Multiple Modes of Fab Purification with Hydroxyapatite

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Trends in antibody therapy

Designer antibody fragments are expected to represent a major new area of growth for therapeutic immunology in the coming decades.

Various constructs, including Fab, F(ab')$_2$, minibodies, diabodies, nanobodies, and others potentially offer:

- *Rapid, high-level targeting*
- *Access to tissues that are poorly accessible by intact antibodies*
- *Timely clearance from blood and normal tissues*
- *Lower immunogenic response*
- *Administration through eye-drops, inhalants, or orally*

Trends in antibody therapy

The potential for this new sector has triggered the formation of many new start-ups, as well as new R&D programs, partnerships, or acquisitions by established pharmaceutical companies and clinical research institutions:

- *Ablynx (Merck)*
- *Adnexus (Bristol-Meyers Squibb)*
- *Avidia (Amgen)*
- *City of Hope*
- *Domantis (Glaxo)*
- *ESBA-Tech*
- *Novartis*
- *Trubion*
- *Wyeth*

Purification of antibody fragments

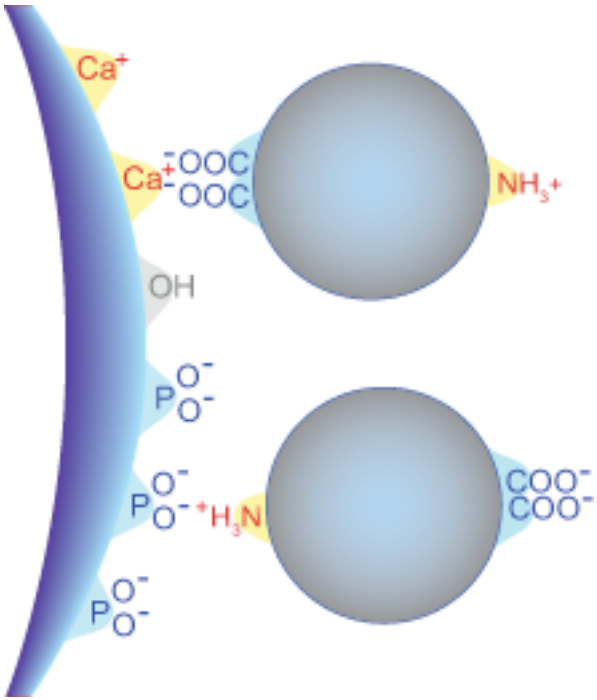
Immunoreactive fragments lack the Fc region that binds protein A.

Fragment purification must therefore rely on methods that achieve fractionation by classical chemical mechanisms, such as ion exchange, hydrophobic interaction, immobilized metal affinity, and mixed mode chromatography methods.

Using Fab as a model, this presentation addresses the potential of one of those mixed mode methods – hydroxyapatite (HA) – as a tool for fragment purification.

Hydroxyapatite

Primary retention mechanisms on HA



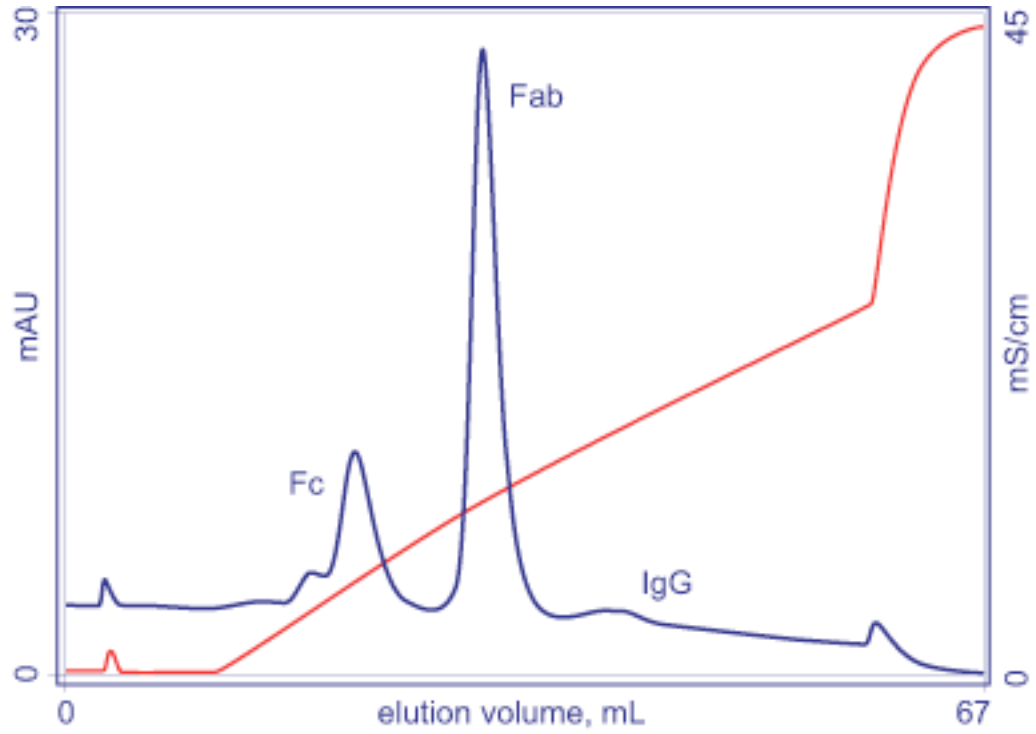
Calcium metal affinity. Protein carboxyl clusters form chelation bonds in the same way as the carboxyl doublets on EDTA. These bonds are stronger than ionic bonds and often survive exposure to high concentrations of NaCl. Elution normally requires an agent with strong calcium affinity, such as phosphate.

