mixed with 50 μL Native Sample Buffer 2x (Biorad, CA, USA) and added up to 100 μL with PBS. Tris/Glycine buffer (Biorad, CA, USA) was used as running buffer. SDS-PAGE was performed for hydrolysates and protein sources as well, both in the absence and
presence of DTT. For the SDS-PAGE without DTT, samples were mixed with 25 μL Laemmli sample buffer 4x (Biorad, CA, USA), and made up to 100 μL with PBS. Tris/Glycine/SDS buffer was used as a running buffer. In the SDS-PAGE containing DTT, the hydrolysates were mixed with 25 μL Laemmli sample buffer 4x and 5 μL 1M Dithiothreitol (DTT), and added up to 100 μL with PBS. The running buffer was also Tris/Glycine/SDS buffer. In the SDS-PAGE Unstained Protein Standard (Biorad, CA, USA) was included. A 5 μL protein standard sample was loaded on the gel.

**Infant in vitro digestion**

The digestibility of hydrolysates was tested in an *in vitro* infant digestion model [25]. Stomach digestion was performed for 40 min, since this was found to be representative for gastric half-emptying time after human milk and infant formula consumption [20]. This model consisted of a compartment mimicking the enzyme activities in the stomach and the duodenum. The stomach juice consisted of sterile H₂O containing 175 mg/L NaCl, 1100 mg/L KCl, 110 mg/L CaCl₂, 62 U/mg protein pepsin and 15 U/mg protein lipase. The solution contained 18 mM HCl, resulting in a pH of approximately 2.0. Duodenal juice contained 1250 mg/L NaCl, 150 mg/L KCl, 55 mg/L CaCl₂, and 80 mg/g protein pancreatin in sterile H₂O supplemented with 1 mM of the bile salts sodium taurocholate and sodium glycodeoxycholate. Before use the pH of the intestinal juice was set at 6.8 using 0.1 M NaHCO₃.

At the start of the experiment, hydrolysates were dissolved in 70 mL sterile H₂O at a concentration of 1.2 % protein by stirring at RT for 15 min. Then, 15 min before the start of the digestion, the hydrolysates were preheated in at 37 °C in a water bath. At the start of the digestion, 50 mL of the preheated hydrolysate was transferred to a Scott bottle in a 37 °C water bath, while the rest of the sample was stored for further analysis (see below) (sample 0). Then, 25 mL preheated stomach juice was added to the Scott bottle. The whole digestion was performed in a 37 °C water bath under continuous stirring. Samples were taken at the start of the stomach digestion (sample 1), after 20 min (sample 2) and after 40 min (sample 3) and stored for further analysis (see below). After 40 min of incubation, the pH of the stomach juice was adjusted to 6.7 with 1M NaHCO₃, after which 47 mL preheated intestinal juice was added. Again, samples were taken at the start of the intestinal digestion (sample 4), after 60 min (sample 5) and after 120 min (sample 6). As a negative control, the digestion was performed with PBS instead of the protein sample. The digestion was performed twice.

**Storage of digestion samples**

Three different samples were taken for further analysis at the 7 mentioned time points. A sample of 400 μL of the digestion products was snapfrozen and stored at -20 °C for testing of TLR activating properties in THP-1 reporter cells as described above. Digestion products were tested undiluted. The response was corrected for the effects of the stomach and intestinal juice by also testing the digestion products of the control, PBS digestion. Another 400 μL of the digestion products was snapfrozen for the purpose of running a Native PAGE, which was performed as described above. Lastly, 400 μL of the digestion samples was added to 400 μL 0.46 M TCA and stored at -20 °C for o-phthaldialdehyde (OPA) analysis (see below).
Analysis of digestion using the OPA method
To assess the level of digestion of the hydrolysates, OPA analysis was performed to identify the amount of amino groups present in the digestion samples collected at different time points during digestion. After thawing, samples were first centrifuged at 14,000g for 10 min. L-Leucine was used as a standard, and a stock of 10 mM L-Leucine was used to prepare a serial dilution in H₂O (0, 1, 2, 3, 4, and 5 mMol/mL). A sample of 400 μL 0.46 M TCA was added to 400 μL of the standards, which were then incubated at 37 °C for 30 min.

