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The influence of small oligosaccharides on the immune system

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Abstract—In this study, oligosaccharides known to enhance the synthesis of penicillin by *Penicillium chrysogenum* have been presented to human immune cells and their effect measured. In addition a range of commercially available oligosaccharides have been tested. Results obtained indicate that oligosaccharides with a degree of polymerisation greater than 6 and with a tendency to form helical structures are most effective at influencing the immune system as measured by the production of reactive oxidising species. Laminariheptaose has been shown to increase reactive oxidising species production by up to 25%, whilst mannan-oligosaccharides with a DP of 6 to 7 decrease production by up to 44%. These and other results show that the immune system can recognise subtle differences in oligosaccharides and that these oligosaccharides could potentially be used to modulate the immune response.

Keywords: Immunomodulation; Oligosaccharides; Carbohydrates; Reactive oxygen species; Reactive oxidising species; 2′,7′-Dichlorodihydrofluorescein diacetate

1. Introduction

The role of oligosaccharides as elicitors of secondary metabolites and defence mechanisms from plant and fungal systems is now a well-established phenomenon.[1] Oligosaccharides can elicit defence mechanisms in plant cultures causing the production of reactive oxidising species (ROS),[2,3] whilst elicitation with oligosaccharides in fungal cultures can achieve the overproduction of secondary metabolites such as penicillin.[4,5]

The production of reactive oxidising species (e.g., H_2O_2, ·OH, HOCl, NO) by cells of the immune system (neutrophils and monocytes) is considered an essential step in the destruction of ingested microorganisms.[6] In most cases ROS production is desirable, however in the case of acute respiratory distress syndrome (ARDS), rheumatoid arthritis and ischemia-reperfusion injury the opposite is true.[7]

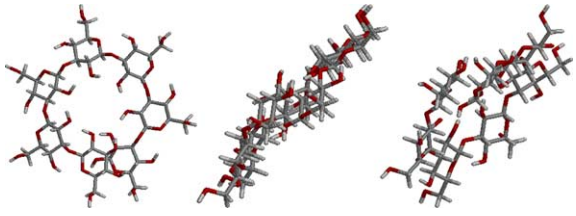

Figure 1. A 3-D representation of laminariheptaose (β-D-1→3-glucoheptaose).

Oligosaccharides such as laminari-oligosaccharides (β-(1→3)-D-glucans) have been shown to have a variety of effects on the immune system, such as inhibition of cancer metastasis,[8] antitumour activity,[9] immunological activity[10] and complement activation.[11] They are commonly found in higher fungi, plants and seaweeds. The α-helical structure of β-(1→3)-D-glucans (Fig. 1) has a history of reported immunomodulatory activity and are probably the most actively tested group of oligosaccharides. Identical in composition yet differing in conformation are malto-oligosaccharides (α-(1→4)-D-glucans, Fig. 3). Commercially produced by hydrolysis of starch by the bacterial enzyme α-amylase,

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malto-oligosaccharides are digested in the small intestine and therefore do not reach the colon intact,[12] but may be produced from undigested starch in the large intestine by microbial action. There is an absence of literature suggesting malto-oligosaccharides are researched for their immunological properties. Cyclodextrins (α, β and γ) are cyclic α-(1→4)-glucans with degrees of polymerisation (DP) of 6, 7 and 8, respectively. Their cyclic structure allows them to clathrate nonpolar molecules. Cholesterol is bound by cyclodextrins (β is the most effective, followed by α and γ[13]), which affects the associated membrane bound proteins.[14] Identical in composition to malto- and laminari-oligosaccharides is an oligoheptaose derived from pullulan (α-D-glucose-(1→6)-maltotriosyl-(1→6)-maltotriose, Fig. 5). Pullulan, an exopolysaccharide from the fungus *Aureobasidium pullulans*, finds applications in the pharmacy and cosmetic industry.[15] Arabinose is often found contained within lipopolysaccharides of *Mycobacterium tuberculosis*,[16] *Salmonella typhimurium*, *Yersinia pestis* and *Proteus mirabilis*,[17] as well as in plant polysaccharides. Arabino-oligosaccharides have a corkscrew-like conformation (Fig. 9). Oligomannuronate (OM, Fig. 11) and oligo-guluronate (OG, Fig. 13) are produced from alginate.[18] Alginate is commercially produced from the brown seaweeds; however, *Pseudomonas aeruginosa* and *Azotobacter vinelandii* also produce alginate, except with a higher ratio of mannuronate to guluronate.[19] The alginate coat of *P. aeruginosa* is known to provide protection from immune defence systems.[20,21] OM and OG are known to elicit the overproduction of Penicillin G from *Penicillium chrysogenum*.[18] Mannan-oligosaccharides (β-(1→4)-D-mannans) were obtained from Locust Bean Gum.[4] The leaf of the *Aloe vera* plant, which is known to have 'soothing' properties when applied to the skin, also contains β-(1→4)-D-mannans.[22]

