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Innate and adaptive immune effects of chicory root dietary fibers

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Document Version

Publisher's PDF, also known as Version of record

Publication date:

2015

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Vogt, L. (2015). *Innate and adaptive immune effects of chicory root dietary fibers*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen.

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CHAPTER 5

THE IMPACT OF LEMON PECTIN CHARACTERISTICS ON TLR ACTIVATION AND T84 INTESTINAL EPITHELIAL BARRIER FUNCTION

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Submitted to Journal of Functional Foods.

ABSTRACT

Scope

Metadata analyses confirm that dietary fibers reduce risk and incidence of disease. This study aimed to investigate underlying structure-response relationships of different degree of polymerization (DP) and degree of methyl esterification (DM) pectins as immunomodulatory and epithelial barrier active dietary fibers.

Methods and Results. 30DM, 56DM and 74DM lemon pectins were applied to test effects of degree of methyl esterification. Enzymatic digests of these pectins were applied to study the effects of chain length modification. NF- κ B/AP-1 activation was measured using TLR and MyD88 reporter assays. The pectins demonstrated TLR dependent activation, and an increasing TLR activation with increasing DM. Pectins induced TLR2, and TLR4 activation, and enzymatic digestion of the polymers into oligomers abrogated their TLR activating potential. Barrier function of human intestinal epithelial cells was quantified by resistance measurements across T84 monolayers. 30DM and 74DM pectins induced a strong barrier protective effect, while 56DM pectins induced moderate protection of T84 TEER.

Conclusion. Activation of immune cells by lemon pectins is TLR dependent through ligation of TLR2 and 4, and the intact polymer backbone is indispensable for activation. Degree of methyl esterification is a determining factor in activation potential and epithelial barrier protective effects, which is strongest for 30 DM and 74 DM lemon pectins.

INTRODUCTION

5.1 Background

An important category of health promoting nutritional compounds, which are under extensive study, is that of dietary fibers [1]. Data from meta-analyses confirm that adequate intake of dietary fibers reduces the risk and incidence of diseases such as type 2 diabetes, cardiovascular diseases, cancer [2-5], several gastrointestinal disorders, and obesity [6]. Although dietary fibers have been shown to have a collective health promoting effect, it is largely unknown which chemical properties of which individual fiber are responsible for which of the observed effects. Dietary fibers comprise a spectrum of different molecules from a broad range of plant species, and can also be produced by chemical or enzymatic synthesis such as polydextrose or galactooligosaccharides (GOS) [7,8]. Moreover, within one category of dietary fibers, the physiological responses can differ depending on backbone chain length [9,10] and other physicochemical properties such as the number and the type of side chains [11].

Pectins form a large family of dietary fiber molecules with a range of different structural properties. They are important structural components of plant cells consisting of linear 1,4- α -D-galacturonan (homogalacturonan) segments and highly branched rhamnogalacturonan segment [12]. Industrially extracted pectins are used by the food industry as gelling agent. When pectin has more than 50% of the acid units in the backbone methyl-esterified (degree of methyl esterification – DM), it is usually classified as "high methyl ester (HM) pectin" [13]. Saponification of pectins will reduce the degree of esterification and will yield "low methyl ester (LM) pectins" (DM<50) [14]. Examples of pectin structures with different DM are depicted in Figure 1. Next to the absolute amount of methyl esters present within the pectin molecule, also the distribution of methyl esters over the galacturonan backbone may determine the techno-functional properties [15,16].

As previously shown, the degree of methyl esterification as well as the chain length of pectin molecules are important factors in inducing different biological effects [17], including effects on the consumer's immune system [18-26]. Up to now these effects on the host immune system were mainly attributed to beneficial effects of the dietary fibers on the consumers' microbiota. However, recently we have shown that dietary fibers can also have direct effects on immune cells. Several prebiotic fibers

have been shown to directly ligate sensors of the innate immune system, the so-called pattern recognition receptors (PRRs) [9,27,28]. Toll-like receptors (TLRs) are the best characterized PRRs and have been shown to be involved in signaling of dietary fibers [9,27,29,30]. We hypothesized that activation of TLRs may be one of the factors involved in the immunomodulatory properties of pectins. To address potential structure-function relationships we compared lemon pectins which can be obtained in defined chemical compositions. We investigated the role of chain length and degree of methyl esterification of pectins on TLRs. Also we studied the effects of pectin with different degree of methyl esterification on barrier function of human intestinal cells in vitro, as pectins might exert health effects by modulating the intestinal barrier [31,32]. To this end we applied a phorbol 12-myristate-13-acetate (PMA) damage model to T84 intestinal epithelial monolayers.

