

Optimization of antibody separation conditions on Size Exclusion Chromatography

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Introduction

Monoclonal antibodies (mAbs) are widely used as a biopharmaceutical and still new mAbs are developed by modifying the complementarity determining regions. There are some analysis technologies for quality control of mAbs such as centrifugation, size exclusion and ion exchange chromatography, etc.

Size exclusion chromatography (SEC) is a powerful and convenient tool for determining mAb monomers and their impurities including aggregates, oligomers and mAb fragments. Although each mAb have different properties on hydrophobicity, hydrophilicity and electro-statics, chromatographic conditions such as pH, ionic strength, and operating temperature play an important role on obtaining better resolutions by narrowing peak widths and enlarging elution gaps among mAbs and their related fragments. Although the pore size for separating mAb monomer and its dimer would be estimated around 100kDa-500kDa, the slope of the calibration curve over this range are more critical property to obtain better resolutions.

We report here the investigation of chromatographic conditions affecting the resolutions between monomer, dimer and fragments of mAb samples in SEC mode.

Experimental

Column

- TSKgel G3000SW_{XL} (7.8mmI.D. x 30cm)
- TSKgel Super3000SW (4.6mmI.D. x 30cm)
- TSKgel CM-STAT (4.6mmI.D. x 10cm)

Instrumentation

- Pump: DP-8020 (Tosoh)
- Detector: UV-8020 (Tosoh)
- Auto-sampler: AS-8020 (Tosoh)
- Data processing: LC-8020 model 2 (Tosoh)

Chemicals and Reagents

- All proteins and reagents were purchased from Kishida Chemicals (Osaka).
- Monoclonal antibodies were purchased from Kaketsuken (mAb-1, Japan) and gifted from Tokyo research center (mAb-2 - mAb-7, TOSOH, Japan). The mAb-2 and mAb-4 samples were digested with papain and cysteine in 10 mmol/L phosphate buffer (pH7.0) at 37°C.
- The protein maker was from Oriental Yeast (Japan).

Conclusion

- Better resolutions between mAb dimer/monomer and monomer/fragment could be achieved on SEC columns having gentle slope in the calibration curve.
- However, SEC column efficiencies for mAbs were lower than those for globular proteins. Although HETPs were improved by elevating temperature (Fig.3), but the HETPs of mAbs were still larger than those of the other proteins. The linear velocity also affects HETP, but HETPs of mAbs still remain higher than those of other proteins of similar size (Fig.1).
- In combination with IEC separation, a high degree of polydispersity of mAb-3 was detected, whereas mAb-2 showed a common isofom pattern in IEC. However lower SEC column efficiencies for all mAb samples compared to globular proteins were recognized regardless of the degree of polydispersity. In a papain digest study, F(ab)₂ of mAb-4 having many analogues showed a similar pattern of the intact sample on IEC. On the contrary, F(ab)₂ and Fab from mAb-2 digest were eluted with single peaks, respectively.
- The SEC column efficiency for Fab from mAb-2 was higher than that for mAb-4. The peak broadening of mAb samples could be caused by the degree of polydispersity, but by also limited accessibility of strict molecules into pores could contribute to this effect.
- Using multiple SEC columns in series provides better resolutions, especially for mAb fragments (Fig. 8 & 10).
- The combination of different separation modes, especially SEC and IEC, should be selected to clarify the reason of broad peak shapes of mAb and fragments on SEC. In further investigation, SEC-MALS measurements will be performed to obtain further information on molecular weights and sizes of analogues and fragments.

1. Fundamental properties of SEC columns

1) Comparison of slopes among proteins on SEC columns

Table 1 Slopes between various proteins on SEC columns

Column	Particle size (um)	TP		Rs				Slop gradient				
		mAb-1	Fab	mAb-1 D/M	mAb-1 D/M	Mab/(Fab) ₂	Mab/Fab	(Fab) ₂ /Fab				
G3000SW _{XL}	5	1,830	4,185	1.676	-0.265	-0.349	-0.291	-0.263				
Commercial SEC	3	2,888	-	1.948	-0.306	-	-0.328	-				
Super3000SW	4	2,398	4,427	2.046	-0.265	-0.333	-0.275	-0.248				

Conditions:

- Eluent: 200 mM Phosphate buffer + 0.05% Na₂P₆H₈
- Flow rate: 1.0 mL/min (except Super3000SW:0.35 mL/min)
- Detector: UV (280nm), Temperature: 25°C
- Samples: mAb-1 and its papain digest.