To start the reaction, 200 μL of standard or sample (samples were 10x diluted in borate buffer) was added to a 15 mL tube, together with 2.5 mL 0.15M borate buffer pH 9.5, 50 μL 50 mM N-acetyl cysteine solution and 50 μL 50 mM OPA. The reaction mix was incubated for 15 min at RT, after which the absorbance was measured at 340 nm using an Epoch 2 spectrophotometer (Biotek instruments, Winooski, USA). Standard curve results were used to create a linear standard curve, which was used to calculate the amount of amino groups in the digested hydrolysates. Results were expressed as μmol/g protein. The results shown were corrected using the control, PBS digestion values.

Statistical analysis
Statistical analysis was performed using Graphpad Prism 6. Normal distribution of the data was tested using the Kolmogorov-Smirnov test. When data were normally distributed, values were expressed as mean ± SD. ANOVA followed by a Tukey's multiple comparison test was used to identify individual differences. To test for significant effects of celastrol on DC cytokine production paired t-tests were done. For data which were not normally distributed, median ± range was shown. The Kruskal-Wallis test followed by a Dunn's test was used to show individual differences. A p-value of <0.05 was considered to indicate a significant difference.

Results
Characteristics of soy and whey hydrolysates
The soy and whey protein hydrolysate were obtained by a two-step hydrolysis of soy isolate and whey protein concentrate, respectively. Peptide patterns determined by reversed phased chromatography and molecular weight distributions are shown in figure 1. Both hydrolysates are mainly composed of peptides, but also contain a fraction of larger (>10 kD), possibly intact, proteins. The soy hydrolysate tested was hydrolyzed more extensively compared to the whey hydrolysate, and therefore contains a larger fraction of small peptides (<500 Da).
Figure 1. Characterization of soy and whey hydrolysate. Peptide and amino acid patterns were obtained with by RP-UHPLC (A), and molecular weight distribution was calculated (B). Both hydrolysates are mainly composed of peptides, but also contain a fraction of proteins larger than 10 kD. The soy hydrolysate tested was hydrolyzed more extensively compared to the whey hydrolysate.

Both soy and whey hydrolysate induce TLR activation

TLR activation has been recognized as a mechanism for cell stimulation by hydrolysates in the intestine [8]. In order to test whether soy and whey hydrolysates are also able to induce TLR activation, samples were tested on a THP-1 reporter cell line.

Both soy and whey hydrolysate showed a significant activating effect on TLRs in the THP-1 cell line compared to unstimulated cells (both $p<0.05$) (figure 2).
A fraction of proteins larger than 1000 kD is responsible for the observed TLR activation induced by soy and whey hydrolysates

In order to identify protein fractions which are responsible for the observed TLR activation induced by soy and whey hydrolysates, we fractionated the hydrolysates based on size using filters with MWCO's of 10 kD, 100 kD, and 1000 kD. Each fraction was subsequently tested for TLR activating capacities in the THP-1 reporter cell line.

Both for the soy and whey hydrolysate, the total hydrolysate sample significantly increased TLR activation compared to the negative control (figure 3). Furthermore, when hydrolysates were fractionated using a 10 kD filter, the retentate of both hydrolysates significantly enhanced the TLR activating capacity, suggesting that the proteins larger than 10 kD ($p<0.05$) are responsible for this effect. By using a 100 kD and a 1000 kD filter, we subsequently demonstrated that TLR activation is only detected in the fractions containing proteins larger than 100 kD and 1000 kD respectively (figure 3).
Figure 3. NF-κB/AP-1 activation in a THP-1-MD2-CD14 reporter cell line after stimulation with soy and whey hydrolysate fractions. The fractions containing protein larger than 1000 kD were found to be responsible for the TLR activating effects of the hydrolysates. Statistical significant differences compared to the negative control were determined by using the Kruskal-Wallis test followed by the Dunn's test and indicated by *.
The >1000 kD fraction induced cytokine production in dendritic cells similar to the complete soy and whey hydrolysate

Activation of TLRs is known to induce cell activation and cytokine production in dendritic cells in the intestine [13]. We therefore next tested whether the hydrolysates induced cytokine production in DCs. Since the >1000 kD fraction of both hydrolysates also induced TLR activation, we also tested this fraction.