METHODS

5.2 Investigational compounds, enzymatic digestion, and chemical characterization with high performance anion exchange chromatography and high pressure size exclusion chromatography

The investigational compounds applied in this study were lemon pectins with different degrees of methyl esterification, i.e. 30% (30DM), 56% (56DM), and 74% (74DM), acquired from CP Kelco; Lille Skensved, Denmark (see also **Figure 1**). The pectins have been described in detail by Daas et al. (2000) with the codes C30, C55, and C74 respectively. These compounds were applied to investigate structure-function relationships for the degree of methyl esterification. In some experiments, the pectins were digested to oligomers to confirm that the biological effects are dependent on an intact backbone of the molecule or whether they can also be achieved by the oligomers. To this end, the pectin substrates (30DM, 56DM and 74DM; 500 mg) were dissolved in 50 mL of 50 mM ammonium acetate buffer, pH 5. The enzymes used were endopolygalacturonase (EC 3.2.1.15) from *Kluyveromyces fragilis* with an activity of 68.68 U/mL, 20.68 μ L was added [33] (for DM 30 and DM55 pectin) and pectin lyase II (PL, EC 4.2.2.10), from *Aspergillus niger*, with an activity of 8.8 U/mL, 161.41 μ L was added [34] for DM74 pectin. Enzyme incubations were performed for 10 h, rotating at 125 rpm at 30°C. Substrate blanks and enzyme blanks were prepared as controls. Following

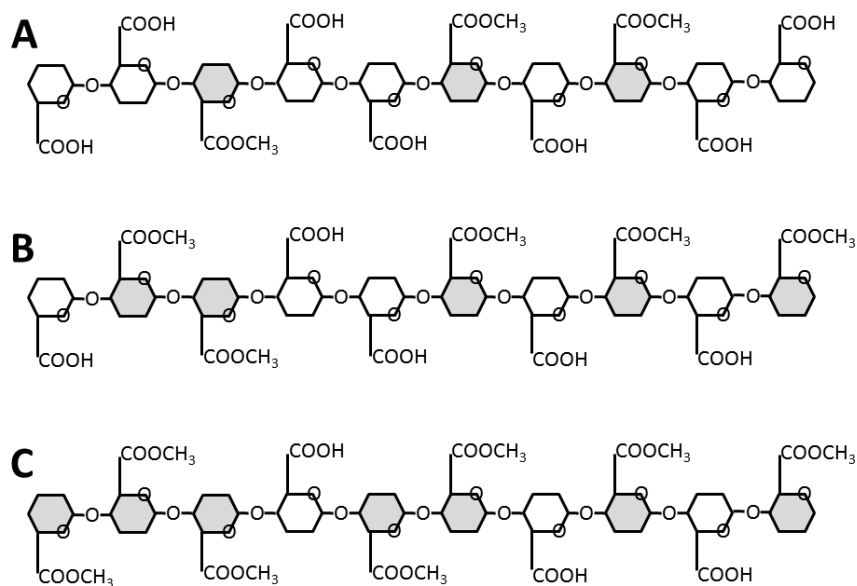


Figure 1. Schematic representation of different DM pectins. A) 30DM pectin. B) 56DM pectin. C) 74DM pectin. Methylated galacturonic-residues are indicated in grey, unmethylated in white.

incubations for 10 h, all samples were boiled for 5 min to inactivate the enzymes.

Chemicals were from Sigma Aldrich Chemie B.V., Zwijndrecht, the Netherlands. Chemical characterization by high performance anion exchange chromatography (HPAEC) and high pressure size exclusion chromatography (HPSEC) of the untreated and treated pectins was performed as previously described [9]. Results of the chemical characterization are shown in **Figure 2**.

5.3 Cell culture

T84 human intestinal epithelial cells were acquired from Sigma Aldrich Chemie B.V. Cells were cultured as previously described [35,36]. THP1 MD2-CD14, THP1 DefMyD, HEK-Blue hTLR2, and HEK-Blue hTLR4 reporter cell lines, Normocin antibiotic, and selection media were all acquired from InvivoGen, Toulouse, France. Reporter cells were cultured as previously described [9]. Briefly, the THP-1 cell lines were cultured in RPMI1640 supplemented with 10% heat inactivated FBS (60°C, 60 min), glucose (4.5 g/L), NaHCO₃ (Boom B.V., Meppel, The Netherlands; 1.5 g/L), HEPES (10

mM), L-glutamine (2 mM), penicillin/streptomycin (50 U/mL and 50 mg/mL), and sodium pyruvate (1 mM) all from Sigma-Aldrich Chemie B.V., and with Normocin (100 mg/mL, from InvivoGen). THP-1 cell lines were maintained at a concentration of 5×10^5 cells/mL. After culturing for 3 passages, THP1 cell lines were maintained in selection media by adding Zeocin (200 $\mu\text{g/mL}$) and G418 (250 $\mu\text{g/mL}$) to the THP1 MD2-CD14 cell culture medium, and by adding Zeocin (200 $\mu\text{g/mL}$) and HygroGold (100 $\mu\text{g/mL}$) to the THP1 DefMyD cell culture medium. HEK-Blue cells were cultured in DMEM (Life Technologies Europe B.V., Bleiswijk, the Netherlands), supplemented with glucose (4.5 g/L), L-glutamine (2 mM), 10% heat inactivated FBS, penicillin/streptomycin (50 U/mL and 50 mg/mL), and Normocin (100 mg/mL). HEK cells were grown to ca. 80% confluency. After culturing for 3 passages, the HEK cell lines were maintained in selection media by adding 1X HEK-Blue Selection to the culture medium.