Phosphoryl cation exchange. Protein amino residues can participate in cation exchange interactions with HA phosphate. As with traditional carboxy- or sulfo- cation exchangers, protein binding becomes weaker with increasing pH and/or conductivity.

The positive charge on HA calcium is theoretically capable of anion exchange interactions with single carboxyls but this has not been shown to contribute significantly to protein binding.

Fab purification with HA

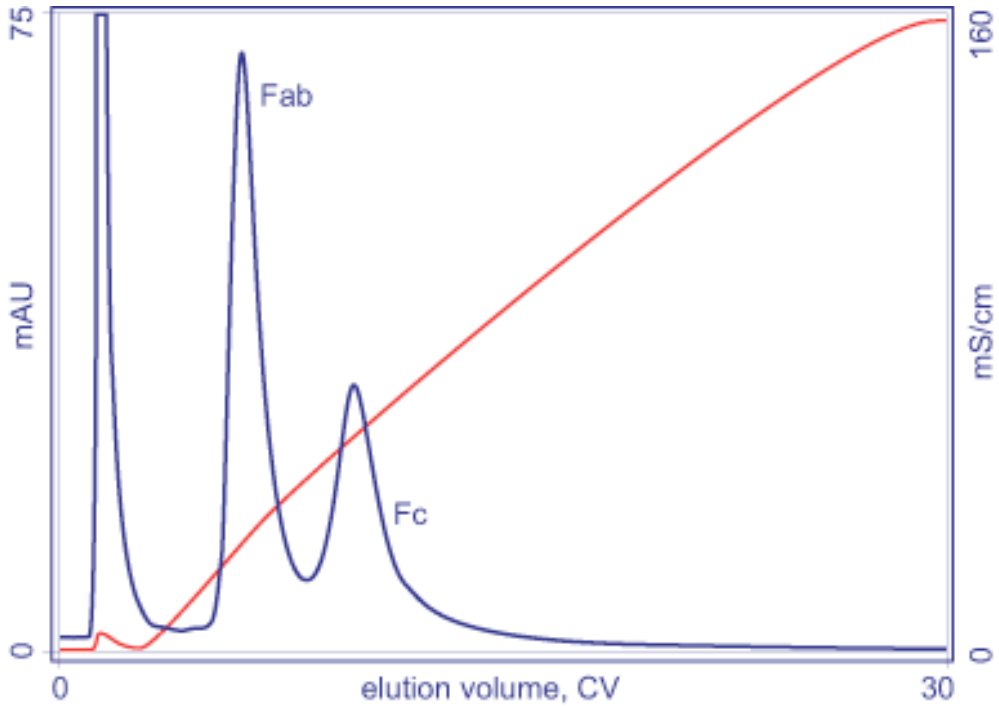
HA, eluted with phosphate gradients, has been known to be an effective tool for Fab purification since the late 1980s.



CHT™ type I, 20 µm, 2.5 mL
MediaScout® 8 x 50 mm,
2.5 mL/min (300 cm/hr)
Equilibrate: 5 mM $NaPO_4$, pH 7.0
Inj: 100 µL papain-digested $hIgG_1$ Mab
Wash: 5 mM $NaPO_4$, pH 7.0
Elute: 20 CV LG to 250 mM $NaPO_4$ pH 7.0
Clean: 500 mM $NaPO_4$ pH 7.0

Fab purification with HA

More recently, HA elution with a sodium chloride gradient at low phosphate concentration has shown promise for Fab purification, but the elution sequence of Fab and Fc are reversed.



CHT type I, 40 µm, 300 cm/hr.
Equilibrate: 5 mM $NaPO_4$, pH 7.0
Inj: papain-digested $hIgG_1$ Mab
Wash: 5 mM $NaPO_4$, pH 7.0
Elute: 30 CV LG to 5 mM $NaPO_4$, 2.0 M NaCl, pH 7.0

For additional details, refer to: Aberin, C., Snyder, M., Ng, P. (2007) Purification and separation of Fab and Fc fragments on IgG monoclonal antibodies on CHT™ Ceramic Hydroxyapatite, Bioprocess International Conference and Exhibition, Boston, Oct.1-4. Chromatogram redrawn from this reference, with permission.