After stimulation with 2 mg/mL soy hydrolysate, the cytokines IL-1β, IL-12, IL-8, TNFα, IL-10, IL-1RA, IL-6, MCP-1, MIP-1α, RANTES, and TSLP were significantly increased compared to unstimulated cells (figure 4). When DCs were stimulated with the >1000 kD fraction, the production of the same cytokines was significantly increased (all \( p < 0.05 \)), except for IL-1RA. After 24 hours of stimulation with the whey hydrolysate, the dendritic cells produced higher amounts of IL-12, IL-8, IL-10, MCP-1, MIP-1α, and RANTES (all \( p < 0.05 \)). The fraction of this hydrolysate containing proteins larger than 1000 kD was responsible for enhanced dendritic production of IL-12, IL-8, IL-1RA, and MCP-1 (all \( p < 0.05 \)) but not of IL-1β, TSLP, and TNFα.

The effects of soy and whey hydrolysates and their large protein fractions were found to be NF-κB dependent, since the NF-κB inhibitor celastrol significantly reduced cytokine production induced by the hydrolysates and/or their fractions (figure 5).
Figure 4. Cytokine production by DCs after stimulation with soy and whey hydrolysates or their >1000 kD protein fraction. After stimulation with 2 mg/mL soy hydrolysate, the cytokines IL-1β, IL-12, IL-8, TNFα, IL-10, IL-1RA, IL-6, MCP-1, MIP-1α, RANTES, and TSLP were statistically significantly increased. After 24 hours of stimulation with the whey hydrolysate, the dendritic cells produced higher amounts of IL-12, IL-8, IL-10, IL-1RA, IL-6, MCP-1, MIP-1α, and RANTES. Similar effects were observed when DCs were stimulated with soy and whey fractions. Statistically significant differences compared to the negative control were determined by using ANOVA followed by Tukey’s post test and indicated by *. 
Figure 5. The NF-κB inhibitor celastrol was used to test the NF-κB dependency of the observed effects of hydrolysate (fractions) in dendritic cells. The NF-κB dependency of the observed cytokine production by hydrolysates and their bioactive fractions was tested by pretreating dendritic cells with the NF-κB inhibitor celastrol before they were treated with hydrolysates or their fractions. Celastrol inhibited the hydrolysate (fraction) induced cytokine production of all cytokines measured. Three representative graphs were shown (IL-6, TNFα and MIP-1α). Statistically significant differences between cells treated and not treated with celastrol were determined by using paired t-tests and indicated by *.

Both SDS and DTT lead to the breakdown of the large protein fraction in the soy and whey hydrolysates

We studied the involvement of charge interactions in the buildup of the large protein hydrolysate proteins in intact soy isolate and intact whey protein and their hydrolysates by comparing the Native PAGE result with an SDS-PAGE gel in which a uniform charge density was established with SDS. To study the presence of disulfide bonds, we used SDS-PAGE in the presence of DTT, which is known to break disulfide bonds.

First, we ran a Native PAGE gel with intact soy isolate and intact whey protein and their hydrolysates (left gel in figure 6). As shown in figure 6, it was found that more of the large protein fraction (arrow on the left side of the gel) was present in the whey hydrolysate compared to the soy hydrolysate. Also, this large protein band of the whey hydrolysate is thicker compared to the band of the intact whey, while the large protein band of the soy hydrolysate is smaller compared to the band of its source material.

Electrostatic interactions were involved in the large protein fraction (figure 6, middle gel) as treatment of the hydrolysates and source materials with SDS reduced the thickness of the bands representing this large protein fraction. Also disulfide bridges were involved as when DTT was used together with SDS (right gel), the specific bands containing the large protein fraction almost completely disappeared.

Finally, while the protein band containing the large protein disappeared after SDS and DTT treatment of whey hydrolysate, two specific bands of 18 kD and 14 kD, corresponding to the two main whey proteins β-lactoglobulin and α-lactalbumin, appeared in the gel [26,27].
Figure 6. Gels of Native PAGE, SDS-PAGE, and SDS-PAGE with DTT to assess the role of electrostatic forces and disulfide bridges in the protein make up. Native gel (left), SDS-PAGE (middle), and SDS-PAGE with DTT (right) were all loaded with from left to right: soy hydrolysate (soy hydr.), soy isolate (soy prot.), whey hydrolysate (whey hydr.) and whey protein concentrate (whey prot.). Electrostatic interactions were involved as treatment of the hydrolysates and source materials with SDS reduced the size of the bands representing the large protein fraction (indicated by arrows left). Also, disulfide bridges were involved as when DTT was used together with SDS, the specific bands containing the large protein fraction disappeared. Arrows on the right show the appearance of bands representing intact whey proteins when the large protein fraction of whey hydrolysates disappeared.