5.4 Reporter cell assays

To establish whether lemon pectin can activate PRRs, and more specifically TLRs, TLR reporter cell assays were performed according to the manufacturer's protocol. Briefly, THP-1 MD2-CD14 cells expressing all TLRs were resuspended to a cell density of 1×10^6 cells/mL, 100 μL of this cell suspension per well was stimulated for 24 h (37°C, 5% CO_2) with untreated and treated pectic compounds (described above), or 100 ng/mL ultrapure *E.coli* LPS (InvivoGen) as a positive control. Culture medium was incorporated in the assay as negative control. Subsequently 20 μL of supernatant of the stimulated reporter cells was incubated with 180 μL of Quanti-blue reagent (InvivoGen; 45 min, 37°C, 5% CO_2) and NF- κB /AP-1 was measured by the amount of excreted SEAP and Quanti-blue color conversion (OD) at 650 nm using a VersaMax microplate reader (Molecular Devices GmbH, Biberach an der Riss, Germany) and SoftMax Pro Data Acquisition & Analysis Software.

To study whether activation of NF- κB /AP-1 in the THP-1 MD2-CD14 cells was fully TLR-dependent, THP-1 cells expressing all TLRs but with a non-functional TLR adapter MyD88 were applied. These cells were resuspended to a density of 2×10^6 cells/mL and the reporter assay was performed as described above for THP-1 MD2-CD14 cells, with the exception that the positive control for MyD88 independent stimulation of THP1-DefMyD cells was 25 $\mu\text{g/mL}$ Tri-DAP (InvivoGen).

According to recent literature reports, TLR2 and TLR4 appear to be two main TLRs by which dietary fibers can induce direct cellular activation

[9,37-40]. Therefore, we next studied the activating capacity of the pectic compounds for activation of TLR2 and TLR4 in reporter cells. HEK-Blue hTLR2 reporter cells were resuspended to a cell density of $2.8 \cdot 10^5$ cells/mL, and HEK-Blue hTLR4 reporter cells were resuspended to a cell density of $1.4 \cdot 10^5$ cells/mL. 180 μ L of cell suspension was stimulated for 24 h (37°C, 5% CO₂) with 20 μ L of the pectic compounds described above, or 20 μ L of culture medium as negative control, or 20 μ L of a suspension of $1 \cdot 10^8$ heat killed *Listeria Monocytogenes* cells per ml (InvivoGen) or 100 ng/mL ultrapure *E.coli* LPS as positive control. Analysis of NF- κ B/AP-1 activation was performed as described for the THP-1 cells.

5.5 TEER of T84 human intestinal cells

Epithelial resistance across differentiated monolayers of T84 intestinal cells was determined using gold-plated electrode chamber slides and Epithelial Cell Substrate Impedance Sensing as described previously [36] with some modifications. Briefly, multiple electrode gold-plated 96 well chamber slides (96W20idf, Applied Biophysics, IBIDI, München, Germany) were coated with 300 μ L/well of a 0.2% L-cysteine (Sigma-Aldrich Chemie B.V.) solution in DMEM (Life Technologies Europe B.V.) for 30 min at room temperature. Wells were washed twice with DMEM, and coated overnight at room temperature with 300 μ L/well of 1% PureCol™ bovine tail collagen (Nutacon B.V., Leimuiden, the Netherlands) and 0.1% BSA (Sigma-Aldrich Chemie B.V.) in culture medium. Wells were then washed twice with culture medium and cells were seeded at a density of $2 \cdot 10^5$ cells per well in a final volume of 300 μ L/well. Prior to stimulation, the cells were maintained in the wells for 14 days to reach a stable TEER, and medium was changed every other day. Resistance was measured continuously at multiple frequencies [41] upon placing the chamber slides in an ECIS incubator (Z-Theta model, Applied Biophysics, Troy, New York, USA). Measurements performed at 500 Hz were used to calculate the AUC, as relatively low frequencies are specifically representative for the tight junction mediated resistance [41]. To establish whether pectins exert protective effects, a damage model was applied based on T84 cell incubation with phorbol 12-myristate 13-acetate (PMA, 10nM, Sigma-Aldrich Chemie B.V.), which is a barrier disrupting agent [42]. T84 cells were incubated with DM30, DM56, and DM70 pectins for 24 h, followed by addition of PMA. Cells were maintained in this stimulation medium for at least 20 h, and this time frame of 20 h was subsequently used to calculate the AUC relative to untreated controls.

5.6 Statistical analysis

GraphPad Prism version 5.0 was used for statistical analysis of all reporter cell data and all data from the ECIS experiments. Significance levels were determined by Wilcoxon signed rank test and one-way ANOVA. Results are expressed as median and interquartile range. p -values < 0.05 are considered statistically significant and are denoted with *.

RESULTS

5.7 Chemical characterization of lemon pectins

The pectins DM30, DM56, and DM74 used in this study have a similar molecular weight (40-100 kDa) (**Figure 2A-C**). All three pectins had a rather random distribution of their methyl esters. To study the effect of size of the pectin molecules, the pectins were degraded by enzymes to oligomeric fragments. Digests were studied with HPSEC and HPAEC (**Figures 2 A-D**). The 30DM digest after treatment with polygalacturonase consists of saturated GalA oligomers of DP2-DP20, with the main fraction being DP2-DP6 (**Figure 2A, 2D**). The 56 DM polygalacturonase digest was less degraded by the enzyme but still consists of lower Mw fragments (**Figure 2B, 2D**). The digestion of 74DM pectin was performed by pectin lyase since polygalacturonase is hindered too much by methyl esters in the 74DM pectin. The HPSEC pattern in Figure 2C demonstrates a successful degradation to low Mw material. However, due to the working mechanism of the enzyme, mainly unsaturated GalA oligomers of DP2-DP14 are present in the digest (**Figure 2D**).