IgG/fragment interactions with HA

Early elution of Fc in phosphate gradients suggests that it has a high sensitivity to phosphate. This could reflect weak cation exchange binding, but since it elutes after Fab in a chloride gradient at 5 mM phosphate, the implication is that it binds HA dominantly by calcium affinity.

Early elution of Fab in chloride gradients at 5 mM phosphate shows that it has very weak affinity for HA calcium. The fact that it elutes after Fc in phosphate gradients suggests that cation exchange is probably its dominant retention mechanism.

IgG elutes later than either fragment, in both systems. This suggests cooperativity between the binding mechanisms.

IgG/fragment interactions with HA

It should be possible to reveal the relative contributions of calcium affinity and cation exchange with greater definition by conducting:

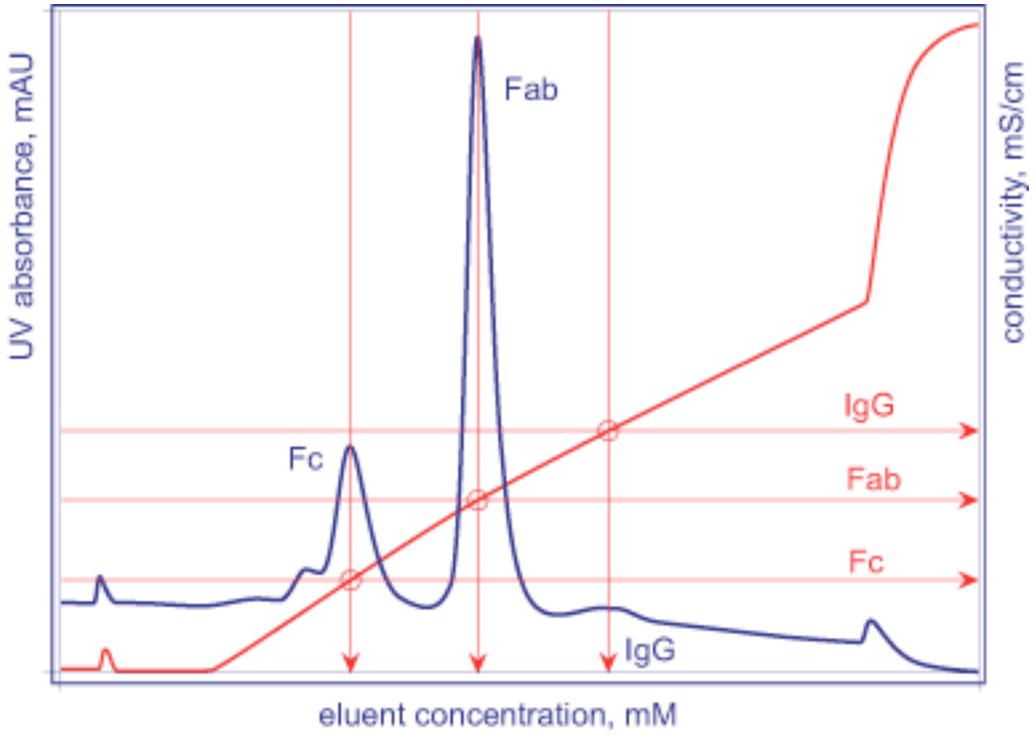
- *Chloride gradients at level phosphate concentration, over a range of phosphate concentrations and different pH values.*

- *Phosphate gradients at level sodium chloride concentration, over a range of chloride concentrations and different pH values.*