2) Relationship between HETP and molecular weights of proteins at various linear velocity

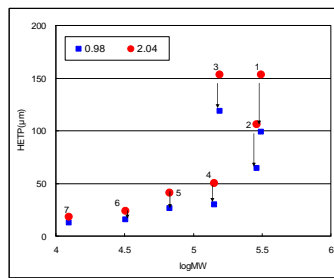


Fig.1 Effect of linear velocity on HETP of proteins

Conditions:

- Column: TSKgel G3000SW_{XL}, Flow rate: 1.0 mL/min, 0.5 mL/min
- Sample: 1. mAb-1 dimer (310,000Da), 2. Glutamate dehydrogenase (290,000Da), 3. mAb-1, 4. Lactate dehydrogenase (142,000Da), 5. Enolase (67,000Da), 6. Myokinase (32,000Da), 7. Cytochrome c (12,400Da)

The other conditions were the same as in Table 1.

2. Effect of operating temperature on column efficiency of proteins on SEC column

1) Temperature dependency on HETP of proteins

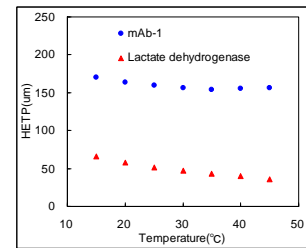


Fig.2 Dependency of temperature on HETP of proteins

Conditions:

- Flow rate: 1.0 mL/min, Temperature: 15-45 °C
- Samples: 1. mAb-1, 2. Lactate dehydrogenase (142,000Da)

The other conditions were the same as in Figure 1.

2) Temperature dependency on HETP of various proteins

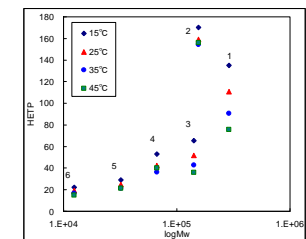


Fig.3 Temperature effect on HETP of proteins

Conditions:

- Eluent: 200 mM Phosphate buffer + 0.05% Na₂P₆H₈
- Samples: 1. mAb-1 dimer (310,000Da), 2. mAb-1 (155,000Da), 3. Lactate dehydrogenase (142,000Da), 4. Enolase (67,000Da), 5. Myokinase (32,000Da), 6. Cytochrome c (12,400Da)

The other conditions were the same as in Figure 1.

3. Polydispersity of antibody

1) Separation of mAb-2 on IEC and SEC

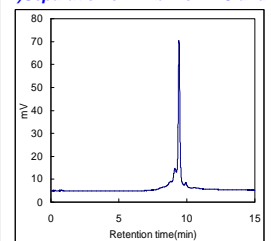


Fig.4 pH gradient separation of mAb-2 on IEC

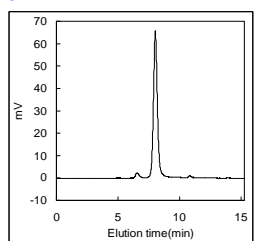


Fig.5 SEC of mAb-2

Conditions for IEC:

- Column: TSKgel CM-STAT (4.6 mmI.D. x 10 cm)
- Eluent: (A) 20 mmol/L MES (pH6.0)
- (B) 20 mmol/L MES + 0.5 mol/L NaCl (pH6.0)
- Gradient: B 10% (0 min) - 30% (30 min) - 100% (30 min) - 100% (32 min) - 10% (32 min) - 10% (36 min)
- Flow rate: 1 mL/min, Temperature: 25°C
- Detector: UV (280nm)
- Sample: mAb-2 (Fig.4), mAb-3 (Fig.6)

Conditions for SEC:

- Column: TSKgel G3000SW_{XL} (7.8mmI.D. x 30cm)
- Flow rate: 1.0 mL/min, Detection: UV (210nm)
- Temperature: 25°C,
- Sample: mAb-2, Fractions described (Figure 5) mAb-3 (Fig.7)

The other conditions were the same as in Figure 1.

