ImmunoModulatory properties of protein hydrolysates
Kiewiet, Mensiena Berentje Geertje

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Soy and wheat hydrolysates possess Toll-like receptor activating and inhibiting properties contributing to their immunomodulatory effects

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Abstract

Hydrolysates have been found to possess a range of immunomodulatory effects. TLR signaling has been identified as an underlying mechanism and was shown to effectively alleviate allergy symptoms. TLR signaling by soy and wheat hydrolysates has never been studied. In this study, the activating and inhibiting effects of six soy hydrolysates and three wheat hydrolysates on TLR2, 3, 4, 5, 7, 8, and 9 was investigated. We also studied effects of hydrolysates on cytokine production of primary peripheral blood mononuclear cells. It was found that two of the soy and two of the wheat hydrolysates could induce TLR activation while TLR inhibiting effects were observed in other, both soy and wheat derived hydrolysates. The effects were highly hydrolysate dependent. The hydrolysates with the strongest TLR activation, were found to enhance production of TNFα and IL-10 in PBMCs. Overall, it was shown for the first time that specific soy and wheat hydrolysates were able to modulate TLR signaling. This knowledge contributes to a better understanding of the possible effects of hydrolysates and gives insights for the design of intelligent hydrolysate formulations for allergy management.
**Introduction**

Due to changed life style factors the prevalence of allergic diseases has increased over the last centuries in industrialized countries [1], and has been recognized as an important health issue [2]. For example, food allergy now likely affects 5% of the adults in Europe [3], while 11% suffer from allergic rhinitis [4]. Due to novel insights in underlying immune responses new treatment options to control or eventually prevent allergic symptoms have become available [5]. Among these are strategies to dampen Th2 response, which is crucial in allergic responses [6].

One promising treatment option is the dampening of the Th2 response via Toll-like receptors (TLRs) [5]. TLRs are a family of pathogen recognition receptors (PRRs) that serve as sensors for the immune system. PRRs are responsible for modulation of the intestinal immune response by interacting with pathogen and food associated molecular patterns in the lumen [7]. TLR signaling is known to induce Th1 cells, which counteract the Th2 response, leading to attenuation of allergic responses [8,9]. Efficacy has been shown by administration of agonists for TLR4, 8, and 9 in allergic rhinitis in phase II clinical trials [5, 10].

TLR signaling can also be achieved with specific food ingredients [11,12], and could potentially also be utilized to dampen allergic responses [13]. Hydrolyzed proteins (hydrolysates) form a promising food product for this purpose, since we and others have recently shown that some hydrolysates from cow’s milk protein were able to stimulate TLRs in vitro, and in this way modulate immune function [14,15]. Besides cow’s milk, protein from crops like soy and wheat are also used for the production of hydrolysates [16]. These hydrolysates also have immunomodulatory effects [10-14, 17, 18], but it is not known whether these effects are induced via TLRs. Therefore, in the present study we tested whether different soy and wheat hydrolysates are able to induce immune effects and signal via TLRs. We tested the TLR activating and inhibiting capacity for TLR 2, 3, 4, 5, 7, and 9 of a range of soy and wheat hydrolysates.

**Materials and methods**

**Ethical statement**

For blood sampling of human volunteers, written informed consent was obtained. Data were analyzed and presented anonymously. The present research and consent procedure was approved by the ethical review board of the University Medical Center Groningen, as stated in the application “2007/255”. All investigations were conducted according to the principles stated in the Declaration of Helsinki.

**Test materials**

All hydrolysates tested were provided by FrieslandCampina (Amersfoort, the Netherlands). In the present study, six different soy hydrolysates and three wheat hydrolysates were tested, with different peptide composition, as illustrated by the different molecular weight distribution of the hydrolysates (table 1). Most peptides in the soy hydrolysates have a molecular weight smaller than 500 Dalton. The fraction between 1000 and 500 Dalton also contained a significant amount of peptides. Larger proteins (>10,000 Dalton) are only present in soy hydrolysate Soy 1. For the wheat hydrolysates it was found that Wheat 1 contained more proteins bigger than 10,000 Dalton compared to the other wheat hydrolysates.
All samples were tested for endotoxins by means of the Limulus amebocyte lysate assay (LAL) [19]. The concentrations measured have no influence on the cells applied in this study.