- *A conductivity gradient on a cation exchanger at the same pH as HA.*

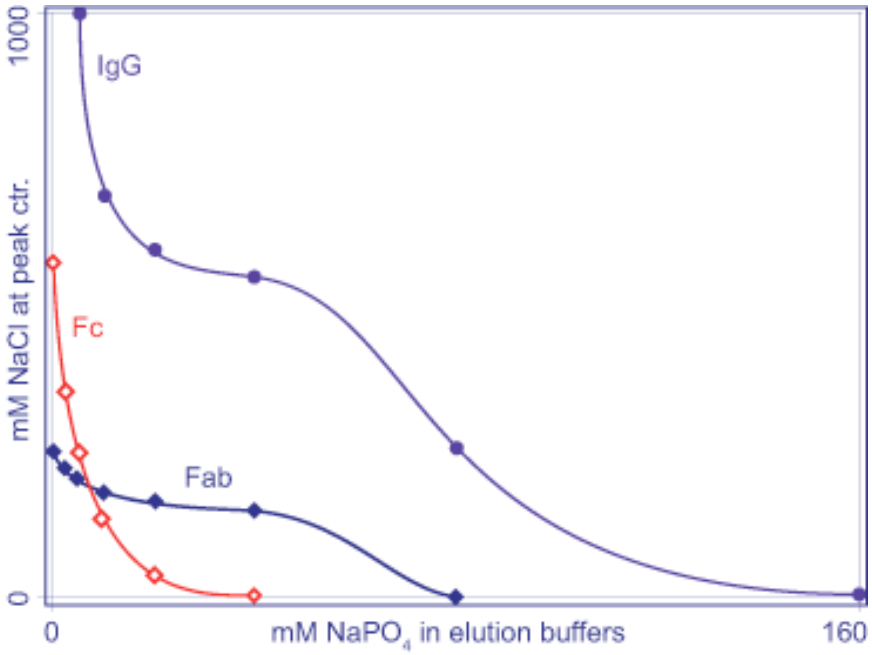
Retention mapping

Selection of data points



Retention mapping

HA retention in NaCl gradients at level phosphate concentrations

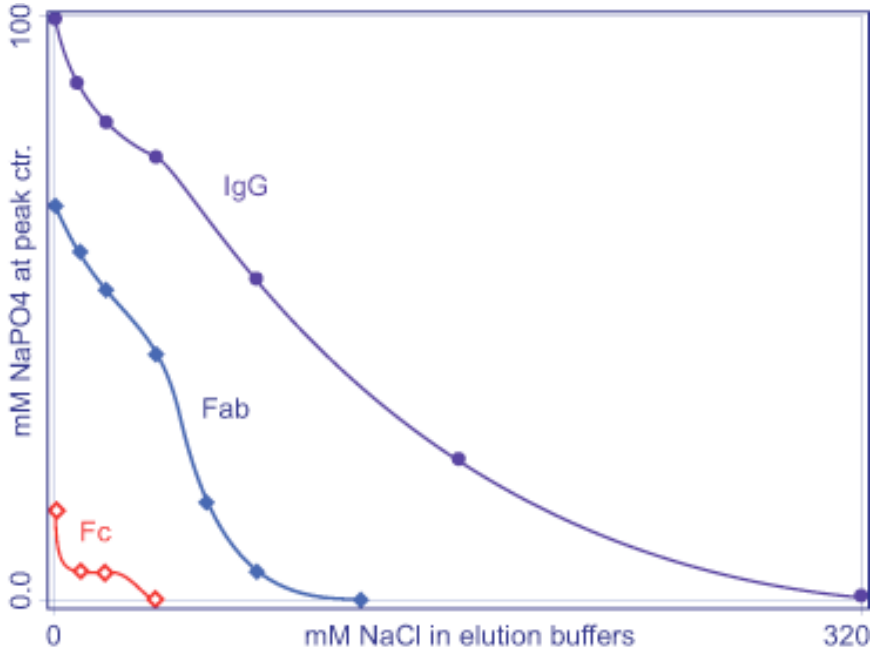


CHT type I, 20 µm, 1.0 mL
MediaScout 5 x 50 mm,
1.0 mL/min (300 cm/hr)
Equilibrate: 50 mM Hepes, pH 7.0
Inj: 50 µL papain-digested $hIgG_1$ Mab
Wash: 50 mM Hepes, pH 7.0
Elute: 20 CV LG to 50 mM Hepes, 1.0 M NaCl, pH 7.0
Clean: 500 mM $NaPO_4$ pH 7.0
Repeat with equal increments of phosphate added to the equilibration and eluting buffers as indicated.

The strong response of Fc to low phosphate concentrations indicates a corresponding dependence on calcium affinity binding. The relatively minor change in Fab binding at low phosphate suggests that calcium affinity plays a relatively small role in its retention. The flattening of the Fab curve up to about 40 mM phosphate indicates a strong contribution by cation exchange. Low phosphate concentrations lack sufficient conductivity to substantially reduce cation exchange binding but higher concentrations eventually cause elution. Note that the shape of the IgG curve is a composite of the Fc and Fab curves.

