

# Stabilization of Proteins

## Lyophilization: Method

### MATERIALS

TREHALOSE SG:	Lot No. 70822
Sucrose:	Special grade, Lot No. CKQ1328 (Wako Pure Chemical Industries, Ltd.)
D-Mannitol:	Special grade, Lot No. ALP7215 (Wako Pure Chemical Industries, Ltd.)

### REAGENTS

Polysorbate 80:	Chemical grade, Lot No. TPL4338 (Wako Pure Chemical Industries, Ltd.)
Anti TNF- $\beta$ 1 monoclonal antibody (MAb-TNF- $\beta$ 1):	Lot No. 980505 (Nagase Viita Co., Ltd.)
TNF- $\beta$ :	Lot No. B-016 (Nagase Viita Co., Ltd.)

### TEST SOLUTION

Buffer:	50 mM Sodium phosphate buffer, pH 6 containing 0.6 mg/mL polysorbate 80
Concentration of carbohydrate:	80 mg/mL
Concentration of antibody:	20 mg/mL
Content:	0.5 mL/vial

### LYOPHILIZATION

Test solutions were transferred to glass vials and frozen at -40°C overnight. The vials were placed in a lyophilization chamber and dried at -30°C, -20°C, -10°C, 0°C, 5°C, 10°C, 20°C and 25°C for 6 hr at each temperature. Thereafter the vials were purged with nitrogen purged and sealed.

### DURATION OF STORAGE

Temperature and Duration: 40°C for 1, 3, and 6 months, and 60°C for 1, 2, 3 and 6 weeks.

### NEUTRALIZING ANTIBODY TITER

Lyophilized antibodies were completely rehydrated with 0.5 mL of distilled water. Antibody solutions were serially diluted and mixed with 30JRU/mL TNF- $\beta$ . Neutralizing antibody titers of the mixtures were measured as a change in cytotoxicity against LM cells.

### GEL FILTRATION CHROMATOGRAPHY

Column: G3000SWXL, ( $\phi$ 7.8mm $\times$ 30cm, Tosoh Corp.)  
Solvent: 50mM Sodium phosphate buffer, pH6.0 containing 0.2M Arginine-HCl  
Flow rate: 1mL/min  
Method: Each lyophilized antibody was completely rehydrated by addition of 0.5mL of distilled water. Antibody solutions were centrifuged at 17,000 $\times$ g for 10 min and 0.5mL of the supernatant were applied to liquid chromatography.

### WATER CONTENT

Equipment: Karl Fischer moisture titrator MKC-210 (Kyoto Electronics Manufacturing Co., Ltd.)  
Method: Water content was measured in accordance with Japanese Pharmacopoeia, General tests, Water determination test by the coulometric titration method.

### POWDER X-RAY DIFFRACTION SPECTROSCOPY

Equipment: X<sup>o</sup> Pert PRO MRD (Spectris plc.)  
X-ray: 40mA, 45kV, scanning angle range: 5 - 65° ( $2\theta$ )  
Method: X-ray analysis was conducted in accordance with Japanese Pharmacopoeia, General tests, X-ray powder diffraction method.