**Table 1. Overview of the characteristics of the hydrolysates studied**

<table>
<thead>
<tr>
<th>samples</th>
<th>source</th>
<th>Molecular weight distribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&gt;10,000 Da</td>
</tr>
<tr>
<td>Soy 1</td>
<td>Soy isolate</td>
<td>4</td>
</tr>
<tr>
<td>Soy 2</td>
<td>Soy isolate</td>
<td>0</td>
</tr>
<tr>
<td>Soy 3</td>
<td>Soy isolate</td>
<td>0</td>
</tr>
<tr>
<td>Soy 4</td>
<td>Soy isolate</td>
<td>0</td>
</tr>
<tr>
<td>Soy 5</td>
<td>Soy isolate</td>
<td>0</td>
</tr>
<tr>
<td>Soy 6</td>
<td>Soy isolate</td>
<td>0</td>
</tr>
<tr>
<td>Wheat 1</td>
<td>Wheat gluten</td>
<td>17</td>
</tr>
<tr>
<td>Wheat 2</td>
<td>Wheat gluten</td>
<td>1</td>
</tr>
<tr>
<td>Wheat 3</td>
<td>Wheat gluten</td>
<td>1</td>
</tr>
</tbody>
</table>

**Culturing of THP-1 and HEK reporter cell lines**

To determine the effects of hydrolysates on TLR signaling, various THP-1 and HEK reporter cell lines, all purchased from InvivoGen (Toulouse, France), were used. In order to quantify TLR activation in the cells, all cell lines contained a construct for Secreted Embryonic Alkaline Phosphatase (SEAP), which was coupled to the nuclear factor κB/Activating protein-1 (NF-κB/AP-1) promoter. NF-κB/AP-1 is a well-known downstream target of TLR receptors [11,20]. To assess the TLR dependent effects of soy and wheat hydrolysates, two THP-1 human acute monocytic leukemia cell lines were used which express all TLRs endogenously. The first THP-1 cell line (THP1-XBlue™-MD2-CD14) expressed MD2 and CD14, thus responds to TLR ligands. To check for TLR dependency, the results obtained with the THP-1 cell line were compared with a second THP-1 cell line (THP1-XBlue™-defMyD). This cell line expresses a truncated, non-functional form of the TLR adapter Myeloid differentiation primary response gene 88 (MyD88), and is therefore unresponsive to TLR2, 4, 5, 6, 7, 8, and 9 activation. THP-1 cell lines were cultured in RPMI1640 medium (Gibco, Life Technologies, Bleiswijk, The Netherlands), containing 10% heat inactivated FBS (Fetal Bovine Serum, HyClone, Thermo Scientific, Breda, The Netherlands), 2 mM L-glutamine, 1.5 g/L sodium bicarbonate (Boom B.V. Meppel, The Netherlands), 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 100 mg/mL Normocin™ (Invivogen, Toulouse, France), and 50 U/mL and 50 μg/mL Penicillin/Streptomycin. All additives were purchased from Sigma Aldrich (Zwijndrecht), unless indicated otherwise. Both THP-1 cell lines were passaged twice a week by inoculating 5x10⁵ cells. To study the effects on individual TLRs, 7 Human embryonic kidney (HEK)293 cell lines (HEK-
Blue™-hTLRX) were used, each containing an inserted construct of either human TLR2, 3, 4, 5, 7, or 9. HEK cells were cultured in DMEM medium (Gibco, Life Technologies, Bleiswijk, The Netherlands), supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 4.5 g/L glucose, 50 U/mL and 50 mg/mL penicillin/streptomycin and 100 mg/mL Normocin. These HEK cells were grown to ~80% confluency. All reporter cell lines were cultured for 3 passages before they were maintained in zeocin containing selection media (InvivoGen, Toulouse, France) as previously described [11,20].

