TOSOH

Application Note

Two-Step Monoclonal Antibody Purification Platform Development Using SkillPak™ Pre-packed Columns

Introduction

Hybridoma technology is an efficient method for production of monoclonal antibodies (mAbs). It uses mice for eliciting an immune response, followed by fusion of IgG-expressing B cells with myeloma cells to allow propagation in cell culture.

This note details a case study where a proprietary mouse mAb was efficiently purified using a two-step purification process from hybridoma cell line supernatant ("feedstock"). The challenge was to obtain a few milligrams of mAb for cell-based functional assays to evaluate the purification process in spite of very low mAb titer (~0.03 g/L) in the feedstock. The two-step purification platform was established using SkillPak™ 1 mL and 5 mL columns from Tosoh Bioscience.

Materials and Methods

Pre-packed columns

The SkillPak 1 mL and 5 mL columns (see specifications listed in *Table 1 and 2* respectively) are designed for fast method development or resin screening. They are pre-packed with TOYOPEARL®, TSKgel® or Ca^{++}Pure-HA™ process chromatography media for bioseparations such as monoclonal antibodies, proteins and oligonucleotides. These columns are designed to be operated with commonly used low or medium pressure liquid chromatography systems.

Table 1. Specifications of SkillPak 1 mL column

Column dimension	7 mm ID × 2.5 cm bed height
Volume	1 mL
Maximum flow rate	4 mL/min (600 cm/hr)
Maximum operating pressure	0.3 MPa
Connections	Standard fittings (10-32 for 1/16 inch capillary)
Shipping buffer	20% ethanol for TOYOPEARL and TSKgel [with the exception of 0.5 mol/L sodium citrate with 20% ethanol for TSKgel SP-5PW (20) and SuperQ-5PW (20)], 20 mmol/L phosphate with 20% ethanol for Ca^{++}Pure-HA

Table 2. Specifications of SkillPak 5 mL columns

Column dimension	8 mm ID × 10 cm bed height
Volume	5 mL
Standard flow rate	1.3 mL/min (150 cm/hr)
Maximum flow rate	5 mL/min (600 cm/hr) for TOYOPEARL M and C grade resins; 2.5 mL/min (300 cm/hr) for TOYOPEARL S-grade, TSKgel, Ca^{++}Pure-HA and TOYOPEARL F grade resins, including TOYOPEARL AF-rProtein A HC-650F, TOYOPEARL AF-rProtein L-650F
Maximum operating pressure	0.3 MPa for TOYOPEARL resins, ≤ 0.4 MPa for TSKgel resins and Ca^{++}Pure-HA
Connections	Standard fittings (10-32 for 1/16 inch capillary)
Shipping buffer	20% ethanol for TOYOPEARL and TSKgel, 20 mmol/L phosphate with 20% ethanol for Ca^{++}Pure-HA
Asymmetry factor (As) specifications	0.8-1.4 for TOYOPEARL and TSKgel, 0.8-2.6 for Ca^{++}Pure-HA

The study used a SkillPak 5 mL column pre-packed with TOYOPEARL AF-rProtein A HC-650F, a polymethacrylate, high dynamic binding capacity protein A affinity media, in the capture step and a SkillPak 1 mL column pre-packed with TOYOPEARL Sulfate-650F, a high salt-tolerant cation exchange (CEX) media, in the polishing step.

Step 1 (Capture). Column characteristics

Media	TOYOPEARL AF-rProtein A HC-650F
Column	SkillPak 5 mL TOYOPEARL AF-rProtein A HC-650F
Bed size	8 mm ID × 10 cm
Particle size	45 µm
Pore diameter	100 nm
DBC* (5 min)	70 g/L
DBC* (2 min)	50 g/L
Caustic stability	>200 CIP cycles (0.1 mol/L NaOH)
Maximum operating pressure	0.3 MPa

*DBC = Dynamic Binding Capacity

Step 2 (Polish). Column characteristics

Media	TOYOPEARL Sulfate-650F
Column	SkillPak 1 mL TOYOPEARL Sulfate-650F
Bed size	7 mm ID × 2.5 cm
Particle size	45 µm
Pore diameter	100 nm
DBC	>120 g/L of IgG
Caustic stability	>200 CIP cycles (0.1 mol/L NaOH)
Maximum operating pressure	0.3 MPa