We report here the results of a study in which the size and shape of oligosaccharides effects the production of ROS by the immune system. We used a variety of oligosaccharides with different degrees of polymerisation (from DP 1 to 8) and structural properties (helices, straight chains). Oligosaccharides were tested against immune cells in the presence and absence of a bacterial peptide (*N*-formyl-methionyl-phenylalanine, FMP), which induces a stimulated state in immune cells. The effects on ROS production were compared to controls.

2. Results and discussion

2.1. Laminari-oligosaccharides

The laminari-oligosaccharides show a marked difference in their effect on ROS production from immune cells depending on their degree of polymerisation (Fig. 2).

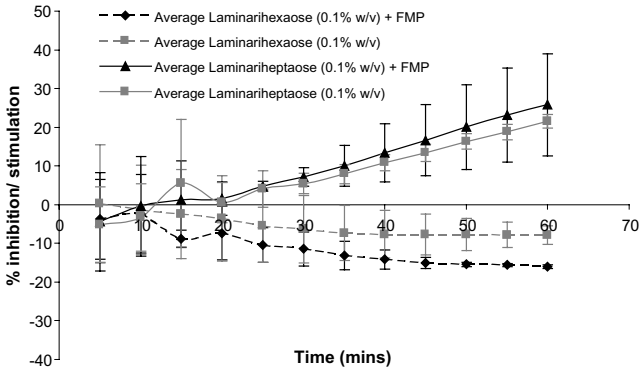

Figure 2. The effect of laminari-oligosaccharides on ROS production from immune cells.

Laminarihexaose (DP 6) had a mild inhibitory effect on ROS production from immune cells. Laminariheptaose (DP 7) however had a stimulatory effect on ROS production. Structurally the difference between laminarihexaose and laminariheptaose is the additional glucose unit in laminariheptaose (Fig. 1), which completes a rotation in the secondary structure (which brings the two 'ends' of the oligosaccharide close together). The effect of an additional glucose unit to ROS production from stimulated immune cells is a 41% change (−16% for laminarihexaose and +25% for laminariheptaose in terms of ROS production compared to control). Unstimulated immune cells show a 28% change (−7% for laminarihexaose and +21% for laminariheptaose in terms of ROS production compared to control).

2.2. Malto-oligosaccharides

The malto-oligosaccharides of DP 6 and below had limited effects on ROS production from immune cells (Fig. 4). Even though at a concentration 0.1% (w/v) maltoheptaose (DP 7) had the lowest molar concentration it gave the only significant result (15% inhibition of ROS production from stimulated and unstimulated cells) in terms of altering the production of ROS from immune cells.

As with β-1→3-glucan oligosaccharides, maltoheptaose is the structure with the two ends of the oligosaccharide chain closest together (Fig. 3).

