Purification of Fab and scFv using High Binding Capacity TOYOPEARL® AF-rProtein L-650F

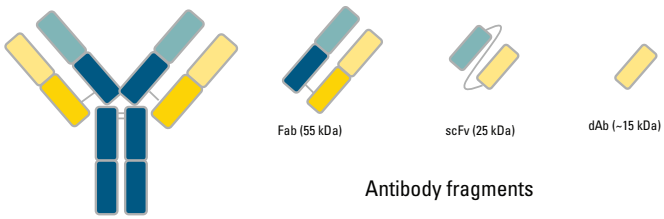
A High Capacity Protein L Resin for the Purification of Monoclonal Antibody Fragments

TOYOPEARL
APPLICATION NOTE

Introduction

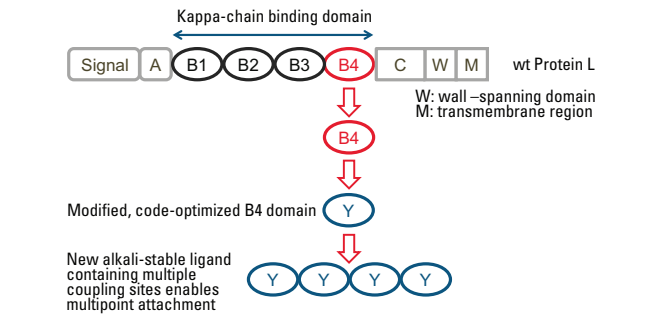
Protein L based affinity chromatography is used for the capture of antibodies and antibody fragments that do not bind to protein A. Unlike protein A and G, which bind to the Fc region of immunoglobulins (IgGs), protein L binds through interactions with the variable region of an antibody's kappa light chain. *Figure 1* shows several possible binding sites of protein L to fragments (Fabs), single-chain variable fragments (scFvs) and domain antibodies (dAbs).

Figure 1. Protein L binds to the variable region of the kappa light chain of IgGs



TOYOPEARL AF-rProtein L-650F is an affinity chromatography resin that combines a rigid polymer matrix with a recombinant ligand, which is derived from the B4 domain of native protein L from *Peptostreptococcus magnus* and is expressed in *E.coli (Figure 2)*. Code optimization of the domain results in high binding capacity and an improved alkaline stability of the ligand compared to the native molecule.

Figure 2. Structure of TOYOPEARL AF-rProtein L-650F



The selected recombinant protein L ligand also has an affinity for a broad range antibody subclasses as demonstrated in *Table 1*.

Table 1. TOYOPEARL AF-rProtein L-650F ligand with a broad affinity range for mAb subclasses

Species	Class	Affinity
General	Kappa light chain	++
	Lambda light chain	-
	Heavy chain	-
	Fab	++
	ScFv	++
	Dab	++
Human	IgG(1-4)	+
	IgA	+
	IgD	+
	IgE	+
	IgM	+
Mouse	IgG_1	+
	IgG_2a	+
	IgG_2b	+
	IgA	+
	IgM	+
Rat	IgG_1	+
	IgG2a,b,c	+
	IgA	+
Hen	IgM	+
	IgY	+

This application note demonstrates the ability of TOYOPEARL AF-rProtein L-650F resin to capture and purify Fabs and scFv fragments with a high yield and high purity. The scFv fragment is expressed in the complex and challenging mammalian cell line.

Materials and Methods

Digestion

Humanized IgG_1, 3 mg/mL, was digested using papain enzymatic protocol as described in the Pierce™ Fab Preparation Kit (catalog number: 44985).

Chromatography

Purification of Fab: 100 µL of the papain digested IgG_1 was loaded onto a TOYOPEARL AF-rProtein L-650F column, 5 mm ID × 2.5 cm (0.5 mL total volume). See chromatogram for operating conditions.

Size analysis of fractions: the collected peaks, 10 µL each, were injected separately onto a TSKgel® UP-SW3000, 4.6 mm ID × 15 cm size exclusion chromatography (SEC) column for size analysis. See chromatogram for operating conditions.

SDS-PAGE

4% to 15% TGXgel under reducing condition (DTT) was used. Gel was stained with silver stain using Dodeca™ Silver Stain Kit for Protein Gels, small, catalog number 1610481.

Western Blot

Proteins were separated using 4% to 15% TGXgel under reducing condition (DTT) and transferred onto a PDF membrane. Western blot procedure was performed with the membrane following the anti-human keppa-HPR protocol in the dAb kit, catalog number 07388-24.