Retention mapping

HA retention in phosphate gradients at level chloride concentrations

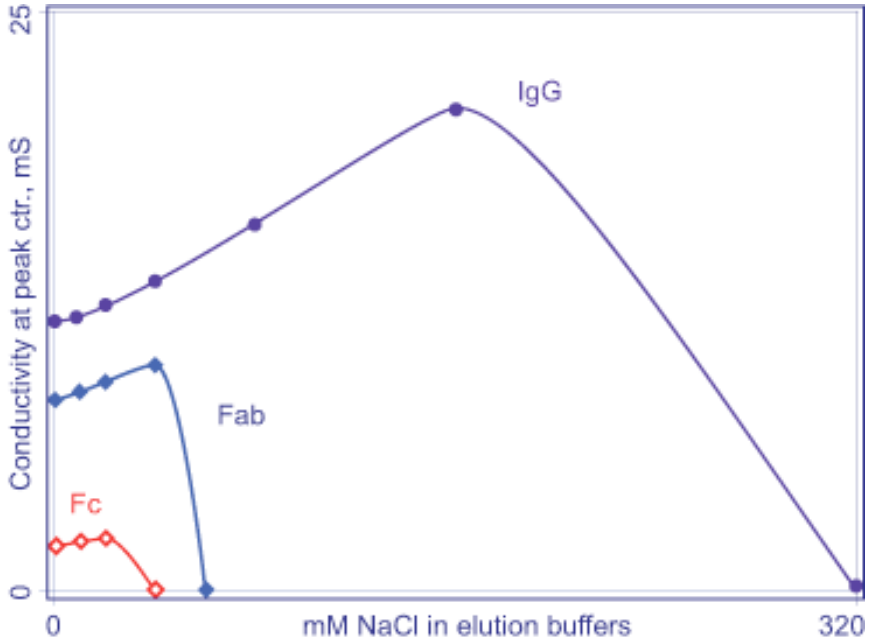


CHT type I, 20 µm, 1.0 mL
MediaScout 5 x 50 mm,
1.0 mL/min (300 cm/hr)
Equilibrate: 50 mM Hepes, pH 7.0
Inj: 50 µL papain-digested $hIgG_1$ Mab
Wash: 50 mM Hepes, pH 7.0
Elute: 20 CV LG to 500 mM $NaPO_4$
pH 7.0
Repeat with increments of NaCl added to the equilibration and eluting buffers as indicated.

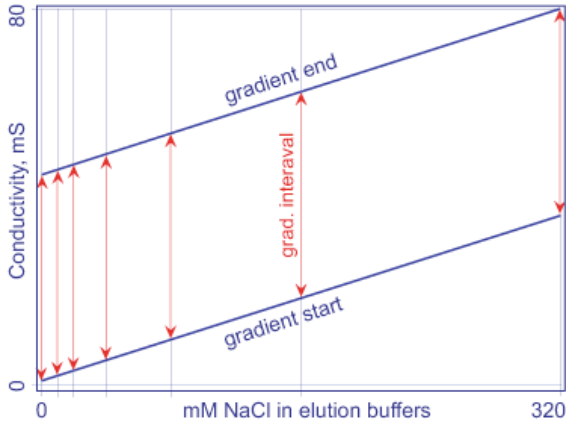
All of the curves show a bimodal response, as in the previous data set, demonstrating the respective contributions of calcium affinity and phosphoryl cation exchange. However, the responses of Fc and Fab are reversed. The substantial reduction in Fc binding caused by 10 mM NaCl shows that its cation exchange binding component is very weak. The level retention segment shows that its calcium binding component is resistant to NaCl from 10 to 20 mM. The same basic pattern is apparent in Fab and IgG at higher salt concentrations.

Retention mapping

HA elution conductivity in phosphate gradients at level NaCl concentrations



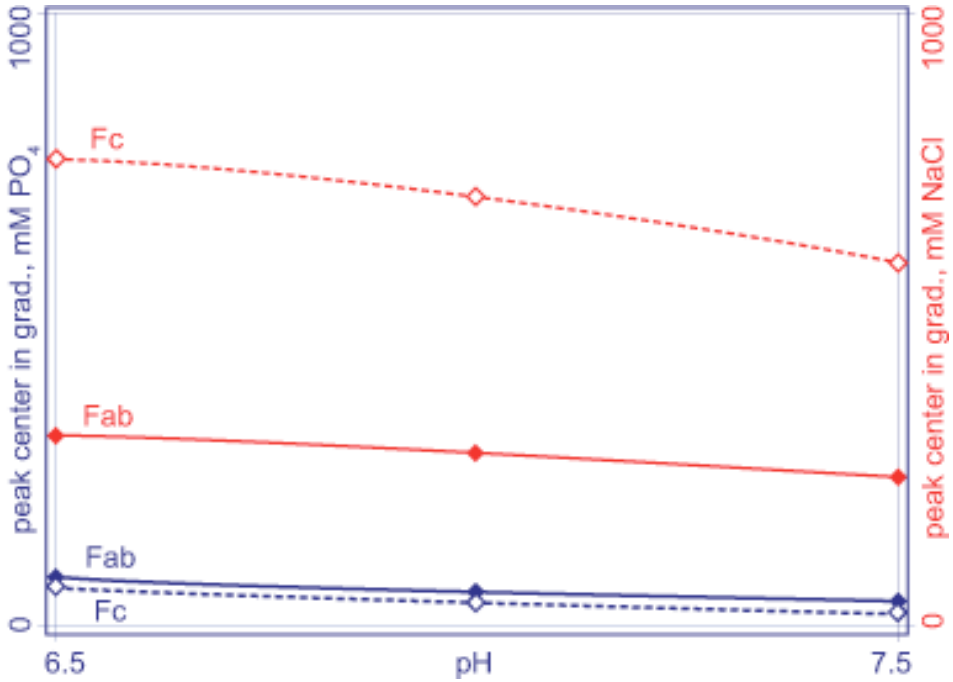
Conditions for data at left as in the previous slide but with retention plotted as a function of conductivity. Conductivity values of gradient start and endpoints illustrated below.