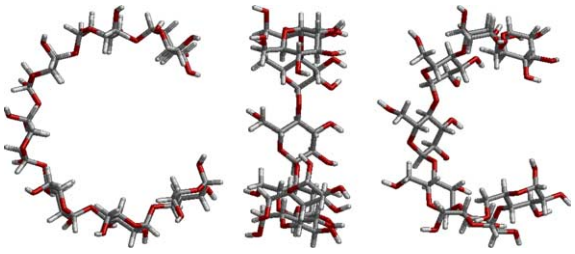

Figure 3. A 3-D representation of maltoheptaose (α-(1→4)-D-glucoheptaose).

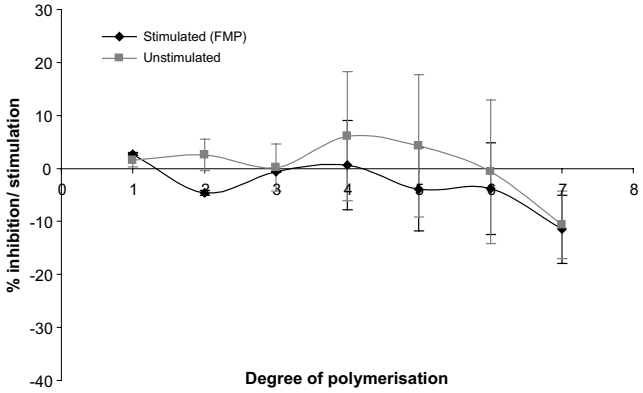

Figure 4. The effect of malto-oligosaccharides on ROS production from immune cells.

This would mean that there would be a possibility of two glucose residues interacting with a receptor (lectin), whilst the smaller malto-oligosaccharides would have these 'ends' further apart. Therefore the 'ends' of a lone oligosaccharide are less likely to be able to interact with a receptor at the same time.

2.3. Pullulan-derived heptaose

When exposed to pullulan-heptaose (DP 7, Fig. 5) immune cells were inhibited in their production of ROS (Fig. 6). Stimulated cells were inhibited slightly more than unstimulated cells.

Whether the pullulan produced by *A. pullulans* aids it in evading the immune system is not known. However, the results do show that production of ROS is reduced in the presence of a pullulan derived oligosaccharide.

2.4. Cyclodextrins

Beta-cyclodextrin (DP 7, Fig. 7) was the most effective at inhibiting ROS production from immune cells (Fig. 8). Alpha- and gamma-cyclodextrin (DP 8 and 9, respectively) were not as inhibitory as beta-cyclodextrin, with alpha-cyclodextrin being slightly more inhibitory than gamma-cyclodextrin (order of effectiveness in inhibition of ROS production beta > alpha > gamma). This followed a similar pattern to cholesterol depletion from membranes,[13] which found the order of effectiveness of cholesterol removal to be beta > alpha > gamma.

Figure 5. A 3-D representation of pullulan-heptaose.

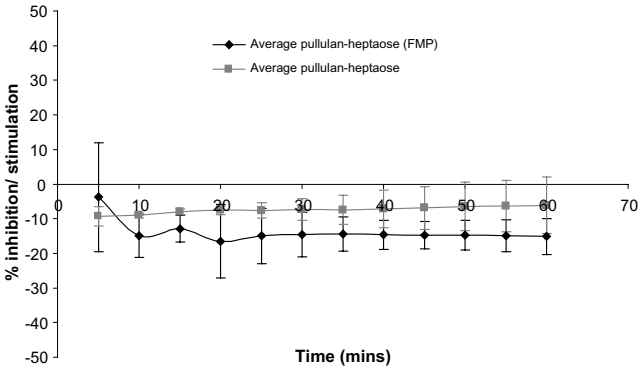

Figure 6. The effect of pullulan-heptaose on ROS production from immune cells.

Figure 7. A 3-D representation of beta-cyclodextrin (cyclic α-(1→4)-D-glucoheptaose).