5.8 Activation of THP1MD2-CD14 cells by lemon pectin polymers is dependent on TLR activation

To study whether lemon pectins can activate immune cells, culminating in NF- κ B/AP-1 induction, DM56 pectin was co-incubated with THP1 MD2-CD14 cells equipped with a reporter gene. As shown in **Figure 3A** we observed that pectin activated the THP1 MD2-CD14 cells with a 4.4 fold-induction (± 0.19 interquartile range, $p < 0.05$) as compared to control.

Next, we studied whether the induced NF- κ B/AP-1 activation in the THP1 MD2-CD14 cells was mediated by MyD88, i.e. the central adapter molecule for TLR signaling. This was done by comparing the activation of the THP1 MD2-CD14 cells which express all TLRs in

conjunction with MyD88 [43], with THP1 DefMyD cells which only express all TLRs, but a truncated, inactive form of MyD88 [9].

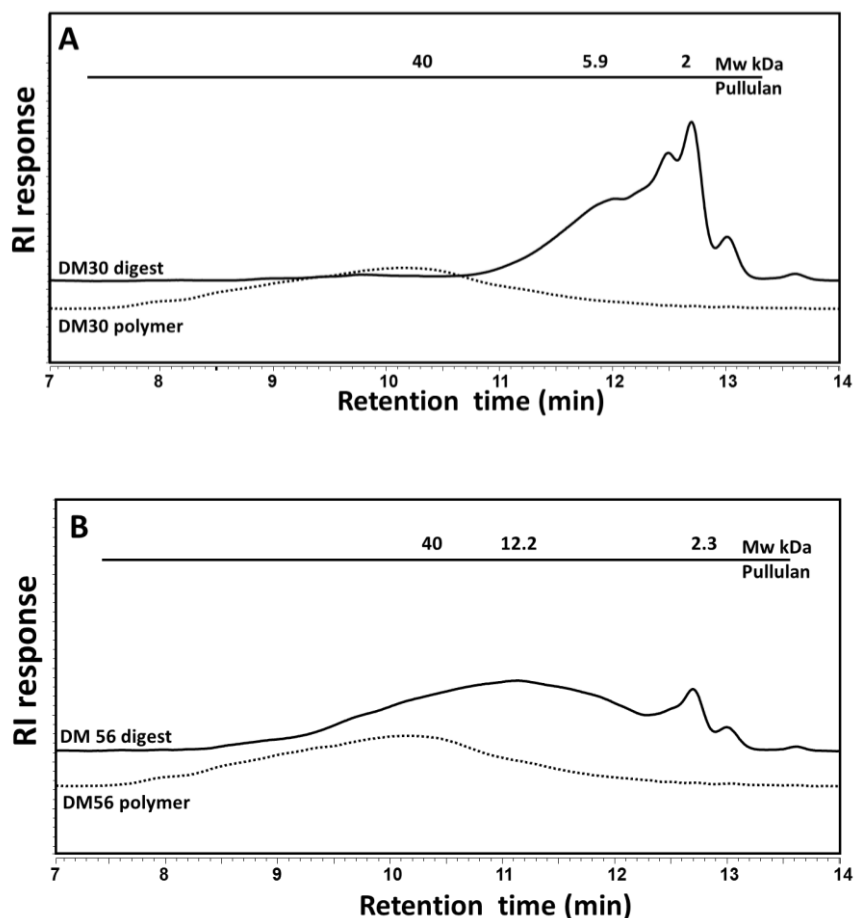


Figure 2 (continued). HPSEC elution patterns of the untreated pectin and of the corresponding enzyme digest, as a measure for their molecular weight distribution profiles in kDa for A) DM30 pectin; B) DM55 pectin; and C) DM74 pectin. D) HPAEC profiles of the pectin oligomers present in the enzyme digests of DM30, DM55, and DM74 pectins. GalA, galacturonic acid, uGalA, unsaturated galacturonic acid Subscript number indicates length of oligomer. Mw; Molecular weight, DM; Degree of Methyl esterification, DDM30; digested 30DM pectin, DDM56; digested 56DM pectin; DDM74; digested 74DM pectin.

As shown in **Figure 3B**, the pectin induced no activation of the MyD88 deficient cell line, indicating that the observed activation in the THP1 MD2-CD14 cell line was dependent on MyD88 signaling and thus TLR-mediated.