These results highlight the bimodality of antibody binding on HA. In a purely ion exchange system, retention would become weaker with increasing conductivity. The ascending segment of each curve reveals the influence of a binding mechanism that is resistant to changes in conductivity, which is understood to be calcium affinity. These data also show that even though chloride weakens cation exchange binding to a greater extent than it weakens calcium affinity, it has a significant effect on calcium affinity as well.

Retention mapping

HA retention in phosphate and chloride gradients as a function of pH



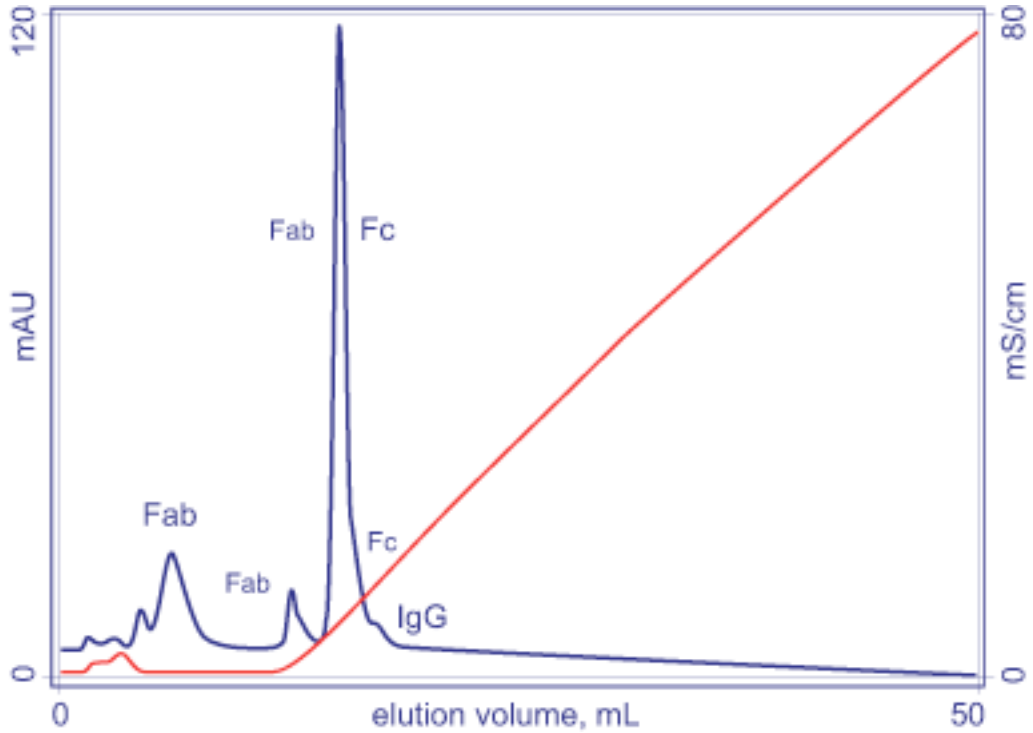
Red traces: NaCl gradient data
Equilibrate: 5 mM $NaPO_4$
Inj: papain-digested $hIgG_1$ Mab
Wash: 5 mM $NaPO_4$
Elute: 30 CV LG to 5 mM $NaPO_4$
1.0 M NaCl. pH as indicated.

Blue traces: phosphate gradient data
CHT type I, 40 µm, 300 cm/hr.
Equilibrate: 5 mM $NaPO_4$
Inj: papain-digested $hIgG_1$ Mab
Wash: 5 mM $NaPO_4$
Elute: 30 CV LG to 500 mM $NaPO_4$
pH as indicated.

Reduced retention with increasing pH for both fragments under both sets of elution conditions is consistent with weakening of cation exchange interactions. The shallowness of the curves suggests that the cation exchange component of HA binding is weak in this pH range. This graph also emphasizes the molar effectivity of phosphate over chloride as an HA eluting ion. Data from Aberin, C., Snyder, M., Ng, P. (2007) Purification and separation of Fab and Fc fragments on IgG monoclonal antibodies on CHT™ Ceramic Hydroxyapatite, Poster, Bioprocess International Conference and Exhibition, Boston, Oct.1-4.