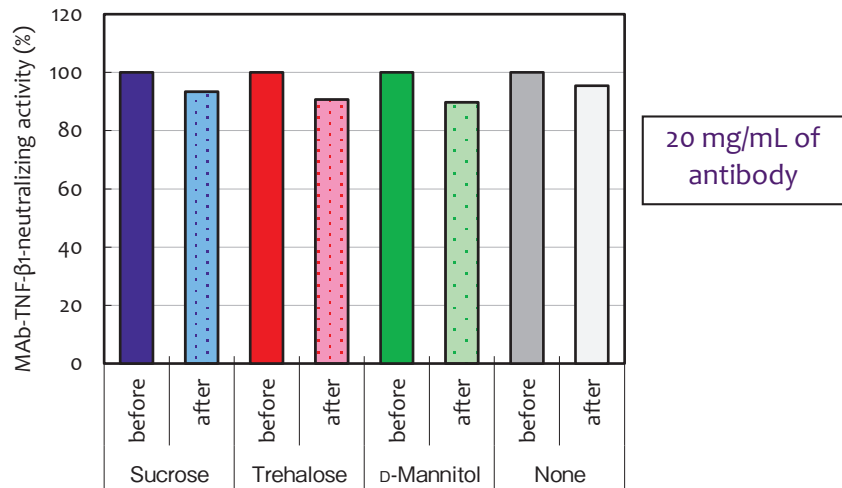
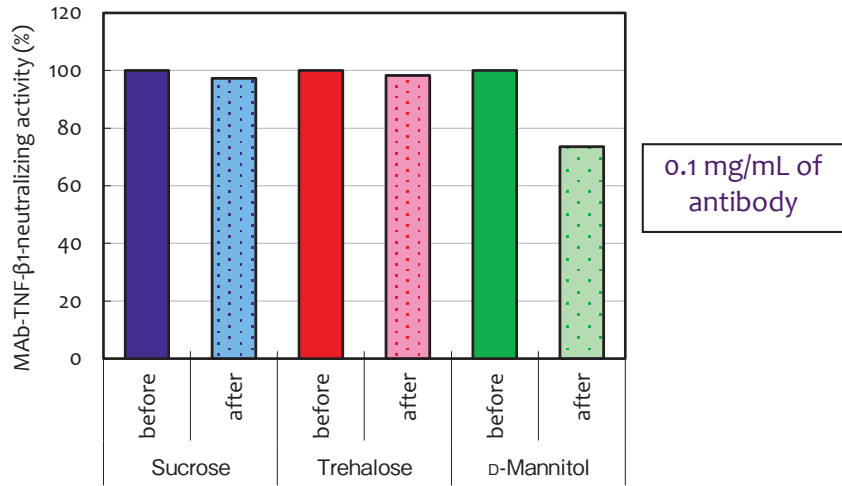
### DSC ANALYSIS

DSC analysis: DSC Q20 (TA instrument)  
Atmospheric gas: Nitrogen at 50 mL/min  
Method: DSC analysis was conducted in accordance with Japanese Pharmacopoeia, General tests, Thermal analysis.

Lyophilization: Results

RESULT 1

The neutralization activity of antibodies before and after lyophilization (no storage) are shown in the following tables:

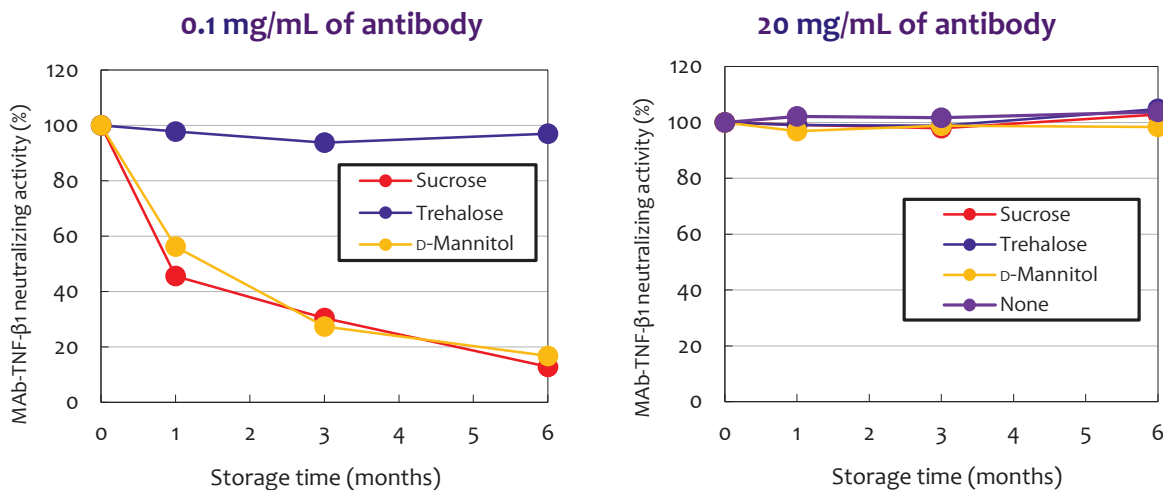


CONCLUSION 1

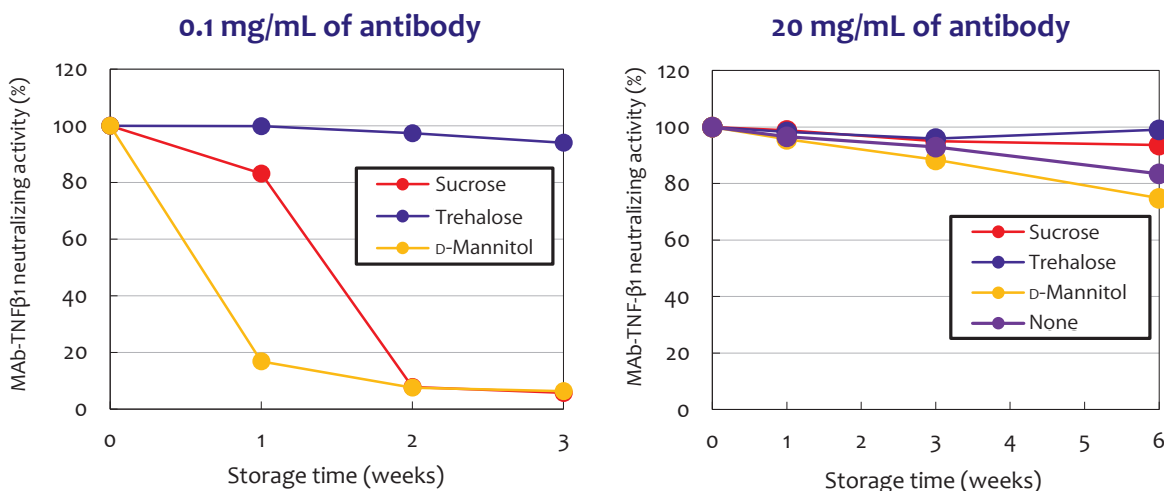
Prominent decrease of the neutralization activity after lyophilization of antibody was not observed also in the presence of TREHALOSE SG.

**RESULT 2**

Residual rates of neutralization activity of lyophilized MAb-TNF-β 1 after storage at 40°C



Residual rates of neutralization activity of lyophilized MAb-TNF-β 1 after storage at 60°C

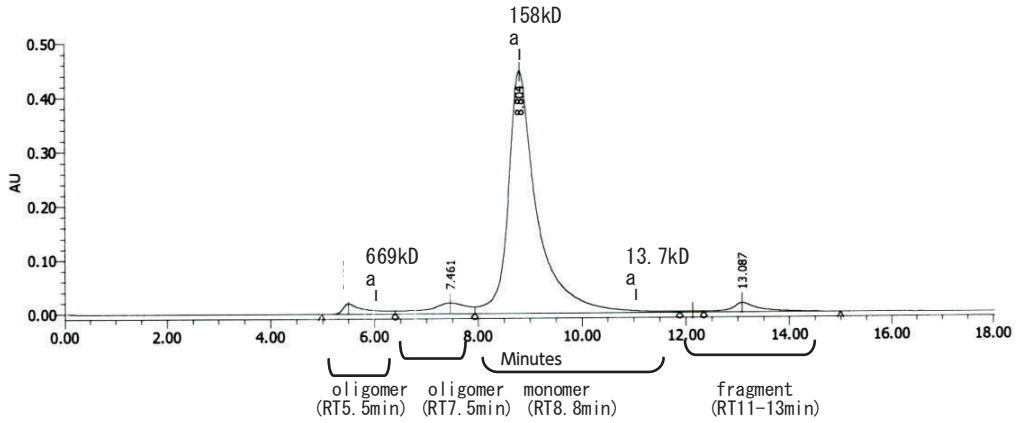


**CONCLUSION 2**

TREHALOSE SG inhibited a decrease of the neutralization activity of the lyophilized antibody at 0.1 mg/mL after storage at 40°C or 60°C. The inactivation of lyophilized antibody at 20 mg/mL after storage at 40°C was hardly observed in all samples including the untreated control. After storage at 60°C, TREHALOSE SG maintained the neutralization activity of the antibody better than Sucrose or D-Mannitol.