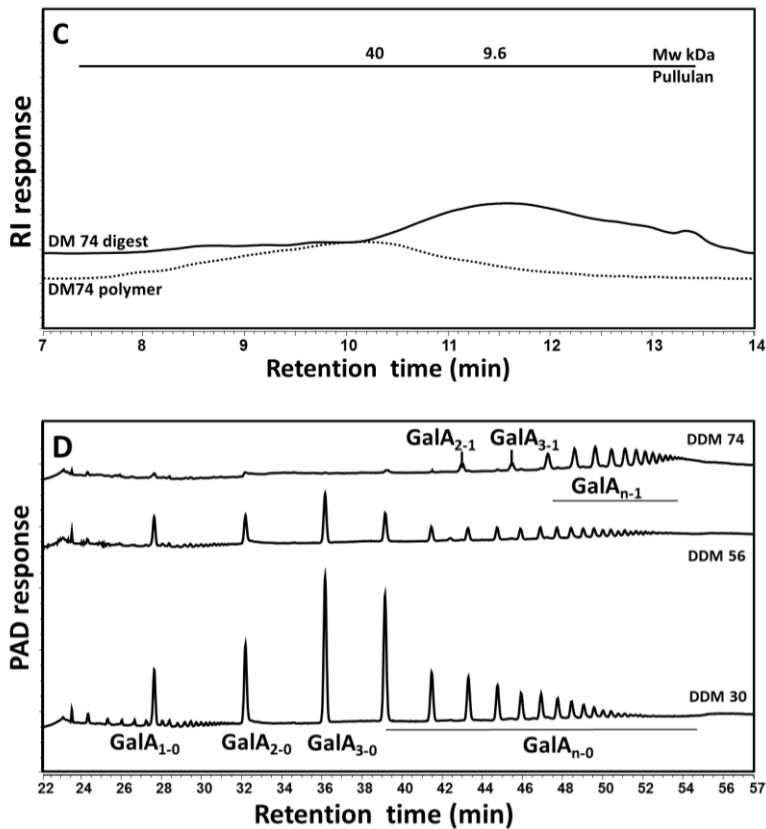


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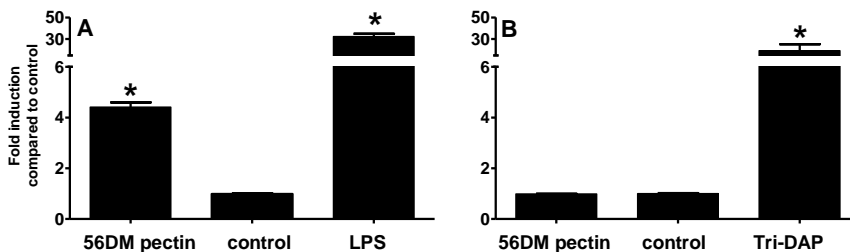


Figure 3. Stimulation of MyD88 proficient-, and deficient TLR reporter cells with 56DM lemon pectin. Figure A shows the results of fully functional THP-1 MD2-CD14 cells expressing all human TLRs, and Figure B depicts the results for THP-1 reporter cells expressing all human TLRs but which are deficient in MyD88 signaling (THP1 DefMyD cells). Both cell lines were incubated with 1 mg/mL 56DM lemon pectin, culture medium as negative control, and 100 ng/mL LPS or 25 µg/mL Tri-DAP as positive controls respectively. Statistical significance was determined with a Wilcoxon signed rank test, and activation compared to control is indicated with an asterisk. Median and interquartile range of activation is plotted as fold induction as compared to control (n = 8).

5.9 TLR induced NF- κ B/AP-1 activation by lemon pectin is dependent on the degree of methyl esterification

Next the effect of the degree of methyl esterification on immunological properties of pectin was investigated by comparing the TLR-mediated NF- κ B/AP-1 activation in THP1 MD2-CD14 cells after exposure to pectin polymers with a DM value of 30, 56, and 74.

As shown in **Figure 4A**, the higher the degree of methyl esterification of the pectin polymers, the higher the TLR-mediated NF- κ B/AP-1 activation in THP1 MD2-CD14 cells. The 30DM pectin polymers induced a 3-fold induction of activation compared to control (\pm 0.86 interquartile range, $p < 0.05$), the 56DM pectin polymers induced a 4.4-fold induction of activation (\pm 0.19 interquartile range, $p < 0.05$) and 74DM pectin polymers induced the strongest activation, i.e. a 5-fold induction as compared to control (\pm 0.92 interquartile range, $p < 0.05$). Comparison between the different DM fractions indicated that 56DM and 74DM pectins induced significantly stronger activation as compared to 30DM pectins and 74DM pectins trended to induce stronger activation compared to 56DM pectins ($p < 0.1$). These results show that the structural property of degree of methyl esterification in pectins is an important feature determining the TLR-activating capacity of lemon pectins.

5.10 TLR dependent NF- κ B/AP-1 activation by lemon pectin requires an intact backbone

To determine whether the TLR-mediated NF- κ B/AP-1 activation by lemon pectin is dependent on its molecular weight or can also be achieved by oligomers of pectins, the degree of polymerization was enzymatically reduced to pectin oligomers. Reduction of the polymer length resulted in loss of TLR activation (**Figure 4B**). The oligomers did not have the same effect as the full pectins with an intact backbone, suggesting that backbone chain length is an important factor for the activating potential of these lemon pectins.

5.11 Different DM pectins show different activation patterns of TLR2 and TLR4

Next it was studied whether TLR2 and TLR4 are involved in the activation. TLR2 and TLR4 can recognize polymeric carbohydrates [44-46] and are both implicated in the recognition of dietary fibers [9,37-40]. Therefore it was investigated whether the NF- κ B/AP-1 activation by pectins is TLR2 or TLR4 dependent. HEK-Blue TLR reporter cells with either human TLR2 or TLR4 constructs were stimulated with concentration series of pectin