**Reporter cell stimulation assays and Quanti-Blue analysis**

All relevant ligands were purchased from InvivoGen (Toulouse, France) (table 2). THP-1 and HEK cells were collected by centrifuging (5 min, 1500 rpm), and resuspended in culture medium following the manufacturer’s protocol (table 2). Cells were seeded in a flat bottom 96 wells plate, and stimulated for 24 hours (37 °C, 95% oxygen, 5% CO₂) with 2 mg/mL hydrolysate or a relevant ligand as a positive control (table 2). We selected 2 mg/mL based on dose response curves performed in THP-1 and HEK-Blue human TLR2 cells [14]. Medium was used as a negative control. After incubation, Quanti-Blue detection medium was used to analyze the cell supernatant as described before [11]. 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. The median and range for each sample were plotted as the fold-change compared to the negative control, which were unstimulated cells. The negative controls were set at 1.

**Table 2. Cell densities and ligands used in the different reporter cell line assays.**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell density for seeding</th>
<th>Positive control (concentration in well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THP-1 MD2-CD14</td>
<td>1*10⁶ cells/ml (100 µl/well)</td>
<td><em>Escherichia coli</em> K12 Lipopolysaccharide (10 ng/ml)</td>
</tr>
<tr>
<td>THP-1 MyD88 deficient</td>
<td>2*10⁶ cells/ml (100 µl/well)</td>
<td>MurNAc-L-Ala-γ-D-Glu-mDAP (M-TriDAP, 100 µg/ml)</td>
</tr>
<tr>
<td>HEK-Blue human TLR2</td>
<td>2.8*10⁵ cells/ml (180 µl/well)</td>
<td>Heat killed Listeria monocytogenes (10⁴ cells/ml)</td>
</tr>
<tr>
<td>HEK-Blue human TLR3</td>
<td>2.8*10⁵ cells/ml (180 µl/well)</td>
<td>Polyninosine-polycytidylic acid (high molecular weight) (5 µg/ml)</td>
</tr>
<tr>
<td>HEK-Blue human TLR4</td>
<td>1.4*10⁵ cells/ml (180 µl/well)</td>
<td><em>Escherichia coli</em> K12 Lipopolysaccharide (10 ng/ml)</td>
</tr>
<tr>
<td>HEK-Blue human TLR5</td>
<td>1.4*10⁵ cells/ml (180 µl/well)</td>
<td><em>Salmonella typhimurium</em> derived flagellin (10 ng/ml)</td>
</tr>
<tr>
<td>HEK-Blue human TLR7</td>
<td>2.2*10⁵ cells/ml (180 µl/well)</td>
<td>Imiquimod (5 mg/ml)</td>
</tr>
<tr>
<td>HEK-Blue human TLR8</td>
<td>2.2*10⁵ cells/ml (180 µl/well)</td>
<td>Single stranded RNA (ssRNA40/LyoVec™, 2 µg/ml)</td>
</tr>
<tr>
<td>HEK-Blue human TLR9</td>
<td>4.5*10⁵ cells/ml (180 µl/well)</td>
<td>Type B CpG oligonucleotide (ODN 2006, 0,25 µM)</td>
</tr>
</tbody>
</table>
Primary PBMC isolation, stimulation, and cytokine (IL-8, IL-10, TNFα) measurement