**RESULT 3**

A typical elution pattern using gel filtration chromatography of lyophilized antibody after storage at 60°C without any stabilizer is shown below:

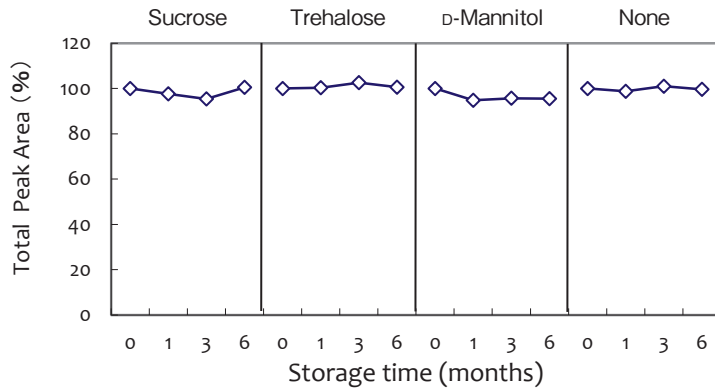


Retention times of peaks of gel filtration chromatogram of lyophilized antibody

Retention time (min)	Component
5.5	oligomer (trimer or more polymerized oligomer)
7.5	oligomer (dimer)
8.8	monomer
11 ~ 13.5	fragments

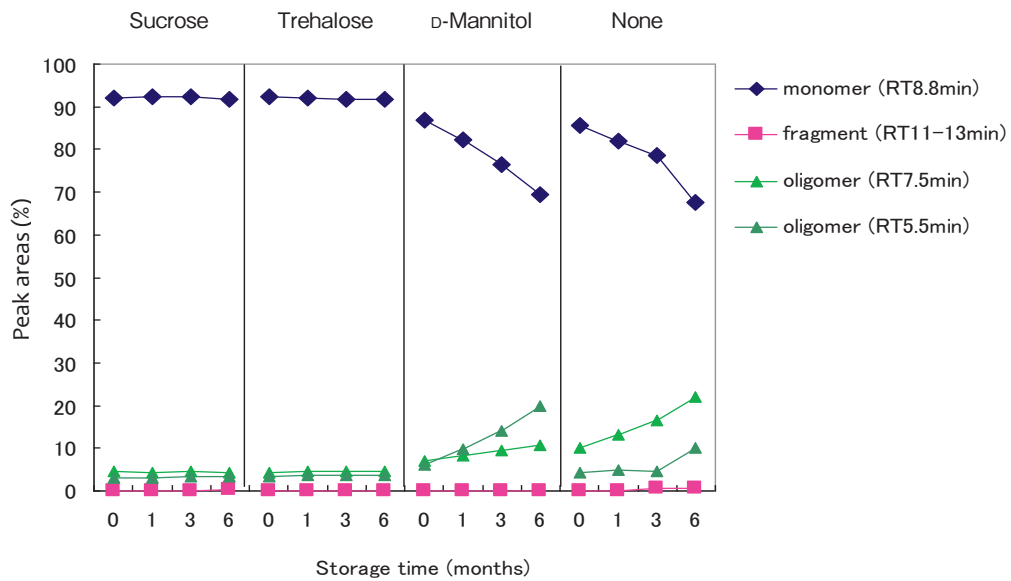
RESULT 4

Total peak area of gel filtration chromatogram of lyophilized MAb-TNF-β 1 (20 mg/mL) after storage at 40°C for up to 6 months