Retention mapping

Cation exchange retention at pH 7.0



CIM™ SO3, 0.34 mL
4.0 mL/min (300 cm/hr)
Equilibrate: 50 mM Hepes, pH 7.0
Inj: 50 µL papain-digested hIgG$_1$ Mab
Wash: 50 mM Hepes, pH 7.0
Elute: 20 CV LG to 50 mM Hepes, 1.0 M NaCl, pH 7.0

This antibody exhibits stronger than average cation exchange binding but even intact IgG is fully eluted at less than 150 mM NaCl. Fab mostly fails to bind and the remainder elutes on the leading side of the Fc peak. None of the components were retained by an anion exchanger (pH 8.5).

Weak binding, or the failure to bind, to a traditional cation exchange ligand indicates that phosphoryl cation exchange – by itself – is a minor contributor to Fab, Fc, and IgG binding on HA.

Characterization of Fab binding

Experimental results indicate that Fab binds HA by both phosphoryl cation exchange and calcium affinity.

Phosphoryl cation exchange appears to be the dominant binding mechanism. This is indicated by relatively unchanged retention in chloride gradients from 10 to 40 mM phosphate.

Despite its apparent dominance, traditional cation exchange results indicate that Fab binding by this mechanism is weak.

This emphasizes the importance of cooperation between the binding mechanisms.

Characterization of Fc binding

Experimental results indicate that Fc also binds HA by both phosphoryl cation exchange and calcium affinity.

The cation exchange component of Fc binding appears to be weaker than the cation exchange binding component of Fab. This is indicated by the 50% reduction in binding strength from 0 to 10 mM NaCl, versus a reduction of about 16% by Fab over the same interval.

Calcium affinity appears to be the dominant binding mechanism for Fc, as indicated by unchanged retention in phosphate gradients at 10-20 mM NaCl. However, Fc binding by this mechanism is weak, again emphasizing the importance of cooperative binding.

Characterization of IgG binding

Cation exchange and calcium affinity appear to be fairly balanced in the binding of this IgG to HA. Retention mapping in NaCl gradients suggests that cation exchange may dominate slightly, while phosphate gradient data suggest that calcium affinity may dominate slightly.

Cooperative binding is apparent in two aspects of the data:

- *The distinctive shapes of the Fab and Fc retention curves are both apparent in the retention curves for IgG (phosphate and chloride gradients).*
- *The individual amplitudes of the Fab and Fc curves are roughly additive in the IgG curves.*

Fab purification with Ca-HA

Overall, these results suggest that calcium affinity in the absence of cation exchange might provide effective purification by allowing Fab to flow through while selectively binding Fc and IgG.

In the absence of phosphate, soluble calcium forms coordination complexes with HA-phosphate groups, converting them into secondary calcium sites.

This abolishes cation exchange and increases availability of surface calcium.

This calcium derivatized form (Ca-HA) is stable in the absence of phosphate, even at high NaCl concentrations.

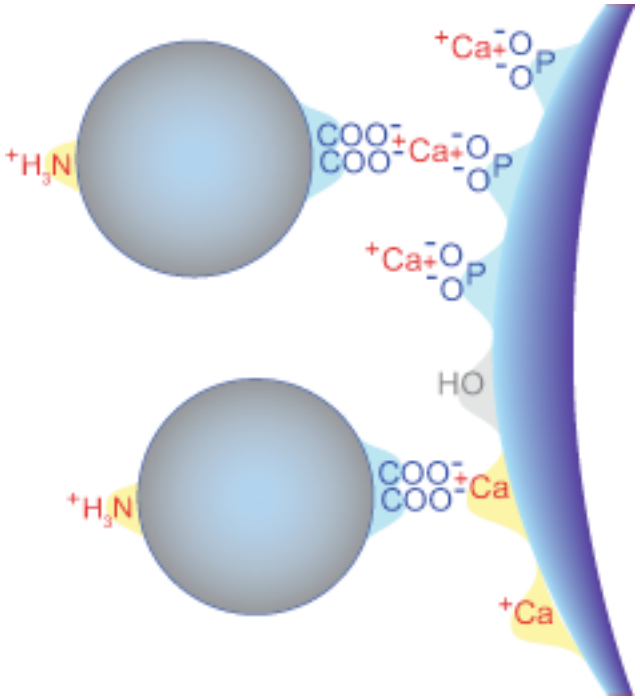
Fab purification with Ca-HA

Ca-HA

Calcium derivatization of HA phosphate abolishes the potential for cation exchange interactions, but increases opportunities for calcium affinity binding.