In order to determine whether soy and wheat hydrolysates with TLR interacting effects could also result in a cytokine response in immune cells, primary PBMCs were isolated from venous blood from a healthy, adolescent, male volunteer. Blood was collected in heparinized tubes (15 IU/mL lithium-heparin, Becton Dickinson B.V., Breda, The Netherlands). Subsequently primary PBMCs were isolated by Ficoll density gradient separation according to the manufacturer’s protocol (Lymphoprep, Axis-Shield, Oslo, Norway). Primary PBMCs were seeded in a 96 wells round bottom plate at a density of 0.4x10⁶ cells/well, in a volume of 200 μL medium, and stimulated with 2 mg/mL hydrolysate for 24 hours (37 °C, 95% oxygen, 5% CO₂). A combination of phorbol myristate acetate (PMA; Sigma Aldrich, Zwijndrecht, the Netherlands) (5 ng/mL) and ionomycin (500 ng/mL) was used as a positive control. Unstimulated cells served as a negative control. All stimulations were performed in technical triplicates. After 24 hours, supernatant was collected and stored at -80 °C. To analyze cytokine levels a self-composed multiplex immunoassay was used containing ProcartaPlex Simplexes for the human anti-inflammatory cytokine Interleukin (IL)-10, and the pro-inflammatory cytokines IL-8, and tumor necrosis factor alpha (TNFα) (Affymetrix, Santa Clara, USA). The immunoassay was performed according to the manufacturer’s protocol. Briefly, cytokine standards of the different Simplexes were mixed, and serial dilutions were prepared, followed by mixing antibody magnetic beads and aliquoting the mixture for analysis. After washing, standards and samples were added (50 μL/well), the plate was sealed, and incubated while shaking (30 min at room temperature (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-phycocerythrin 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. The data were plotted as the fold change compared to the positive control, which was set to 1.

HEK reporter cell inhibition assays

Besides activating TLRs, TLR binding molecules can also inhibit pattern recognition receptors [21]. To assess inhibition of the TLR signaling by hydrolysates, HEK cells were resuspended, and seeded in the same way as described above for the stimulation assays. Then, cells were stimulated with the appropriate TLR ligand (table 2), together with 2 mg/mL hydrolysate. The TLR ligand alone served as a positive control, medium alone as a negative control. Cells were incubated for 24 hours (37 °C, 95% oxygen, 5% CO₂), and afterwards SEAP activity was measured as described above. The median and range for each sample were plotted as the fold change compared to the positive control, which were cells stimulated with its relevant ligand. The positive controls were set at 1.
Statistical analysis
Statistical analysis was performed using Graphpad Prism. Normal distribution of the data was tested using the Kolmogorov-Smirnov test. Data were not normally distributed and expressed as median ± range. Significance levels were assessed using the Kruskal-Wallis test followed by the Dunn's test to show individual differences. A p-value of <0.05 was considered to indicate a significant difference.

Results
Both soy and wheat hydrolysates induced TLR activation
We investigated whether pattern recognition receptors could be involved in immune modulating effects by the soy and wheat hydrolysates. To test this, we used a technology platform of cell lines expressing pathogen recognition receptors [11, 20].

The effects of soy hydrolysates are shown in figure 1. The hydrolysate Soy 1 showed the most pronounced TLR activating effect (p<0.0001 vs negative control). Also, Soy 4 showed clear TLR activation in the THP-1 MD2-CD14 cell line (p<0.01 vs negative control). All other soy hydrolysates did not show a significant TLR activating effect.

Some wheat hydrolysates were found to induce TLR activation as well. The hydrolysate Wheat 1 showed a slight but significant (p<0.01 vs negative control) increased TLR activation. Wheat 2 showed the most pronounced effect among wheat hydrolysates (p<0.0001 vs negative control). The hydrolysate Wheat 3 did not induce TLR activation.

After stimulation of the MyD88 deficient THP-1 reporter cell line with the soy and wheat hydrolysates, it was shown that only Soy 1 and Wheat 1 slightly activated this cell line (figure 1).
Figure 1. NF-κB/AP-1 activation in THP-1-MD2-CD14 and THP-1-MyD88 deficient reporter cells after stimulation with soy and wheat hydrolysates. Two soy hydrolysates and two wheat hydrolysates induced TLR signaling, while all other hydrolysates had no effect (left panel). These effects were TLR dependent, since a suppressed activation was observed in the stimulated MyD88 deficient cell line (right panel). Significant differences compared to the negative control were indicated by * (p<0.05), ** (p<0.01), *** (p<0.001) or by **** (p<0.0001).