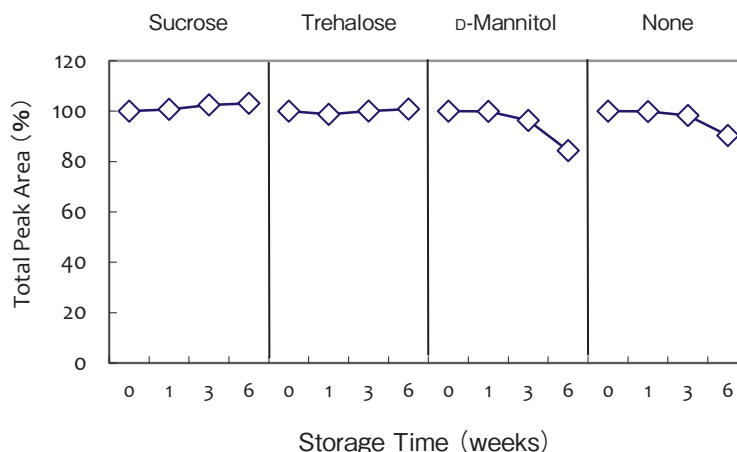
RESULT 5

Peak areas corresponding to lyophilized antibody monomer, oligomers or fragments of MAb-TNF-β 1 (20 mg/mL) using gel filtration chromatography after storage at 40°C for up to 6 months



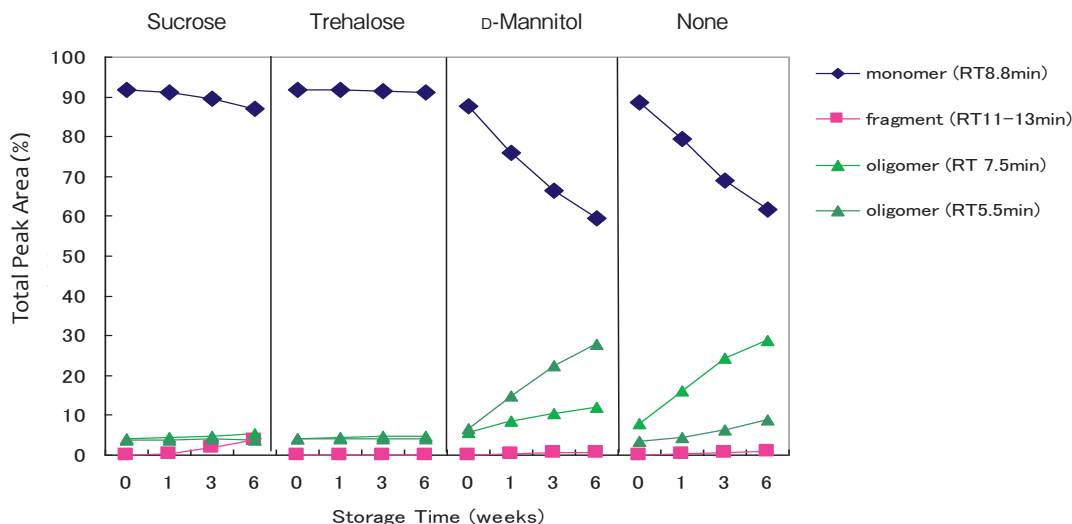
**RESULT 6**

Total peak area of gel filtration chromatography of lyophilized MAb-TNF-β 1 (20 mg/mL) after at 60°C storage for up to 6 weeks



**RESULT 7**

Peak areas corresponding to monomer, oligomers or fragments of MAb-TNF-β 1 (20 mg/mL) using gel filtration chromatography of lyophilized samples after storage at 60°C for up to 6 weeks



**CONCLUSION 3**

Time-dependent change of total peak area and peak areas of monomer, oligomers and fragments of lyophilized antibody after storage at 40°C or 60°C was investigated using gel filtration chromatography. TREHALOSE SG inhibited the decrease of the monomer of the lyophilized antibody by inhibiting oligomerization and fragmentation of the antibody during storage at 40°C for 6 months or 60°C for 6 weeks.

