

# Application of NMR techniques to identify compounds with binding affinity to macromolecules

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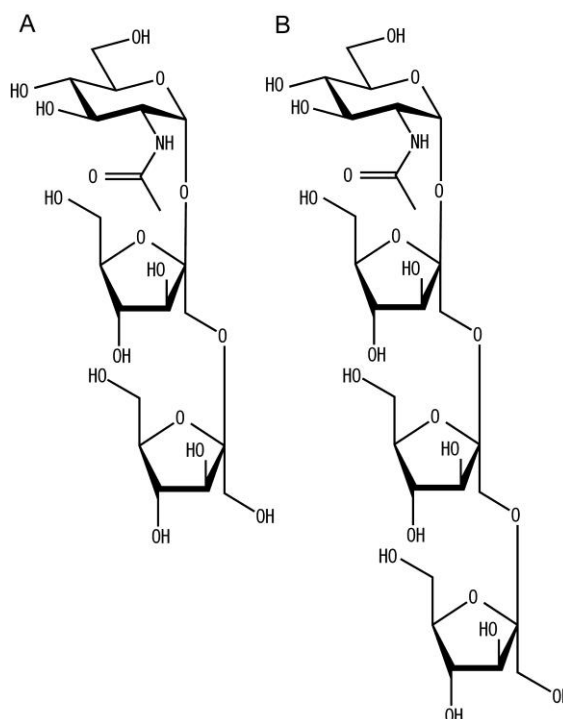
## Abstract

The recent developments of NMR techniques, including high-field magnets and super-cooled probes, enables us to perform sensitive NMR experiments. Various NMR experiments, such as measuring diffusion coefficients, enables to analyze the mixture samples. In the present research, inter-molecular interactions between proteins and ligands have been investigated, and selective detection of bound ligands has been tried using NMR spectroscopy. The structure determination of novel sugars and multivariate analysis of tea components have also carried out.

## Section 1. Structure determination of small compounds

### Structure determination of novel oligosaccharides from *N*-acetylsucrosamine using $\beta$ -fructofuranosidase from *Aspergillus oryzae*

Using a transfructosylation-catalyzing  $\beta$ -fructofuranosidase (FFaseI) extracted from *Aspergillus oryzae* NBRC100959 mycelia, two novel oligosaccharides consisting of GlcNAc and fructose, *N*-acetyl-1-kestosamine (1-KesNAc) and *N*-acetylnystosamine (NysNAc) were synthesized from *N*-acetylsucrosamine (Fig. 1-1), and structures were determined using various 2D NMR experiments. The reaction was carried out also using dried *A. oryzae* mycelia containing FFaseI as the major  $\beta$ -fructofuranosidase, and the yields of 1-KesNAc and NysNAc were 22.3% and 5.42% (molar yield from SucNAc), respectively. This whole-cell catalysis method facilitates the synthesis of 1-KesNAc and NysNAc because extraction and purification of FFaseI from mycelia are unnecessary.