The TLR activating pattern differed between soy and wheat hydrolysates

Then, we studied which individual TLRs can be activated by soy and wheat hydrolysates. To this end, HEK reporter cell lines, each containing a construct for a specific TLR, were stimulated with soy or wheat hydrolysates (figures 2 and 3). The results for the soy hydrolysates are shown in figure 2. Hydrolysate Soy 1 and 6 were the most potent soy hydrolysates. These hydrolysates activated TLR2, 3, 4, 5, 7, and 9 (for Soy 1 TLR2, 4, 7, and 9: p<0.0001; TLR3: p<0.001; TLR5: p<0.05, for Soy 6 TLR2 and 7: p<0.05; TLR3 and 9: p<0.01; TLR4: p<0.001; TLR5: p<0.0001). Soy 4 only activated TLR2 and 4 (TLR2: p<0.001; TLR4: p<0.0001). All other soy hydrolysates were not found to activate individual TLRs. We also observed differences in TLR activation patterns between wheat hydrolysates. The hydrolysate Wheat 2 showed the most pronounced TLR activating capacity; TLR2, 3, 4, 5, 7, and 9 were significantly activated by Wheat 2 (TLR2, 3, and 4: p<0.01; TLR5, 7 and 9: p<0.0001). Wheat 1 only activated TLR4 (p<0.05), while Wheat 3 was only able to induce TLR2 activation (p<0.001).
Figure 2. NF-κB/AP-1 activation in HEK reporter cells carrying individual TLRs after stimulation with soy hydrolysates. Soy 1 induced signaling of all tested TLRs. This pattern of TLR activation was different for the other hydrolysates. Soy 6 activated all TLRs except for TLR4, while Soy 4 only activated TLR2. Other soy hydrolysates did not activate TLRs. Significant differences compared to the negative control were indicated by * (p<0.05), ** (p<0.01), *** (p<0.001) or by **** (p<0.0001).
Figure 3. NF-κB AP-1 activation in HEK reporter cells carrying individual TLRs after stimulation with wheat hydrolysates. Hydrolysate Wheat 2 stimulated TLR2, 3, 5, 7, and 9. Wheat 1 only activated TLR4, while Wheat 3 only activated TLR2. Significant differences compared to the negative control were indicated by * (p<0.05), ** (p<0.01), *** (p<0.001) or by **** (p<0.0001).
Several soy and wheat hydrolysates induce cytokine production in primary PBMCs

To determine whether the TLR activating effects of soy and wheat hydrolysates modulated cytokine responses in immune cells, the induction of pro- (TNFα and IL-8) and anti- (IL-10) inflammatory cytokines in human primary PBMCs was measured after incubation with hydrolysates for 24 hours (figure 4).

Soy 1 induced a slight increase in TNFα. None of the other soy hydrolysates induced TNFα production. IL-10 was also increased most by hydrolysate Soy 1. Other soy hydrolysates did not have an effect on IL-10 production. For IL-8, all hydrolysates showed a similar increase compared to the negative control, which was of the same magnitude as the effect of the positive control.