The large protein fraction in the soy hydrolysates resist digestion, while the large protein fraction of the whey hydrolysate is digested in the intestine

Next, we investigated whether the large protein fraction stayed intact during digestion.

Figure 7A shows Native gels for the digestion products of PBS (negative control), soy hydrolysate and whey hydrolysate at different time points. The bands present in the gel showing the PBS controls (see arrows left) contain bands of the enzymes applied or their breakdown products (left, figure 7A). For the soy hydrolysate, the protein bands representing the large proteins in the hydrolysate (see arrow right) do not significantly change during the stomach or
duodenum digestive circumstances (middle, figure 7A). This was different with whey hydrolysate (right, figure 7A). The whey protein fraction resisted the stomach digestive circumstances but readily disappeared from the digest simulating the duodenum.

OPA analysis was applied as well to study the level of digestion of the hydrolysates under stomach or duodenum circumstances. This OPA analysis showed no digestion in the stomach phase, but an immediately and extensively digestion of the whey hydrolysate when added to the duodenal circumstances (figure 7B). The digestion of soy also started under duodenum circumstances, but to a much lesser extent compared to the whey hydrolysate.

Figure 7. Native PAGE gel and OPA analysis of digestion products at different time point during simulated infants stomach and duodenal digestion. Figure A shows a Native PAGE of digestion products. PBS control indicates bands representing the digestive enzymes used in stomach and duodenal digestion (arrows left). Large proteins in the soy hydrolysate stayed intact, both in the stomach and duodenal digestion phase (arrow left). Large proteins in the whey hydrolysates also stayed intact in the stomach, but started to degrade at the start of the duodenal phase (4), and totally disappeared halfway the duodenal digestion (5). OPA analysis also showed minor digestion of the soy hydrolysate, while the digestion level of whey hydrolysate increased at the beginning of the duodenal digestion phase (6). Numbers in the graphs correspond with sample numbers in the gel.
Digested soy hydrolysates activate TLRs, while digested whey hydrolysates did not

Finally, we tested whether the digested soy and whey hydrolysates maintained their TLR activating capacity during digestion by testing digested samples with appropriate controls on THP-1 reporter cells as described above.

TLR activation was significantly increased in THP-1 reporter cell lines stimulated with undigested soy hydrolysate (sample 0), and digested soy hydrolysate samples 1, 3, and 4 (figure 8). TLR activation was also increased by Soy hydrolysate sample 6, although not significantly. Furthermore, undigested whey hydrolysate (sample 0) and whey hydrolysate samples 1 and 3 significantly increased TLR activation, while whey hydrolysate sample 4 and 6 had no effect.

**Figure 8. NF-κB/AP-1 activation in a THP-1-MD2-CD14 reporter cell line after stimulation with soy and whey hydrolysate digestion products.** The digestion products of the stomach digestion of both the soy and whey hydrolysate induced TLR signaling after stimulation of THP-1 cells for 24 hours. Digestion samples 1, 3, 4, and 6 of the soy hydrolysate still showed TLR activation (although not significant for sample 6), while digestion samples 4 and 6 of the whey hydrolysate did not. Statistical significant differences compared to the negative control were determined by using the Kruskal-Wallis test followed by the Dunn’s test and indicated by *.

Discussion

Hydrolysates have been found to possess immune modulating effects such as a contribution to generation of Treg and Breg cells [28]. For an optimal utilization of these beneficial effects in for example hypoallergenic infant formulas, the working mechanisms and responsible proteins or peptides underlying the observed effects should be elucidated. Therefore, in this study the immunomodulatory effects of a whey and soy hydrolysate were tested, after which we identified and further characterized the bioactive fraction. Lastly, we assessed the fate of this fraction in an infant in vitro digestion model, in order to determine whether the bioactivity is maintained under passage through the stomach and duodenum. Both in soy and whey hydrolysates TLR activation and cytokine production in DCs was found to be induced by the complete hydrolysate as well as the fraction of proteins larger than 1000 kD, which are formed by both electrostatic interactions and disulfide bonds. Both >1000 kD proteins of soy and whey proteins resisted
stomach digestion, while whey proteins were readily digested in the duodenum, resulting in the loss of the TLR activating capacity.

