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Study on the analytical evaluation of immunoactive sulfated polysaccharides by ligand-assisted nuclear magnetic resonance spectroscopy

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（リガンド支援型核磁気共鳴法による免疫賦活活性を持つ硫酸化多糖の分析評価に関する研究）
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Thesis Summary

Sulfate groups in sulfated polysaccharides, particularly fucoidan, play a key role in the recognition of sulfated polysaccharides in immune cell such as macrophage, participate to exhibit various physiological functions of sulfated polysaccharides such as anti-tumor and immunostimulatory effects. The activated macrophage by recognizing fucoidan produces proinflammatory molecules such as nitric oxide (NO) and tumor necrosis factor (TNF)-α. Fucoidan is expected to cooperate with other ligands such as β-glucan to activate multiple receptors on macrophage membrane, enhancing immune-stimulation activity. However, due to an absence of clear information about receptors and an appropriate method for determining sulfate content in natural sulfated polysaccharides without destructive pretreatments such as acid hydrolysis and acid-based extraction procedure, the relationship between sulfate group and physiological functions of fucoidan is still uncertain. Therefore, the present study largely aimed to two aspects; one is the investigation of the synergistic immune-stimulation activity of fucoidan with β-glucan, and the other is the establishment of a new protocol for estimating sulfate content in seaweed polysaccharides.

Firstly, RAW264 cells were simultaneously treated with *Cladosiphon okamuranus*-derived fucoidan and zymosan, *Saccharomyces cerevisiae*-cell wall derived β-glucan-rich particle, to investigate the synergistic effect between fucoidan and β-glucan. As a result, fucoidan was located on cell surface, and stimulated RAW264 cells to secrete proinflammatory molecules including NO and TNF-α. Additionally, fucoidan cooperated with zymosan to enhance the immune function in RAW264 cell. Lipid rafts, which scaffold for recruitment of pattern-recognition receptors (PRRs), such as dectin-1 and toll-like receptors (TLRs), during the activation of macrophage, were revealed to be involved in the synergistic immune-stimulation activity. Notably, dectin-1 was essential for the synergistic effect between fucoidan and zymosan.

Secondly, ^{1}H NMR titration method was applied for establishing a new assay to estimate sulfate content in sulfated saccharides including natural polysaccharides. By screening several candidates, imidazole, showing a well-resolved ^{1}H NMR signal largely shifted toward down-field in the presence of sulfated disaccharide (NB4S), was selected to explore a stoichiometric complex formation between sulfate group in saccharides and ligand. The observed chemical shift (δ) value of imidazole in the presence of NB4S was constant until molar ratios of imidazole to sulfate group were 1:1, whereas at > 1:1 molar ratio, the δ values of imidazole gradually shifted toward up-field, indicating that imidazole formed the 1:1 stoichiometric complex with sulfate group in saccharides. Therefore, by schematization of δ values of imidazole in the presence of sulfated saccharide against the molar concentration of imidazole, the molar concentration of imidazole at the predicted inflection point of the plot may corresponding to sulfate content in saccharides.

On the other hand, the stoichiometric complex between imidazole and sulfate group in saccharides was not affected by non-sulfated saccharides. Carboxylated saccharide (galacturonic acid) ionically interacted with imidazole, while after the elimination of carboxy group in galacturonic acid, the interaction between imidazole and carboxy group was vanished. Therefore, to apply the present ^{1}H NMR assay for estimating sulfate content in natural polysaccharides, the elimination of carboxy group in polysaccharides should be prioritized. To remove carboxy group in polysaccharides, alginate (carboxy group rich polysaccharide) was eliminated by centrifugal precipitation with calcium chloride, and remaining carboxy group in the alginate-free extract was chemically reduced using EDC and $NaBH_4$. Totally, twenty-one samples, including a commercially available *Fucus vesiculosus*-derived fucoidan and twenty seaweed crude polysaccharides extracted from seaweeds spanning nine species and various geographic locations, were used for the application of the proposed imidazole-assisted ^{1}H NMR assay. As a result, the estimated sulfate contents of all samples by the present assay were matched with those by a conventional barium-rhodizonate assay. This result indicated that imidazole was an appropriate ligand to form 1:1 stoichiometric complex with sulfate group in sulfated saccharides, and the proposed imidazole-assisted ^{1}H NMR assay can be extensively applied for estimating sulfate content in various natural polysaccharides.

In conclusion, the present study describes for the first time that fucoidan cooperates with β-glucan, simultaneously activating multiple receptors such as dectin-1 on lipid rafts to enhance immune-stimulation activity. In addition, the findings demonstrate that the imidazole-assisted ^{1}H NMR can be widely applied for estimating sulfate content in diverse sulfated saccharides including natural polysaccharides without destructive pretreatments, which may affect to structure of polysaccharides.