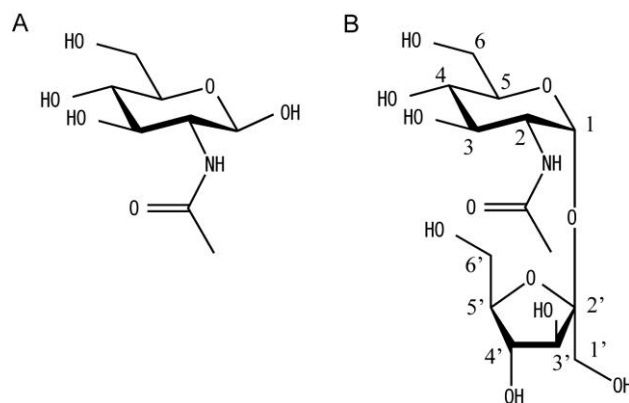


**Fig. 1-1** Structures of  
(A) *N*-acetyl-1-kestosamine,  
(B) *N*-acetylnystosamine

## Section 2 Analysis of inter-molecular interactions

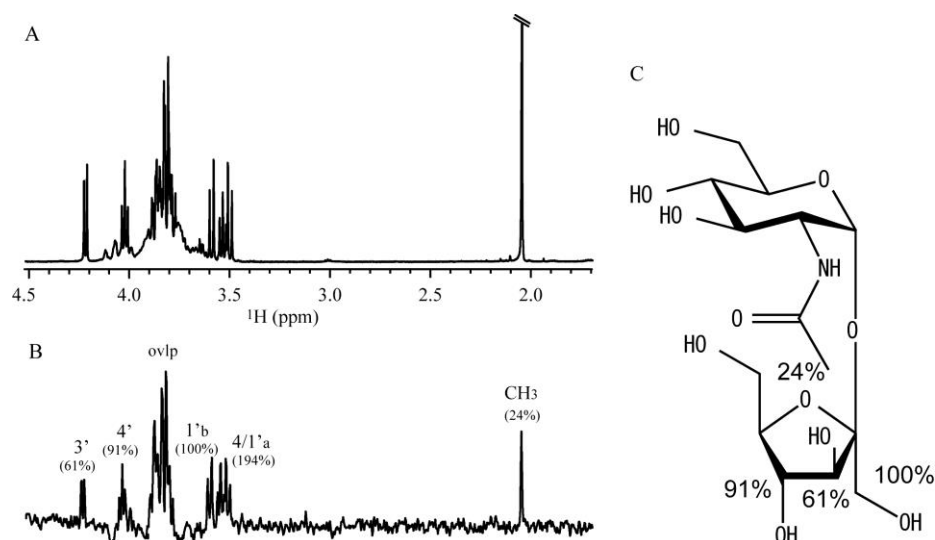
### 2-1 Analysis of affinity of $\beta$ -D-fructofuranosyl-(2 $\leftrightarrow$ 1)-2-acetamido-2-deoxy- $\alpha$ -D-glucopyranoside for yeast $\beta$ -fructofuranosidase using NMR spectroscopy

$\beta$ -D-fructofuranosyl-(2 $\leftrightarrow$ 1)-2-acetamido-2-deoxy- $\alpha$ -D-glucopyranoside (*N*-acetylsucrosamine; SucNAc, Fig. 2-1) is an enzymatic product from sucrose and 2-acetamido-2-deoxy-D-glucose (GlcNAc), and cannot be hydrolyzed by yeast  $\beta$ -fructofuranosidase. Although the distinct binding of SucNAc with the  $\beta$ -fructofuranosidase could not be identified by isothermal titration calorimetry or  $^1\text{H}$  NMR titration, unambiguous signals indicating molecular interactions were observed by STD NMR experiments. An inhibitory activity of SucNAc in hydrolysis of sucrose was also confirmed by  $^1\text{H}$  NMR spectroscopy. Because hydrolysis of sucrose was unaffected in the presence of GlcNAc, a disaccharide moiety including an acetamido group is considered to be crucial to show an inhibitory activity for the  $\beta$ -fructofuranosidase.