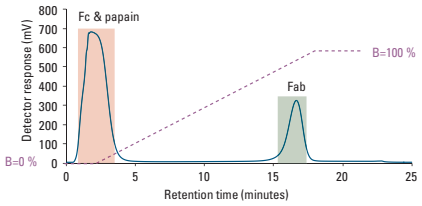
Results and Discussions

Purification of Fab

Figure 3, panel A, shows the purification result of Fab from the IgG_1 papain digested sample using the TOYOPEARL AF-rProtein L-650F column. Only two peaks are observed in the chromatographic profile - the flow through peak and the eluted peak. The eluted peak is eluted with 0.1 mol/L citrate buffer, pH 2.05 at ~90% gradient. The data suggests that Fab is bound strongly onto the TOYOPEARL AF-rProtein L-650F resin. Both peaks were collected for further analysis using a TSKgel UP-SW3000, 4.6 mm ID × 15 cm SEC column. The eluted peak is expected to contain only the Fab fragment because this peak is eluted from the TOYOPEARL AF-rProtein L-650F column.

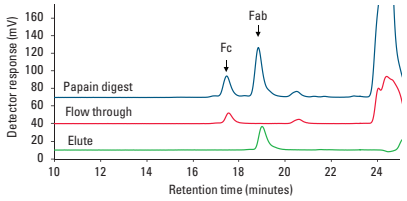
Figure 3. Purification of Fab using TOYOPEARL AF-rProtein L-650F

A: Purification of Fab by Protein L resin



Resin:	TOYOPEARL AF-rProtein L-650F
Column size:	5 mm ID × 2.5 cm
Mobile phase:	A : 0.1 mol/L Na-citrate, pH 6.5
	B: 0.1 mol/L citrate, pH 2.05
Gradient:	B= 0% (0 to 1 min)
	B= 0% to 100% (1 min to 16 min)
Flow rate:	0.5 mL/min (residence time = 1 min)
Dectection:	UV @ 280 nm
Injection:	10 µL, 3 mg/mL humanized mAb papain digest

B: SEC analysis of fractions



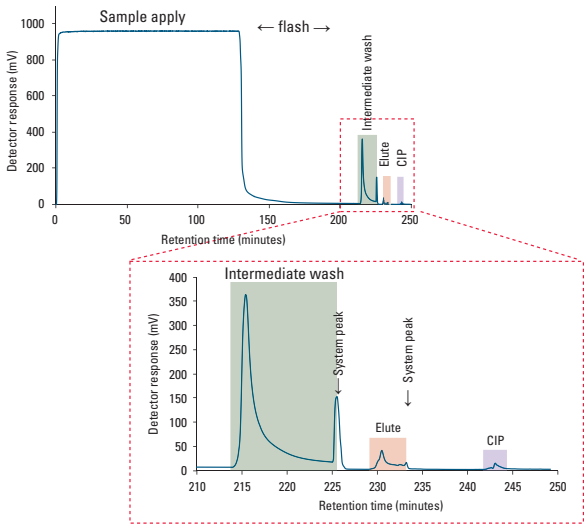
Column:	TSKgel UP-SW3000, 4.6 mm ID x 15 cm x 2
Mobile phase:	20% acetonitrile,
	0.1% trifluoroacetic acid
Flow rate:	0.2 mL/min
Detection:	UV @ 280 nm
Sample:	2 g/L mAb2,
	2 g/L digested mAb2

Figure 3, panel B, shows results of the size exclusion analysis of the digested sample, the flow through and eluted peaks. The SEC profile showed that the digested sample contained two different molecular weight products based on the estimated retention times. As mentioned above, the papain digestion dissociates Fc and Fab regions of IgG and the protein L ligand only has an affinity for the Fab fragment. Therefore, there is no binding of the Fc fragment to the protein L resin and only Fab can be bound and eluted from the protein L column. Data from *Figure 3*, panel B, shows that the first/flow through peak from the papain digested sample has a retention time of 17 minutes and the second/eluted peak has a retention time of ~19 minutes. The 17 minute retention time peak is suggested to be the Fc fragment because it has the same retention time with the flow through peak. And the 19 minute retention time peak is suggested to be the Fab fragment due to it having the same retention time as the eluted peak from the TOYOPEARL AF-rProtein L-650F column.

Purification and analysis of scFv fragment

scFv fragments were expressed in a mammalian cell line. After harvesting, the sample was spun and filtered as described earlier. Approximately 2 mg of total protein (including scFv fragments) was loaded onto a TOYOPEARL AF-rProtein L-650F column (0.5 mL volume). The approximate residence time was 1.4 minutes. A step gradient protocol was used. The intermediate wash peak, system peak, eluted peak, and CIP peak were collected for further analysis as shown in *Figure 4* (zoom in view). The bound sample was eluted with 0.1 mol/L Na-citrate, pH 2.3.