The effects of wheat hydrolysates on TNFα production were also small. The production of IL-10 was more affected by wheat hydrolysates. Both hydrolysate Wheat 1 and Wheat 2 seemed to increase IL-10 production. IL-8 production was also increased after PBMC incubation with hydrolysates Wheat 1 and Wheat 2, and the cytokine level was similar to the positive control. Incubation of PBMCs with the hydrolysate Wheat 3 did not induce any of the cytokines measured.
Figure 4. Human primary PBMCs produced cytokines after stimulation with soy and wheat hydrolysates. Hydrolysate Soy 1 induced a significant increase in IL-10 production in PBMCs (upper panel). Hydrolysate Wheat 1 and 2 increased both IL-10 and IL-8 production (lower panel).
The TLR inhibiting pattern differed between soy and wheat hydrolysates
Since the interaction between binding molecules and TLRs can also lead to inhibition of TLR stimulation, we investigated whether soy and wheat hydrolysates could inhibit TLR ligand induced TLR activation [21]. This was done by adding hydrolysates to cells together with a relevant TLR ligand (figures 5 and 6). All soy hydrolysates tested showed inhibition of specific TLRs. However, the combination of TLRs that could be inhibited differed per hydrolysate. Hydrolysates Soy 2, Soy 3, and Soy 4 possessed the broadest inhibiting capacity, and were both able to inhibit 4 of the tested TLRs. Soy 2 inhibited TLR2, 4, 5, and 9 (TLR2: $p<0.01$; TLR4 and 5: $p<0.05$; TLR9: $p<0.0001$), while Soy 3 inhibited TLR2, 4, 7, and 9 (TLR2: $p<0.0001$; TLR4: $p<0.05$; TLR7: $p<0.0001$; TLR9: $p<0.005$) and Soy 4 inhibited TLR3, 4, 7, and 9 (TLR3: $p<0.05$; TLR4: $p<0.0001$; TLR7: $p<0.01$; TLR9: $p<0.01$). Soy 5 was able to inhibit the activation of TLR2, 4, and 7 (TLR2: $p<0.01$; TLR4: $p<0.05$; TLR7: $p<0.05$). Hydrolysate Soy 6 only inhibited TLR3 and 4. The least potent inhibiting soy hydrolysate was Soy 1, which showed most TLR activating effects. This hydrolysate only inhibited TLR4 activation ($p<0.0001$), like all other hydrolysates. Wheat hydrolysates also showed TLR inhibiting capacities after TLR stimulation with a known ligand. The hydrolysate with the most inhibition effects was Wheat 1. This hydrolysate inhibited all TLRs tested (TLR2, and 7: $p<0.0001$; TLR3, 5, and 9: $p<0.01$; TLR4: $p<0.001$). Interestingly, especially the TLR2 inhibiting effect was very strong. Also, hydrolysate Wheat 3 inhibited most of the TLRs, including TLR3, 4, 5, 7, and 9 (TLR3, 5, and 7: $p<0.0001$; TLR4: $p<0.001$; TLR9: $p<0.01$). Wheat 2, the wheat hydrolysate with the most TLR activating capacities, only showed TLR4 inhibition ($p<0.01$).
Figure 5. NF-κB/AP-1 activation in HEK reporter cells carrying individual TLRs after simultaneous stimulation with its relevant ligand and soy hydrolysates. Soy hydrolysates were able to inhibit TLR activation. Soy 2 and 4 showed most inhibiting effects. Soy 1 only inhibited TLR4. Significant differences were determined by using the Kruskal-Wallis test followed by the Dunn’s test. Significant differences compared to the negative control were indicated by * (p<0.05), ** (p<0.01), *** (p<0.001) or by **** (p<0.0001), significant differences compared to the positive control were indicated by # (p<0.05), ## (p<0.01), ### (p<0.001) or by #### (p<0.0001).
Toll-like receptor activating and inhibiting properties of soy and wheat hydrolysates

Figure 6. NF-κB AP-1 activation in HEK reporter cells carrying individual TLRs after simultaneous stimulation with its relevant ligand and wheat hydrolysates. Wheat hydrolysates were able to inhibit TLR activation. Hydrolysate Wheat 1 and 3 inhibited activation in many TLRs, while Wheat 2 only inhibited TLR4. Significant differences were determined by using the Kruskal-Wallis test followed by the Dunn's test. Significant differences compared to the negative control were indicated by * (p<0.05), ** (p<0.01), *** (p<0.001) or by **** (p<0.0001), significant differences compared to the positive control were indicated by # (p<0.05), ## (p<0.01), ### (p<0.001) or by #### (p<0.0001).
Discussion

Treatments of allergic diseases focus on modulation of the immune system in order to dampen the Th2 response [5]. A promising strategy to do this is by modulating TLR signaling, which is known to be able to stimulate Th1 and decrease Th2 response [8,9]. Since it was shown before that cow’s milk hydrolysates are able to induce immune effects via TLRs [14], the question arose whether hydrolysates from soy and wheat, which have immune stimulating effects [22,23], also have the TLR stimulating potential. To the best of our knowledge we show here for the first time that specific soy and wheat hydrolysates are able to induce TLR activation, while other hydrolysates induced more TLR inhibiting effects. In a subsequent experiment we confirmed immune modulation as we demonstrate that soy and wheat hydrolysate induced cytokine production in PBMCs.