**Fig. 2-1** Structures of  
(A) *N*-acetylglucosamine,  
(B) *N*-acetylsucrosamine

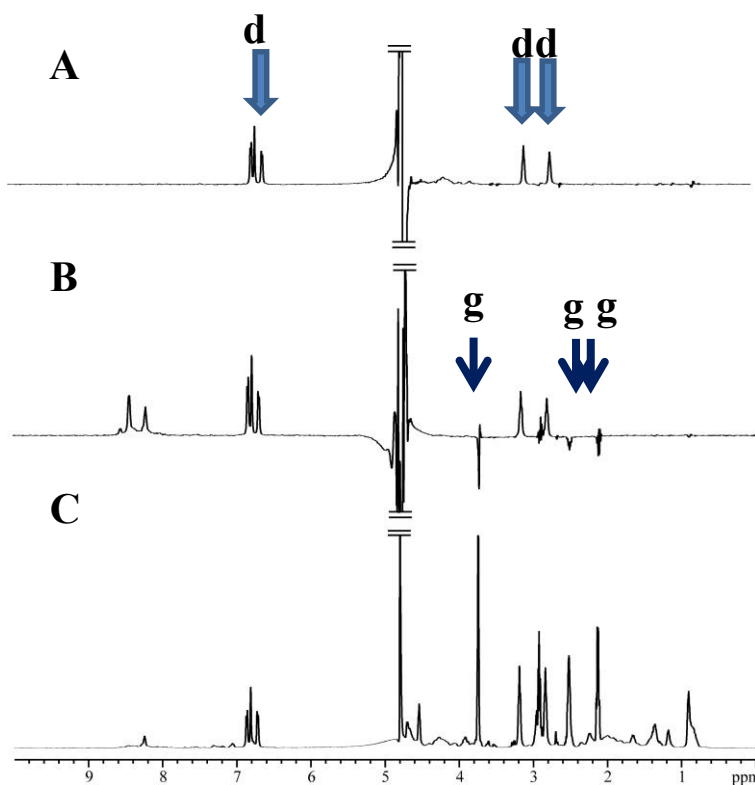
Hydrolysis of sucrose in the presence of SucNAc was observed using  $^1\text{H}$  NMR spectroscopy, and time course of H1 signal area of sucrose resonating at 5.40 ppm was monitored. Hydrolysis of sucrose was slightly inhibited in the presence of equal amount of SucNAc, and it was distinctly inhibited in the presence of 10-fold molar excess of SucNAc. These results indicate that SucNAc can be recognized by  $\beta$ -fructofuranosidase and possesses an inhibitory activity for this enzyme. Because the structural difference between sucrose and SucNAc is the acetamido group at the C2 position, it can be considered that an acetamido group should play a role in the inhibition effect. To observe the effect of GlcNAc moiety on the point of the inhibitory activity, hydrolysis of sucrose in the presence of GlcNAc was also investigated. It was shown that GlcNAc had almost no inhibitory activity for  $\beta$ -fructofuranosidase, indicating that the GlcNAc moiety alone is ineffective to show the inhibitory activity. The saturation transfer difference NMR experiment using WET-pulse sequence was carried out to identify segments of SucNAc in direct contact with  $\beta$ -fructofuranosidase (Fig. 2-2).



**Fig. 2-2** (A) The expanded reference spectrum of a mixture of 3 mM SucNAc and 6  $\mu$ M  $\beta$ -fructofuranosidase. (B) The expanded WET-STD spectrum of the same sample. (C) A structure of SucNAc and the relative degrees of saturation of the isolated protons normalized to that of H1'b as determined from the WET-STD.

## 2-2. Molecular interactions between $\alpha$ -synuclein and dopamine in the oligomerization

$\alpha$ -Synuclein is the major component of the filamentous Lewy bodies and Lewy neurites that define neuropathological features of Parkinson's disease and dementia with Lewy bodies. To investigate the oligomerization process of  $\alpha$ -synuclein in association with dopamine (DA), the structural propensities to form oligomers were studied using NMR (Fig. 3-1) and other biophysical techniques. The  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra indicated that both N- and C-termini interacted with DA. Although interactions with DA were also observed in the presence of glutathione by ESI-MS (Fig. 3-2), the significant suppression of oligomerization was observed in the size exclusion