Monoclonal antibody

Proprietary mouse mAb

Purification protocol

Determination of hybridoma mAb elution pH on protein A resin

Column:	SkillPak 5 mL TOYOPEARL AF-rProtein A HC-650F
Equilibration buffer:	0.1 mol/L Na_2HPO_4/NaH_2PO_4, 0.15 mol/L NaCl, pH 7.3
Mobile phase A (gradient):	0.1 mol/L acetate (NaOH), pH 4.5
Mobile phase B (gradient):	0.1 mol/L acetic acid, pH 2.9
Elution gradient:	linear from pH 4.5 to 2.9 over 10 CV
Flow (load):	150 cm/hr (1.25 mL/min), 4 min residence time
Flow (wash/gradient):	240 cm/hr (2.0 mL/min) (AKTA™ avant 25 instrument)
Sample:	45 mL hybridoma cell culture supernatant (buffer-adjusted)

Capture method for purification of hybridoma mAb

Column:	SkillPak 5 mL TOYOPEARL AF-rProtein A HC-650F
Equilibration buffer:	0.1 mol/L Na_2HPO_4/NaH_2PO_4, 0.15 mol/L NaCl, pH 7.3
Post-loading 1st wash:	equilibration buffer (5 CV)
Post-loading 2nd wash:	0.1 mol/L acetate (NaOH), pH 5.0 (5 CV)
Elution:	0.1 mol/L acetate (NaOH), pH 3.5 (5 CV)
Column strip:	0.1 mol/L acetic acid, pH 2.9 (3 CV)
Column cleaning:	0.2 mol/L NaOH (3 CV), 15 min hold
Flow (load):	150 cm/hr (1.25 mL/min), 4 min residence time
Flow (wash/elution):	240 cm/hr (2.0 mL/min) (AKTA avant 25)
Temperature:	ambient (room temperature)
Sample:	340 mL hybridoma cell culture supernatant (buffer-adjusted)

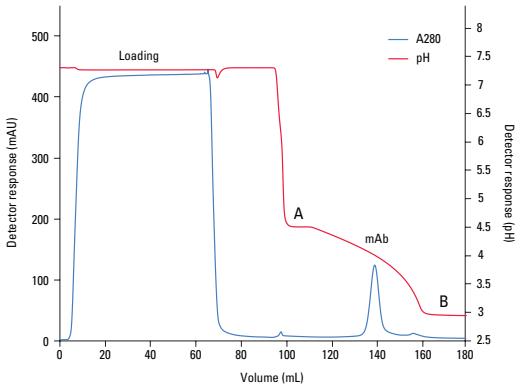
Polishing method to remove mAb aggregates and impurities

Column:	SkillPak 1 mL TOYOPEARL Sulfate-650F
Equilibration buffer:	0.1 mol/L acetate (NaOH), pH 5.0
Post-loading 1st wash:	equilibration buffer (5 CV)
Post-loading 2nd wash:	0.1 mol/L acetate (NaOH), 0.1 mol/L NaCl, pH 5.0 (10 CV)
Elution:	0.1 mol/L acetate (NaOH), 0.35 mol/L NaCl, pH 5.0 (10 CV)
Column strip:	0.1 mol/L acetate (NaOH), 1.0 mol/L NaCl, pH 5.0 (7 CV)
Flow (all steps):	156 cm/hr (1.0 mL/min) 1 min residence time (AKTA avant 25 instrument)
Sample:	21 mL (8.6 mg total mAb) diluted protein A eluate
Temperature:	ambient (room temperature)