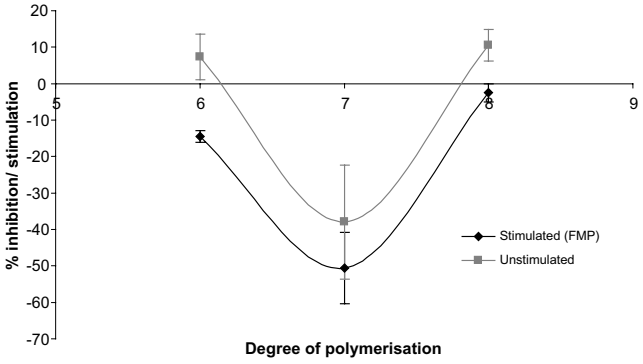

Figure 8. The effect of alpha-, beta- and gamma-cyclodextrin on ROS production from immune cells.

2.5. Arabino-oligosaccharides

The arabino-oligosaccharides (Fig. 9) also showed a correlation between degree of polymerisation and effect on ROS production (Fig 10). As the DP of the arabino-oligosaccharides increases, ROS production by immune cells decreases. Arabino-oligosaccharides have a mild inhibitory effect on ROS production from immune cells. Stimulated cells are more affected by the addition of the arabino-oligosaccharides. However, for both stimulated and unstimulated cells there was a correlation between degree of polymerisation and effect on ROS production.

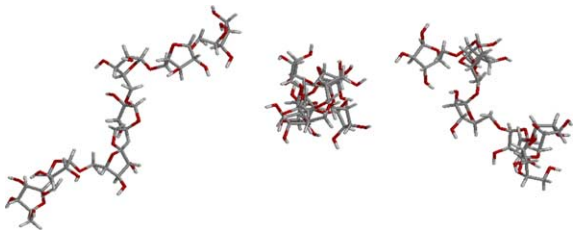

Figure 9. A 3-D representation of arabinoheptaose (α-L-1 → 5-arabinoheptaose).

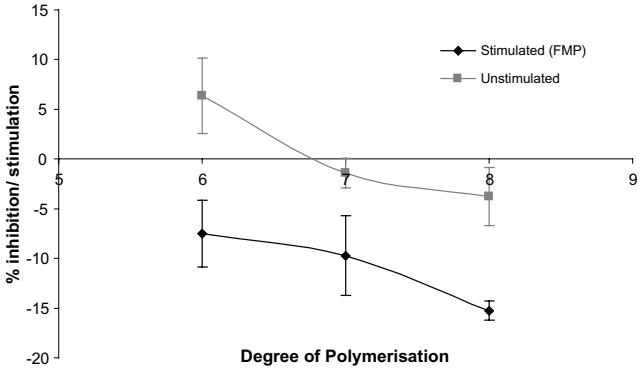

Figure 10. The effect of arabino-oligosaccharides on ROS production from immune cells.

2.6. Alginate-derived mannuro-oligosaccharides and gulurono-oligosaccharides

Oligo-mannuronate (a mixture of DP 7 and 8) had an inhibitory effect on ROS production from immune cells (Fig. 12). These inhibitory effects were mainly seen on stimulated cells. Whether one of the two DP sizes had more effect on ROS production, or both had similar effects is not known.

Oligo-guluronates of DP 6 and DP 10 were tested (separately) for effects on ROS production from immune cells. Neither of the samples had any significant effect on ROS production (data not shown).

The 3-D structure of mannuronate oligosaccharides and guluronate oligosaccharides show them both to be 'straight chains' (Figs. 11 and 13). Guluronate oligosaccharides are known to form an 'egg-box' when exposed to calcium ions.[23] As there will be calcium ions within the cell medium, it is possible that if oligo-guluronate has any immunomodulatory properties they would be masked by the formation of 'egg-boxes'.

Alginates from *Pseudomonas* spp., only have single units of L-guluronate (i.e., not in 'blocks') present.[24] The

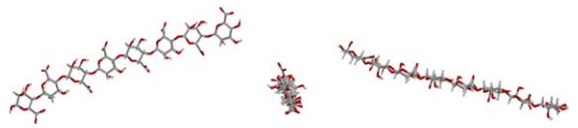

Figure 11. A 3-D representation of β-(1 → 4)-D-mannuronoheptaose.