**RESULT 8**

Water content of lyophilized MAb-TNF-β 1 (0.1 mg/mL) after storage at 60°C for 3 weeks

Duration	Sucrose		Trehalose		D-Mannitol	
	Water content (%)	Water weight per vial (mg)	Water content (%)	Water weight per vial (mg)	Water content (%)	Water weight per vial (mg)
0 weeks	4.19	1.82	2.40	1.04	0.98	0.43
3 weeks	2.47	1.08	2.18	0.95	0.47	0.20

Water content of lyophilized MAb-TNF-β 1 (20 mg/mL) after storage at 60°C for 3 weeks

Duration	Sucrose		Trehalose		D-Mannitol		None	
	Water content (%)	Water weight per vial (mg)	Water content (%)	Water weight per vial (mg)	Water content (%)	Water weight per vial (mg)	Water content (%)	Water weight per vial (mg)
0 weeks	2.41	1.29	2.18	1.16	1.92	1.03	7.31	0.98
3 weeks	2.55	1.36	2.01	1.07	1.58	0.84	6.44	0.87



Sucrose



Sucrose Trehalose D-Mannitol



Trehalose



D-Mannitol

Appearance of lyophilized antibody (0.1 mg/mL) after storage at 60°C for 3 weeks

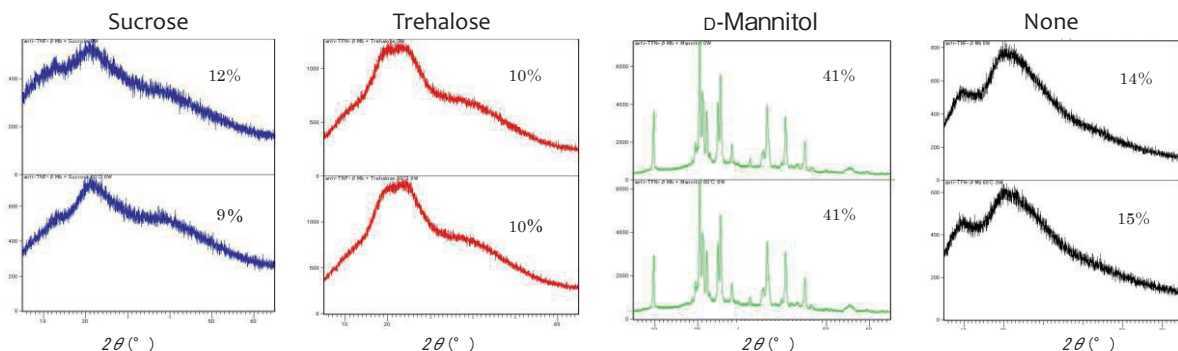
**CONCLUSION 4**

A lyophilized sample containing 0.1 mg/mL of antibody prepared with sucrose was wet after 3 weeks of storage at 60°C. Conversely lyophilized antibody prepared with TREHALOSE SG was dry and its water weight per vial was stable.



**RESULT 9**

Powder X-ray diffraction pattern of lyophilized MAb-TNF-β 1 (20 mg/mL) after 6 week of storage at 60°C



Upper Fig. Before storage; Lower Fig. After storage at 60°C for 6 weeks

Percentages in graphs were degrees of crystallization of lyophilized antibodies. A diffraction pattern of which degree of crystallization was 20-70% was recognized as reliable.

**CONCLUSION 5**

The Powder X-ray diffraction pattern of the lyophilized antibody using TREHALOSE SG or Sucrose did not have a peak corresponding to carbohydrate after 6 weeks of storage at 60°C, indicating that these two saccharides remained amorphous state during this storage condition. Antibody prepared with D-Mannitol crystallized during lyophilization and remained crystallized after 6 weeks of storage at 60°C.

**RESULT 10**

Glass transition temperature of lyophilized MAb-TNF-β 1 (20 mg/mL) after 6 weeks of storage at 60°C

Duration	Sucrose	Trehalose	D-Mannitol	None
0 weeks	50.6	121.7	-	-
3 weeks	54.5	121.5	-	-

- = glass transition shift was not observed

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