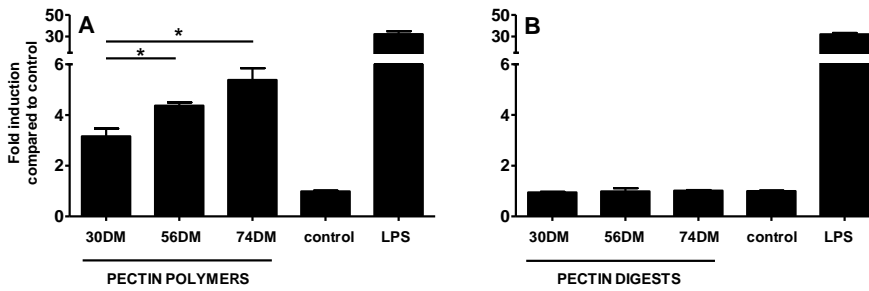


Figure 4. Activation of THP1 MD2-CD14 cells with polymeric pectins and digests of pectins of different DM (1 mg/mL). Figure A depicts activation of the cell line upon stimulation with different DM pectin polymers. Figure B shows the effect of oligomeric digests of the different DM fractions on THP1 activation. Statistical significance was determined with a one-way ANOVA and differences between the different DM fractions are indicated with an asterisk. Median and interquartile range of activation is plotted as fold induction as compared to control (n = 8).

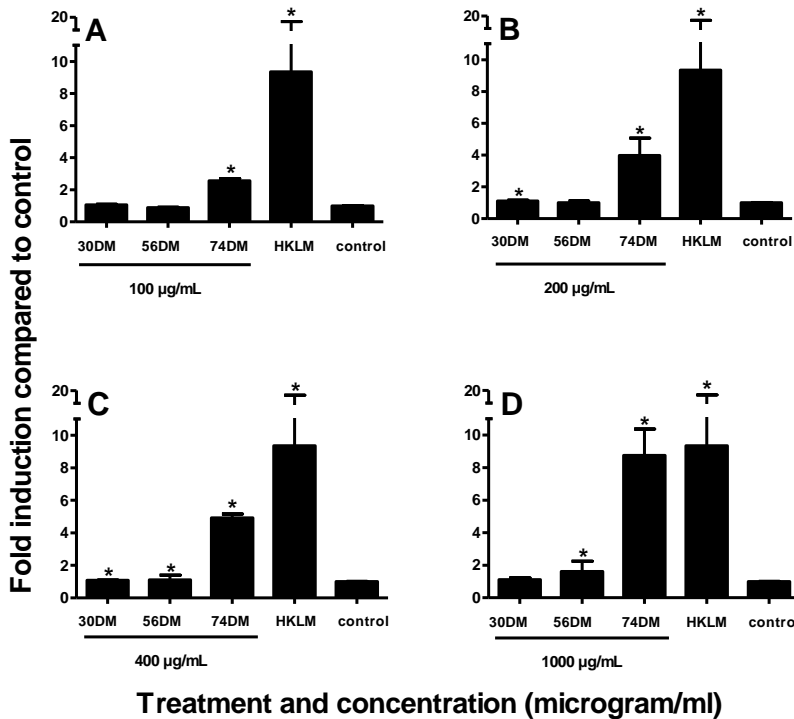


Figure 5. Stimulation of HEK-Blue hTLR2 cells with a concentration series of different DM pectin polymers. A) 100 µg/ml dose. B) 200 µg/ml dose. C) 400 µg/ml dose. D) 1000 µg/ml dose. Differences compared to control were assessed with Wilcoxon signed rank test, and are indicated by an asterisk. Median and interquartile range of activation is plotted as fold induction as compared to controls (n = 8).

polymers. As depicted in **Figure 5A-D**, DM30 pectins only activated TLR2 at the concentrations 200 and 400 $\mu\text{g}/\text{mL}$, and DM56 pectins only activated TLR2 at the concentrations of 400 and 1000 $\mu\text{g}/\text{mL}$. In addition, both DM30 and DM56 pectins only induced moderate activation. However, DM74 pectins induced a relatively strong, and dose dependent TLR2 activation over the whole concentration range tested, reaching a ca. 9-fold induction of activation at the highest concentration (1000 $\mu\text{g}/\text{mL}$, $p < 0.05$).

Figure 6 illustrates that low dosed pectins of different DM, all induced a ca. 4-fold induction of TLR4 activation, and that upon increase of the dose, only a slight increase in activation was observed as compared to the lowest dose. A subtle DM effect appears to be present suggesting that with increasing DM, pectins induce increased activation, however upon testing for multiple parameters with one-way ANOVA, these differences proved not significant. Taken together, these results demonstrate that high DM pectins can dose-dependently activate TLR2, and can also activate TLR4 in a less pronounced manner, and that lower DM pectins can activate both TLR2 and TLR4, but TLR2 activation was much less pronounced as compared to high DM pectins.

5.12 Effect of different DM pectins on TEER of T84 intestinal epithelial cells

To study whether lemon pectins protect T84 cells against PMA-induced loss of TEER and whether the DM value is important in TEER modulation, T84 cells were incubated with lemon pectin polymers with a degree of methyl esterification of 30, 56, and 74, for 24 h. Then PMA was added and the AUC for a 20-h time period after PMA addition was plotted for the different pectin treatments, as a percentage of the AUC of untreated controls, which was set to 100% (**Figure 7**).

PMA treatment caused a decrease in T84 TEER resulting in an AUC of $54.3 \pm 3.4\%$ ($p < 0.01$) as compared to control, confirming that damage was induced to the epithelial barrier. Upon 24 h of preincubation of T84 cells with 100 $\mu\text{g}/\text{L}$ DM30, DM56, and DM74 polymers, all compounds conferred protection against PMA-induced loss of resistance with TEER AUC values reaching $93.9 \pm 24.6\%$ ($p < 0.05$), $59.9 \pm 2.2\%$ ($p < 0.05$), and $77.6 \pm 15.2\%$ ($p < 0.05$) respectively, as compared to control.