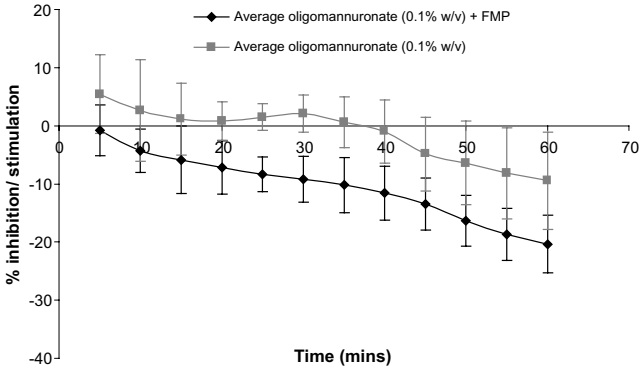

Figure 12. The effect of oligomannuronate (mixture of DP 7 and 8) on ROS production from immune cells.

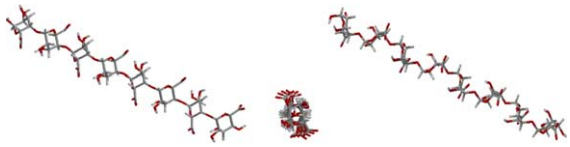

Figure 13. A 3-D representation of α-(1 → 4)-L-guluronoheptaose.

fact that *P. aeruginosa* produces alginate with a higher amount of oligo-mannuronate than the algal alginates and that the alginate provides protection from the host's innate immune system,[19–21] suggests there may be a link between the mannuronate content and the organisms ability to evade the immune system. There is also a possibility that the oligo-mannuronate has a ROS 'scavenging' ability.

2.7. Manno-oligosaccharides

Mannan-oligosaccharides (a heterogenous mix of DP 5 to 7) had an inhibitory effect on ROS production (Fig. 14) from both mixed immune cell and neutrophil suspensions. The 3-D structure of manno-oligosaccharides is similar to that of oligo-mannuronates, in that the secondary structure of both is straight chain.

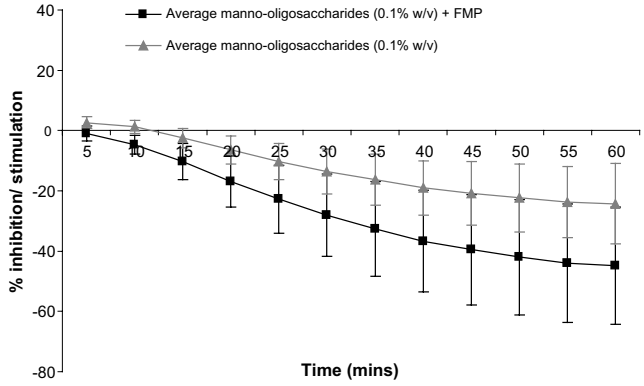

Figure 14. The effect of mannan-oligosaccharides on ROS production from immune cells.

Mannan-oligosaccharides inhibited ROS production to a greater extent in stimulated cells. This suggests a cell membrane receptor based mechanism behind the observed effects. This is because stimulated cells have more receptors displayed from the up-regulation, of membrane receptors, caused by the stimulant (FMP).

The *Aloe vera* leaf gel used in cosmetic products is well known to possess 'soothing' properties for inflammations. This gel contains β-(1→4)-D-mannan chains, thus suggesting a link between β-(1→4)-D-mannan content and the *Aloe vera* 'soothing' ability.