Although TLR dependent signaling was detected after stimulation with hydrolysates both derived from soy and wheat (figure 1), we found that the type of TLRs which were activated or inhibited was hydrolysate dependent. For soy hydrolysates, Soy 1 and Soy 4 significantly increased TLR activation in THP-1 cells, while the other 4 soy hydrolysates had no effect. For wheat hydrolysates, only Wheat 1 and 2 showed TLR activation. The hydrolysate dependent effect on TLR signaling was even more pronounced when investigating the individual TLR types that were influenced by hydrolysates. For instance, the TLR activation by Soy 1 was induced by activation of TLR2, 3, 4, 5, 7, and 9, while Soy 4 only activated TLR2 and 4 (figure 2). Wheat 2 also activated TLR2, 3, 4, 5, 7, and 9, while Wheat 1 only activated TLR4 (figure 3).

In order to determine whether the TLR activating hydrolysates also induced cytokine production in immune cells, we did an experiment using PBMCs which were stimulated with hydrolysates (figure 4). TLR activating hydrolysates Soy 1, Wheat 1 and Wheat 2 also induced cytokine production. TNFα and IL-10 were enhanced mostly by Soy 1, as well as by Wheat 1 and 2. However, Soy 4 also showed TLR activation but no enhancement of TNFα and IL-10 suggesting that the cytokine induction is not strictly TLR dependent or that the total effect of activation and inhibition may result in a neutral effect. As discussed below Soy 4 was a hydrolysate with a broad inhibiting effect which supports the argumentation that the overall effect of inhibition prevents upregulation of TNFα and IL-10. The observation that wheat hydrolysates induce cytokine production in immune cells is in line with results of Lammers et al., who found that cytokine production, including TNFα, IL-10 and IL-8, was induced in PBMCs of healthy volunteers after wheat hydrolysate administration [24]. The increase of TNFα may indicate that Soy 1 and Wheat 1 were able to activate the innate immune system, which is associated with a Th1 response [25,26].

Remarkably soy and wheat hydrolysates were not only able to induce TLR activation, but to inhibit ligand induced TLR signaling. Soy 2, 3 and 4 were the soy hydrolysates with the broadest inhibiting effects. Most studies only focus on activation and not on inhibiting effects of food components. Soy 2 inhibited TLR2, 4, 5, and 9, while Soy 4 inhibited TLR3, 4, 7, and 9 (figure 5). Wheat 1 and 3 both inhibited many TLRs. Wheat 1 inhibited all tested TLRs, Wheat 3 inhibited TLR2, 3, 4, 5, 7, and 9 (figure 6). The most pronounced inhibitory effect was observed for Wheat 1 which inhibited TLR2 signaling with 82%. Direct inhibition of TLR signaling, as shown here, might be a mechanism explaining the anti-inflammatory effects of hydrolysates as reported in many studies [27-29]. What combined activation and specific inhibition of pattern recognition receptors...
implies for allergic response management is not known, but TLR inhibiting capacities should not be ignored because it might influence the overall effects on immune cells as demonstrated with Soy 4 hydrolysate. It is therefore imperative to always test the final effects on immune cells.

Overall, in this study we show that specific soy and wheat hydrolysates are able to induce TLR activation or inhibition. We suggest that this could be a mechanism involved in immunomodulatory effects of soy and wheat hydrolysates. However, small differences in hydrolysate composition might change the immune effects, and the effects of individual hydrolysates should therefore be thoroughly studied. This study contributed to increasing knowledge about immunomodulatory effects of hydrolysates and could ultimately contribute to the use of hydrolysates in management of allergy.
References