Results and Discussions

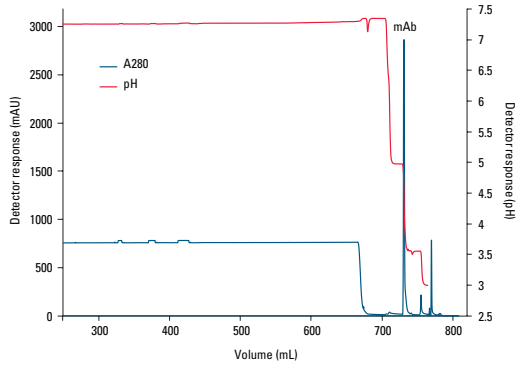
Finding the appropriate buffer and pH for mAb elution reduces the risk of increasing aggregation in the mAb sample. To identify optimized conditions for mAb binding and elution, a buffer-adjusted mAb-containing hybridoma cell line supernatant was loaded onto a SkillPak 5 mL column pre-packed with TOYOPEARL AF-rProtein A HC-650F media. *Figure 1* demonstrates that a sharp mAb peak with elution max at pH 4.0 was obtained using a linear pH gradient. To maximize recovery, pH 3.5 was selected for step elution.

Figure 1. Determination of mAb elution pH on TOYOPEARL AF-rProtein A HC-650F media



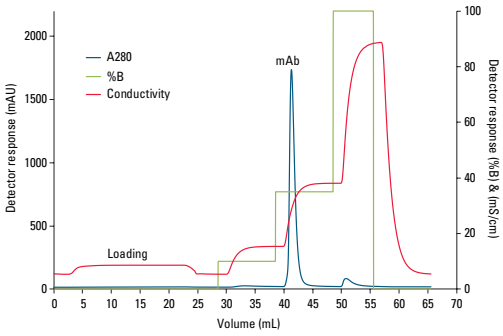
For prep scale purification, 340 mL of hybridoma cell line supernatant (titer ~0.03 g/L) was loaded on the SkillPak 5 mL column. After a short wash at pH 5.0, a sharp and efficient elution peak was obtained at the start of the pH 3.5 elution *(Figure 2)*. Total mAb recovery was 9.8 mg in the elution peak (4.8 mL). Eluate was prepared for a polishing step on a SkillPak 1 mL column packed with TOYOPEARL Sulfate-650F by adding four eluate volumes of 0.2 mol/L acetate, pH 5.0.

Figure 2. mAb capture on TOYOPEARL AF-rProtein A HC-650F media



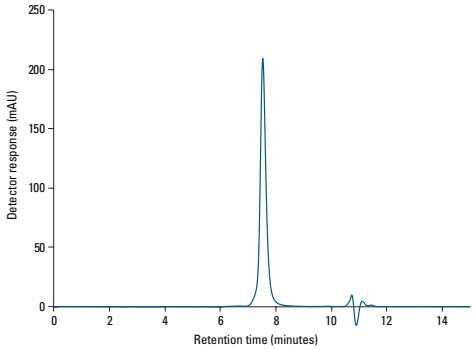
Aggregate and other impurities in the protein A eluate were removed by using the SkillPak 1 mL TOYOPEARL Sulfate-650F column. *Figure 3* shows an efficient elution of mAb at a 0.35 mol/L NaCl step in the equilibration buffer. Impurities were removed by the polishing step as can be seen before and after the elution of the mAb peak.

Figure 3. Polishing chromatography on TOYOPEARL Sulfate-650F media



The mAb eluate from the cation exchange step was subjected to size exclusion chromatography (SEC) to confirm purity and the monomeric state of the collected mAb. The elution peak at ~7.5 minutes indicated a largely monomeric mAb with >98% purity *(Figure 4)*.

Figure 4. SEC analysis of mAb purity



Column:	TSKgel® UP-SW3000, 2 µm, 4.6 mm ID × 30 cm
Mobile phase:	0.1 mol/L KH_2PO_4/Na_2HPO_4, 0.1 mol/L Na_2SO_4, 0.05% NaN_3, pH 6.7
Flow rate:	0.35 mL/min (Agilent 1100 HPLC)
Detection:	UV @ 280 nm
Temperature:	ambient (room temperature)
Injection vol:	10 µL
Sample:	mAb from hybridoma cells, final product

Conclusion

The two-step process presented here using SkillPak pre-packed columns was effective for purification of this mAb from hybridoma cell line supernatant and the process can feasibly be scaled up to a pilot plant and eventually to manufacturing scale.