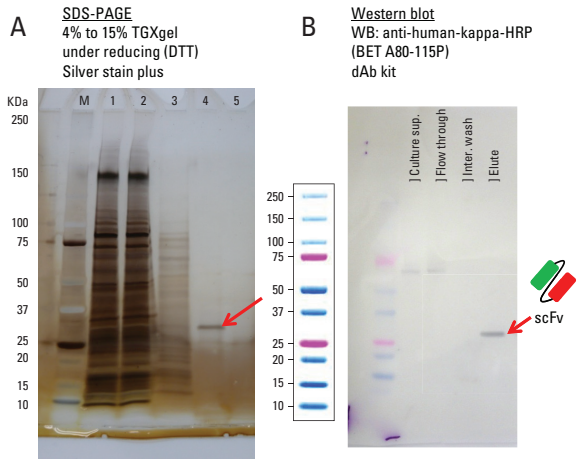
Figure 4. Purification of scFv fragments using TOYOPEARL AF-rProtein L-650F



Resin:	TOYOPEARL AF-rProtein L-650F
Column size:	5 mm ID × 2.5 cm (0.5 mL)
Equib. & flash wash:	0.1 mol/L Na-citrate, pH 6.5
Intermediate wash:	0.1 mol/L Na-citrate, pH 6.5 + 1 mol/L Arg-HCl, 10% iPrOH
Elute:	0.1 mol/L Citrate, pH 2.3
CIP:	0.05 mol/L NaOH
Flow rate:	0.35 mL/min (residence time = 1.4 min)
Detection:	UV @ 280 nm
Sample:	hu-scFv1 CHO culture sup. 40 mL

Figure 5, panel A, shows the results of silver stain from the collected fractions after the sample containing scFv fusion protein was injected onto a TOYOPEARL AF-rProtein L-650F column. 10 µL from each fraction was loaded onto the 4-15% TGXgel under a reduced condition with DTT. The gel was stained with silver stain plus kit. Data from the silver stain gel shows that there is only a single band from the eluted peak (*Figure 5*, panel A, lane 4) with a molecular weight of approximately 26 kDa. This indicates that only the sample containing a molecule of about 26 kDa is captured by the resin. The data suggests that this is the scFv.

Figure 5. Purification of scFv fragments using TOYOPEARL AF-rProtein L-650F



M prestained size marker (Bio-Rad 1610374)
1 Applied sample (hu_scFv_1)
2 Flow through
3 Intermediate wash (Arg+iPrOH)
4 Elute (pH 2.3) 1114series
5 CIP (50 mmol/L NaO_4H)

Figure 5, panel B, shows the Western blot data using anti-human-kappa-HRP from a dAb kit to determine whether the eluted peak of 26 kDa is the scFv. The result from the Western blot analysis reconfirmed that the anti-human-kapp-HRP interacts with this single 26 kDa band (see *Figure 5*, panel B). Based on the data from the silver stained SDS-PAGE and the Western blot, this 26 kDa molecule is confirmed to be the scFv fusion protein. The estimated yield of the scFv fusion protein was estimated >98%.

Dynamic binding capacity

A purified Fab sample was loaded onto the TOYOPEARL AF-rProtein L-650F resin, 0.83 mL column volume with 10% breakthrough at 3.4 minutes and 4.0 minutes residence times to determine the dynamic binding capacity (DBC) of a Fab fragment on the resin. Table 1 shows the comparison data between TOYOPEARL AF-rProtein L-650F resin to its competitor 85 µm agarose-based particle size protein L resin at various residence times. Due to the excellent mass transfer characteristics of TOYOPEARL AF-rProtein L-650F resin, dynamic binding capacities at 1 to 3 minutes residence time excel capacities obtained with the agarose-based resin (nearly double) as shown in *Table 2*. As the molecular weight of fragments is much smaller compared to full length IgGs, a dynamic binding capacity of about 50 mg/mL for a Fab with a typical molecular weight of 55 kDa equals a DBC of >130 mg/L for a ~150 kDa IgG when considering molar binding capacities.

Table 2. Dynamic binding capacity of the TOYOPEARL AF-rProtein L-650F resin for Fab

	IgG SBC (g/L-resin)	Fab DBC (g/L-resin)	
		Residence time	
		3.4 min	4.0 min
rProtein L-650F	72	33	50
Competitor product	42	19	26

Column: TOYOPEARL AF-rProtein L-650F/competitor resin, 4.6 mm ID × 50 mm (0.83 mL)
Detection: UV @ 280 nm
Sample: 2 g/L human Fab in 0.1 mol/L Na-phosphate, pH 6.5

DBC measured at 10% breakthrough

Conclusions

The dynamic binding capacity of TOYOPEARL AF-rProtein L-650F resin exceeds the protein L agarose-based, 85 µm chromatography media currently available. Due to the high binding capacity, high yield and ease of use, TOYOPEARL AF-rProtein L-650F resin can considerably improve process economics of protein L capture steps. With high affinity antibody fragments, single chain variable fragments and domain antibodies, it is suited for the purification of new antibody formats that cannot be purified with protein A media.

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