These results suggest that 30DM and 74DM pectins confer considerable protection on T84 cells against a PMA-induced decrease in TEER, and that 56DM pectins exert moderate barrier protective properties.

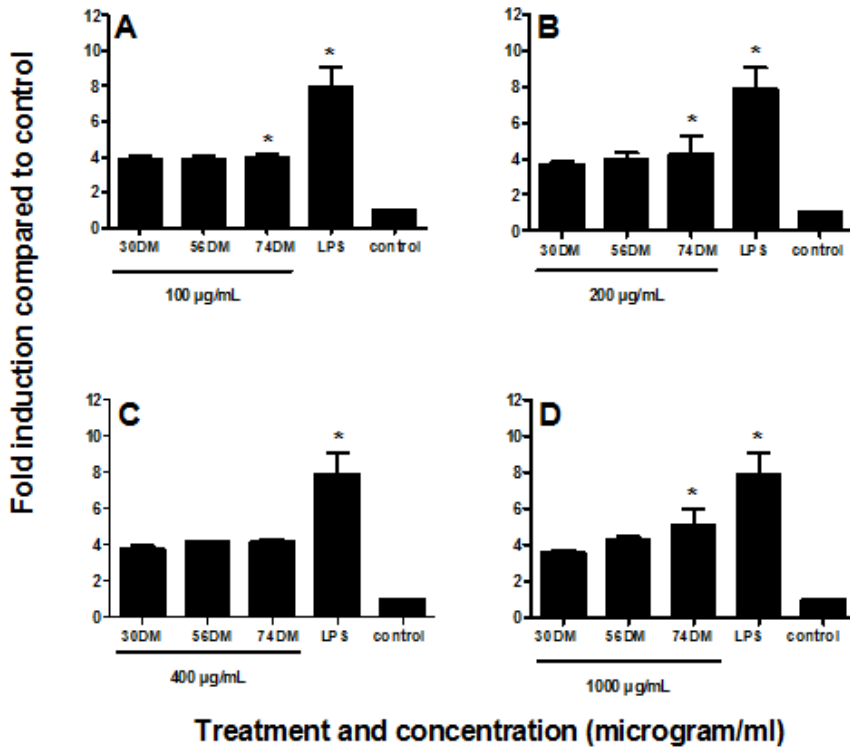


Figure 6. Stimulation of HEK-Blue hTLR4 cells with a concentration series of different DM pectin polymers. A) 100 µg/ml dose. B) 200 µg/ml dose. C) 400 µg/ml dose. D) 1000 µg/ml dose. Differences compared to control were assessed with Wilcoxon signed rank test, and are indicated by an asterisk. Median and interquartile range of activation is plotted as fold induction as compared to controls ($n = 8$).

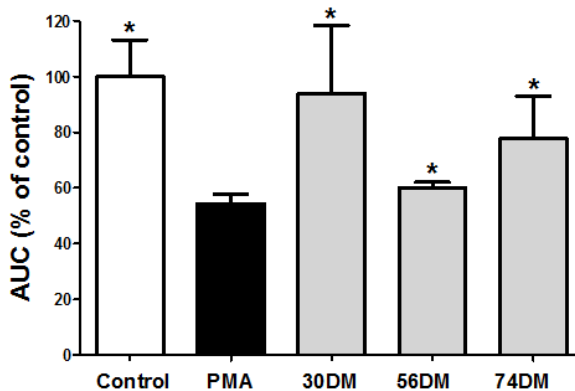


Figure 7. Barrier function of T84 human intestinal epithelial cells represented by AUCs of TEER values in time. Statistical significance levels compared to PMA were determined with a Wilcoxon signed rank test, and differences are indicated by an asterisk. AUCs are plotted as percentage of control, which was set to 100% ($n = 3$).

DISCUSSION

The aim of this study was to investigate the structure-function relationship of lemon pectins by studying the effects of DP and DM on TLR-mediated cell activation, and on the barrier function of human intestinal epithelial cells. Since several dietary fibers are ligands for innate immune receptors [9,37-40], we hypothesized that pectins could also be one of the dietary fiber types capable of activating TLRs, and that this would be one of the mechanisms by which pectins exert their immunomodulatory effects. Upon stimulating a THP1 reporter cell line, which expresses all TLRs, we observed that lemon pectins activate these human monocytic cells. Neither of the pectin compounds activated the THP1 DefMyD cells, confirming that the observed activity was mediated via TLRs. TLRs mediated immune activation can also explain the *in vivo* described immune effects of pectin. Immune activating properties of lemon pectin have been reported in animal studies which showed that supplementation prevented the induction of oral tolerance to OVA in rats, which was preceded by enhanced protein antigen penetration to the blood and activation of macrophages [47]. Moreover, in that study, lemon pectin enhanced IFN γ and TNF α production by peritoneal macrophages. In a study by Suh et al. [48], a lemon derived pectic-type polysaccharide was orally administered to mice, causing increased secretion of GM-CSF and IL-6 from Peyer's patches, indicating immune cell activation.