3. Conclusions

To conclude, laminariheptaose shows a distinct change in activity from laminarihexaose. 3-D molecular modeling shows that the two 'ends' of laminariheptaose are in close proximity to one another, and therefore would be preferably situated with an appropriate lectin. Malto-oligosaccharides with a DP <6 showed no activity but DP 7 inhibited ROS production. The two 'ends' of maltoheptaose, like laminariheptaose, are situated in close proximity. The pullulan-heptaose also has its two 'ends' close to one another and, similar to maltoheptaose, has an inhibitory effect towards ROS production. The activity of the cyclodextrins may be attributed to its cholesterol depleting ability. The arabino-oligosaccharides also show a change in effect with a change in DP size. As the DP increases from 6 to 8 a greater inhibitory tendency towards ROS production is shown. Of the alginate derived oligosaccharides only oligo-mannuronate affected ROS production (however, if the oligoguluronates had any potential effects they may have been negated by 'egg-boxing' with any calcium). Mannan-oligosaccharides had a greater inhibitory effect on ROS than the oligo-mannuronate. Overall it can be seen that there is a direct relationship between size and effect, and there also appears to be a link between structure and effect. Review of these results shows that the immune system can recognise the subtle differences in oligosaccharide composition. Interpretations of these results are speculative; it is most likely that interactions between oligosaccharides and human cells would occur in the gastrointestinal tract. It is here where oligosaccharides will occur as dietary components or resulting in microbial hydrolysis of polysaccharides.

4. Experimental

4.1. Immune cell separation

Whole blood was taken from healthy volunteers and placed on to a histopaque solution (1.119 g/mL [Sigma]). Centrifugation forces erythrocytes to travel through the histopaque whilst immune cells reside above the solution, immune cells were carefully removed. Residual erythrocytes were removed through hypertonic lysis. The immune cells were then washed (twice) in phosphate buffered saline (PBS [Sigma]), counted and prepared to a concentration of 8×10^8 cell/L in PBS. Further details on immune cell separation can be obtained from Sigma.

4.2. DCFH-DA preparation

2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA [Sigma], 0.002 g) was dissolved in 1 mL of ethanol. Once dissolved this was added to a 1 L solution of PBS with 0.17% (w/v) sucrose added.

4.3. Oligosaccharide preparation

Oligosaccharides were prepared at 0.1% (w/v) concentrations in PBS solution. Oligosaccharides were obtained from the following sources: laminari-oligosaccharides (Dextra laboratories), malto-oligosaccharides (Sigma), pullulan derived oligoheptaose (Megazyme), cyclodextrins (Sigma), arabino-oligosaccharides (Megazyme), mannan-oligosaccharides (produced as in Ref. 18), oligo-mannuronate and oligoguluronate (produced as in Ref. 4).

4.4. Stimulant preparation

The bacterial peptide *N*-formyl-methionine-phenylalanine (FMP [Sigma]), was used as a stimulant and thus an activator or ROS production from immune cells. The FMP concentration used was approximately 0.0033 M/L. The FMP was dissolved in RPMI 1640 (cell culture medium [Sigma]).

4.5. DCFH-DA method

All oligosaccharides were tested both 'positively' [in the presence of a bacterial peptide (e.g., *N*-Formyl-Met-Phe or *N*-Formyl-Met-Leu-Phe)] and 'negatively' (in the absence of a bacterial peptide). Positive and negative controls had no oligosaccharide present. The test was performed in a Dynatech 'white clear bottom microplate'. Each well on the microplate contained 90 μL of oligosaccharide solution (controls had PBS), 90 μL of neutrophil solution (these two solutions were left together for 5 min to sensitise the immune cells), 90 μL RPMI 1640 (positive wells had LPS in the RPMI) and 90 μL DCFH-DA solution. Fluorescence was recorded every 5 min for 60 min on a Packard fluorescent microplate reader at 485 nm excitation and 530 nm emission. The gain was set at 1 unit and the PMT (photomultiplier tube) was set at 1100 units. No maximum was set for any well. Further details on the 'DCFH-DA method' can be found in Bland et al.[7]

4.6. Molecular modeling

All 3-D figures were generated on Hyperchem software using the 'sugar builder' package. All bond lengths and angles are automatically calculated within the program. Hence the 3-D structures shown should be used as a guide and not definitive.

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