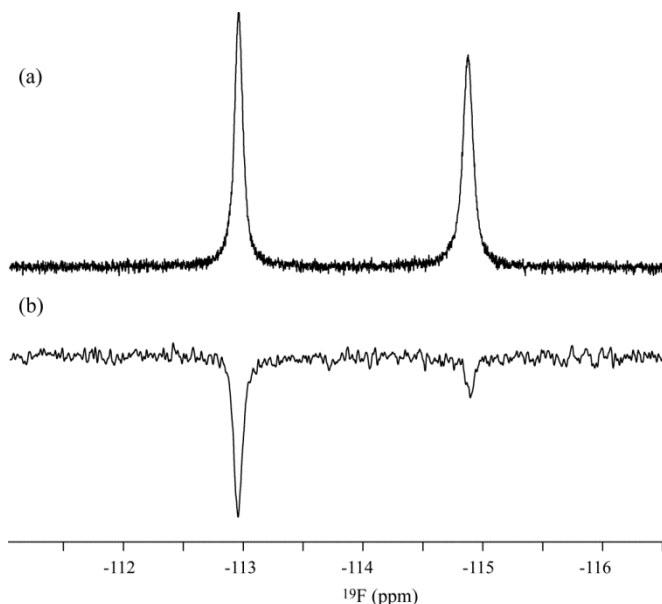
**Fig. 2-3** WaterLOGSY spectra of (A) 0.1 mM  $\alpha$ -syn+8 mM dopamine (d), (B) 0.1 mM  $\alpha$ -syn+8 mM dopamine+8 mM glutathione (g), (C)  $^1\text{H}$  spectrum of (B).

chromatography, suggesting that oxidations of  $\alpha$ -synuclein are required for its oligomerization.

### 2-3. Analysis of interactions between diflunisal and human serum albumin

In analysis of fluorinated compounds, some versatile NMR techniques, which are applicable to the NMR screening as well as structure determination, are required to be developed. Although  $^{19}\text{F}$  NMR spectroscopy is one of the most useful techniques in analyzing the fluorinated compounds, the conventional NMR spectrometer consoles consist of a single high and a few low band amplifiers, which is incapable of performing  $^1\text{H}$ - $^{19}\text{F}$  heteronuclear experiments. Here, we propose a simple and efficient approach to achieve  $^1\text{H}$ - $^{19}\text{F}$  heteronuclear experiments using a conventional spectrometer equipped with a  $^1\text{H}/^{19}\text{F}/^{13}\text{C}$ -double tuned probe. Its efficiency was demonstrated in application to the  $^1\text{H}\{^{19}\text{F}\}$  and  $^{19}\text{F}\{^1\text{H}\}$  saturation transfer difference (STD) experiments using the complex of diflunisal and human serum albumin (HSA).

In the  $^{19}\text{F}\{^1\text{H}\}$  STD experiment, the proton resonance at ca. -1.0 – 0.0 ppm, corresponding to the protein resonance, was initially excited, and during the subsequent process, the proton magnetization of the protein was transferred to fluorine of the ligand via direct or indirect relay processes. The  $^{19}\text{F}\{^1\text{H}\}$  STD spectrum of the diflunisal-HSA complex indicated that  $^{19}\text{F}$  nuclei resonating at -112.9 ppm of diflunisal were in close proximity to protons of HSA (Fig. 2-4).



**Fig. 2-4 5.0mM diflunisal + 0.1mM HSA (a)  $^{19}\text{F}$  spectrum, (b)  $^{19}\text{F}\{^1\text{H}\}$  STD spectrum. In (b),  $^1\text{H}$  irradiation was carried out at 0.0 ppm.**

### Section 3. Multivariate analysis: Analysis of tea ingredients using NMR spectroscopy

Quality assessments of tea ingredients, harvested in several elevation places in Sri Lanka, were performed using multivariate analysis techniques. An analysis of ingredients in tea was also performed using 1D/2D NMR techniques. Various ingredients, such as theanine, caffeine, thea

flavin and theaflavin 3,3'-digallate, were identified, and characteristic difference among the harvest places of tea leaves were also investigated. The present results are expected to be applied to the food and pharmaceutical sciences on the point of quality control.

#### Publication list

- 1) *Carbohydr. Res.*, 2013, 382, 108-112.
- 2) *J. Carbohydr. Chem.*, 2014, 33, 498-505.
- 3) *J. Biophy. Chem.* 2013, 4, 110-114.
- 4) *Magn. Reson. Chem.* 2015, 53, 327-329
- 5) *J. Agric. Food Chem.*, 2011, 59, 5181-5187.