2) Separation of mAb-3 on IEC and SEC

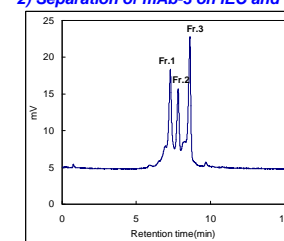


Fig.6 pH gradient separation of mAb-3 on IEC

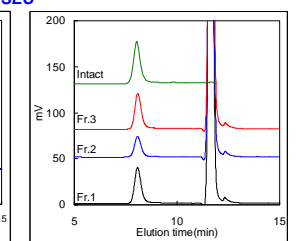


Fig.7 SEC of mAb-3 fractions on IEC

Table 2 Elution time and TP of various mAbs on SEC

mAb	Elution time (min)	TP
mAb-2	8.01	3,323
mAb-3	8.08	2,648
mAb-4	7.62	3,280
mAb-5	7.79	3,898
mAb-6	7.77	3,560
mAb-7	8.04	3,519

Table 3 Elution time and TP of fractions in Fig.7

mAb-3	Elution time (min)	TP
Intact	8.08	2,648
Fr.1	8.07	3,130
Fr.2	8.07	2,988
Fr.3	8.08	3,183

4. Comparison of antibody fragments on SEC and IEC columns

1) Chromatograms of mAb-4 fragments on SEC and IEC

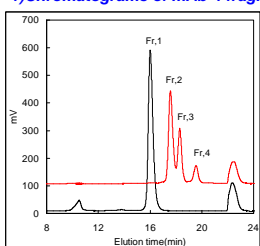


Fig.8 Antibody fragment separation on SEC

Conditions:

- Column: TSKgel Super3000SW (4.6 mmI.D. x 30 cm x 2)
- Flow rate: 0.35 mL/min,
- Sample: (A) mAb-4, (B) Papain digestion

The other conditions were the same as in Figure 1.

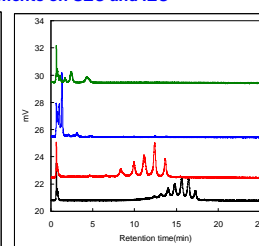


Fig.9 IEC separation of fragments

- Conditions for IEC:
- Column: TSKgel CM-STAT (4.6mmI.D. x 10cm)
- Eluent: (A) 20 mmol/L MES (pH6.0)
- (B) 20 mmol/L MES + 0.25 mol/L NaCl (pH6.0)
- Gradient: B 5% (0 min) - 30% (30 min) - 100% (33 min) - 5% (35 min)
- Sample: Fraction 1-4 in Figs.8

The other conditions were the same as in Fig.7.

2) Chromatograms of mAb-2 fragments on SEC and IEC

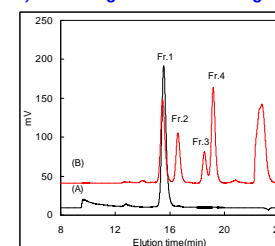


Fig.10 Antibody fragment separation on SEC

- Conditions:
- Sample: (A) mAb-2 (Fr.1)
- (B) Papain digestion (Fr.2-4)

The other conditions were the same as in Fig.8.

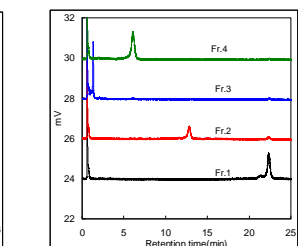


Fig.11 IEC separation of fragments

- Conditions for IEC:
- Eluent: (A) 20 mmol/L MES (pH6.0)
- (B) 20 mmol/L MES + 0.25 mol/L NaCl (pH6.0)
- Gradient: B 20% (0 min) - 45% (30 min) - 100% (33 min) - 20% (35 min)
- Sample: Fraction 1-4 in Fig.10

The other conditions were the same as in Fig.9.