Some studies report immunomodulatory effects of the oligomer fractions which remain after enzymatic digestion of pectins [11]. Although these oligomers often only induced immunomodulation in combination with galactooligosaccharides and fructooligosaccharides [49,50] we decided to test such digests for their TLR activating capacities. By incubating THP1 MD2-CD14 cells with untreated and digested pectin polymers we observed that the intact polymers induced NF- κ B/AP-1 activation, but the digested fractions showed no activating capacity. The absence of activation by the digests indicates that the intact polymeric lemon pectin molecules are required for activation, and that the oligomers derived after digestion had lost this property. This suggests that the direct immunostimulatory effects of pectins will probably occur in the small intestine, as the pectins are likely to be digested by bacteria in the colon. The importance of the backbone of pectins has been thoroughly reviewed by Popov et al. [11]. In a range of vegetables, the types of pectins were characterized and the effects on immune cells were evaluated. Important

conclusions from the different types of pectins of different origins were that structural differences within pectin subtypes can induce different effects on immune cells. More specifically for lemon type pectins, the aforementioned study by Suh et al [48] also confirmed that the intact backbone was necessary for immunostimulatory effects in Peyer's patches cells, as the hydrolysate of the pectin lost its activating potential. Our experiments subscribe that the intact backbone of lemon pectins is an important structural and functional feature, as it determines the TLR-activating capacity.

Next, when comparing the untreated polymers with different DMs for their activating potential, we observed that higher DMs were associated with higher activation of THP1 MD2-CD14 cells. This activating effect for high DM lemon pectins, and inhibitory effect on immune cells was previously reported by Popov et al., [51,52] and Chen et al. [51,52]. In the latter study, different DM pectins were studied for their LPS-inhibiting effect on macrophages, and the highest DM pectin demonstrated the greatest inhibition. As the pectins were not studied without the presence of LPS, the intrinsic activation capacity of the pectins themselves was not determined in that study. Nevertheless, the inhibitory mechanism suggests that there may be a competitive interaction from the high DM pectins with TLR4. From our results it can be concluded that besides DP, also DM is a structural feature, which plays a role in the immunomodulatory potential and outcome upon immune cell contact with this particular type of pectins.

Subsequently, we studied whether the TLR-mediated activation could be attributed to TLR2 and/or TLR4 by applying the pectin compounds to HEK-Blue hTLR2 and HEK-Blue TLR4 cells. TLR2 and TLR4 are the TLRs which have so far been identified as innate receptors important in carbohydrate fiber recognition. In addition, for apple pectin an LPS-inhibiting interaction with TLR4 has been reported [53]. As the digests showed no TLR-activating properties in the THP1 cells, only the intact polymeric pectins of different DMs were applied to the HEK reporter cells. We observed that only the higher DM pectins were capable of TLR2 activation, but that all pectins, already at low doses, readily activated TLR4. These results suggest that the activating potential of lemon pectins as observed in the THP1 cells could be attributed to both TLR2, and TLR4 activation. Several mechanisms for TLR-mediated immune modulation by pectins can be suggested. As a TLR4 ligand, pectin could bind to TLR4 and in this way cause competitive inhibition of LPS-mediated activation. Although TLR4 ligation is best known for its stimulating effect on immune

reactions, and TLR2 is involved in defense against several categories of pathogens, TLR2 ligation can also induce anti-inflammatory responses, depending on its co-signaling molecules and also depending on tissue type or even type of organism [36,54-56]. In addition, TLRs have been shown to interact strongly with each other's signaling pathways [57,58] so the different outcomes in immune modulation by different DM pectins could also be related to the ratio between TLR2/TLR4 activation.

TLR2 serves as an an important barrier regulatory receptor [56], and TLR2 ligation by certain types of fibers can induce beneficial effects on intestinal epithelial cells [36]. Therefore we investigated whether incubation of T84 intestinal epithelial cells with pectins could protect the barrier function against the barrier damaging agent, PMA. In previous studies in our group, the TLR2-activating capacity of the dietary fibers short chain inulin-type fructans exerted preventive effects against barrier loss in human intestinal epithelial cells [36], and TLR2 is an important innate immune receptor with a crucial role in intestinal barrier regulation [56]. When we applied PMA to the T84 cells a decrease in TEER was observed, and by preincubating with pectins, this decrease was ameliorated, confirming a protective effect of pectins on epithelial barrier function. In two studies, bacterial infection or NSAID treatment were applied to broiler chicks and cats respectively, as challenge to the intestinal barrier [59,60]. In these studies protective effects of pectin supplementation were also observed regarding intestinal integrity and barrier function [59,60], suggesting that pectin-mediated protection of the intestinal barrier is not restricted to one damage model and can be beneficial against several toxins or pathogens.

In conclusion, DP as well as DM are physicochemical properties of lemon pectin which can determine their immunostimulatory properties and therefore may be relevant in the use of pectins for improvement of immune status. The implications of our results for functional food applications are that pectin fibers are promising immunomodulatory agents, but that studies into the physiological and more specifically the immunomodulatory effects of pectins should be meticulously designed. The origin, the extraction method, and the full chemical characteristics such as molecular weight and level and distribution of methyl esters may explain deviating observations for the immunomodulatory effects of pectins and should be included in publications in the future. Ultimately, this will facilitate side-by-side comparisons of reported immunomodulatory effects of all the different types of pectins available, providing an opportunity to develop tailored pectin fortified foods, with

well described effects supported by knowledge of the underlying physiological responses.

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