We identified a bioactive protein fraction in soy and whey hydrolysates containing proteins with a size larger than 1000 kD (figure 3). Since intact whey and soy proteins are smaller than 1000 kD [27,29], we concluded that hydrolysates must contain protein aggregates. Heating of whey and soy proteins is known to induce aggregate formation [30,31]. This is probably the step responsible for generation of the immune modulating fraction in whey. Heating is already applied in the production of the source material (in which we also detected an aggregate fraction) and again used in preparation of the whey hydrolysate, where the aggregate amount was even higher than in the source material. In intact soy protein source material we also observed aggregates, but surprisingly the amount was lower in the soy hydrolysate despite application of heating. One explanation for this could be the pertinent structural differences between soy and whey proteins [30,32], that make that soy proteins form less aggregates than whey during a second heating step. Another reason could be the higher degree of hydrolysis of the soy hydrolysate compared to the whey hydrolysate, since this means that the soy hydrolysate contains less large, intact proteins which probably form the aggregates.

When studying the characteristics of the aggregates in more detail, it was found that both electrostatic forces and disulfide bonds were involved in the aggregate formation in both soy and whey hydrolysates, which corroborates findings of Havea et al. [33,34]. Heat induced protein denaturation is the first step towards protein aggregation [35]. During heat denaturation of the proteins, hydrophobic regions and thiol groups, which are normally enclosed in the folded protein, are exposed [36,37]. This leads to both non-covalent bonds and stronger covalent disulfide bonds between denatured protein, resulting in polydisperse aggregate formation [38,39]. These aggregates were predominantly formed from intact whey proteins, since bands corresponding to β-lactoglobulin and α-lactalbumin appeared in the gel when aggregates were treated with SDS and DTT (right arrows, figure 6).

When digesting the bioactive aggregate containing hydrolysates in an infant in vitro digestion model, both soy and whey aggregates were found to resist stomach conditions (figure 7), as was observed before [25,40]. Soy aggregates also resisted the duodenal digestion, but whey aggregates disappeared quickly in the duodenal phase. This corresponded with a fast increase in whey hydrolysate digestion in the beginning of the duodenal digestion as confirmed by OPA analysis (figure 7B). This was not observed for the soy hydrolysate, which had already a high OPA value in line with the more hydrolyzed nature of the material. These results suggest that soy and whey aggregates differ, and therefore suggest that digestibility and bioavailability should be assessed for each hydrolysate individually.

TLR activating capacities were preserved up to 2 hours of duodenal soy hydrolysate digestion (figure 8), indicating that immune effects can be induced by soy aggregates in the subsequent part of the small intestine. Since we showed in vitro that cytokine production in DCs (including IL-6, IL-12, IL-10 and chemokines IL-8, MIP-1α, and MCP-1) was induced by the hydrolysate fraction with protein bigger than 1000 kD (i.e. the fraction containing the aggregates (figure 4)), we hypothesize that DCs in the jejunum and ileum can be stimulated by aggregates in a similar way. One DC type that might be affected is the conventional DC1, since these cells are present in
high numbers in the lamina propria of the jejunum of infants [41]. This is a specific dendritic cell subtype migrating to the mesenteric lymph nodes [42], where it is specialized in Th1 polarization [43]. Furthermore, in these parts of the intestine Peyer’s patches are present [44], where mucosal immune functioning is regulated by sampling of luminal molecules by M-cells and dendritic cells, which then further orchestrate adaptive immune effects [45]. By increasing levels of the mentioned cytokines and chemokines in these intestinal DCs [46,47], Th17 and Th1 responses might be triggered [48,49]. Enhanced Th1 attenuates allergy associated Th2 responses [50,51]. Therefore, stimulation of these cells might be a mechanism to induce Th1 polarization and avoid too strong allergy associated Th2 responses.

In summary, this study shows that both soy and whey hydrolysates contain immunomodulating aggregates of 1000 kD or larger. However, only the soy aggregates were found to resist stomach and duodenal digestion, and are therefore expected to stimulate DC activation and cytokine production via TLR signaling in the small intestine in vivo. This knowledge is important for a better understanding of the hypoallergic effects of hydrolysates. Whether soy aggregate-induced Th1 response is a mechanism by which aggregates in hydrolysates contribute to the dampening of a Th2 skewed allergic reactions should be subject of future studies.
References