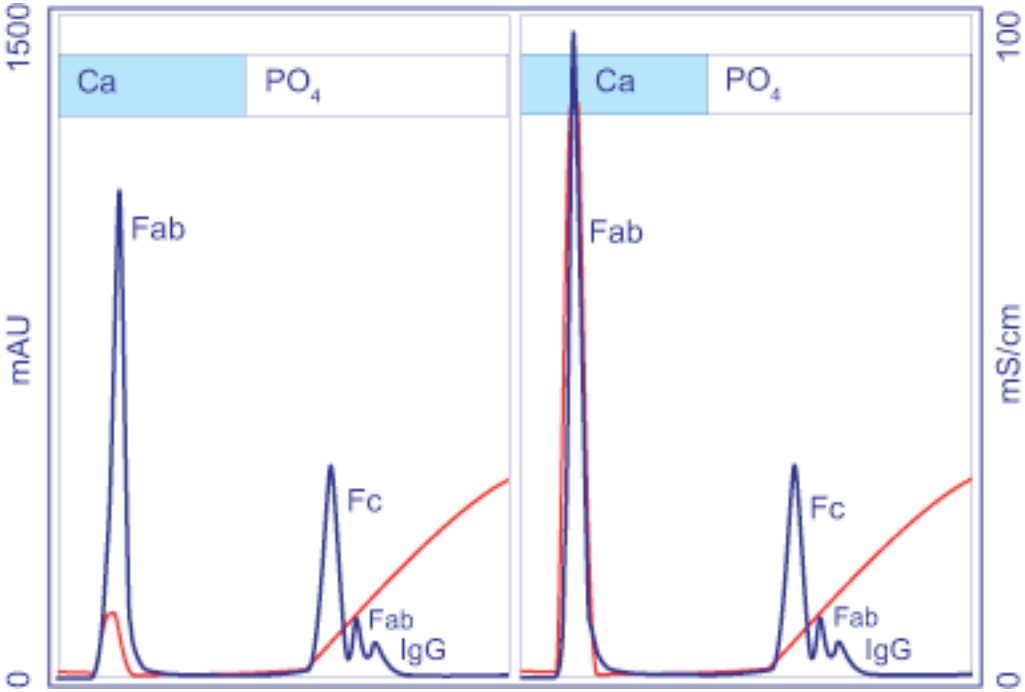
Since calcium affinity is relatively unaffected by conductivity, selectivity should be likewise unaffected, creating a high degree of salt tolerance.

Restoration of native HA can be achieved by washing with phosphate.



Fab purification with Ca-HA

Fab flow-through, Fc binding, at high and low salt concentrations

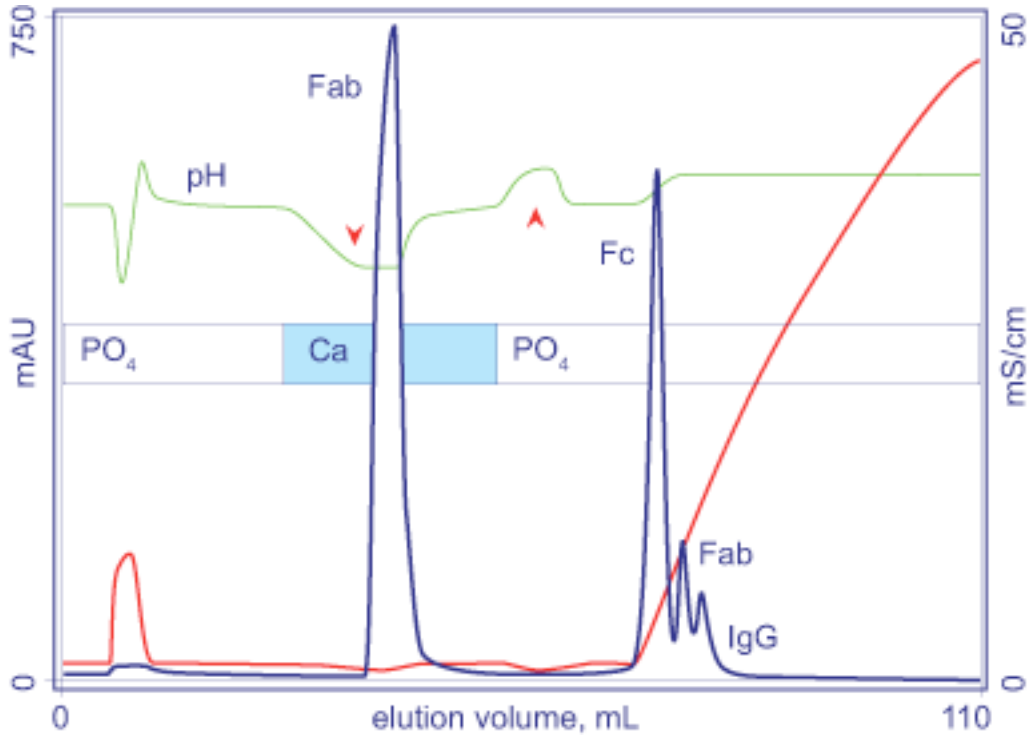


CHT type I, 20 µm, 2.5 mL
MediaScout 8 x 50 mm,
2.5 mL/min (300 cm/hr)
Convert to Ca-HA and equilibrate with
50 mM Hepes, 2.5 mM $CaCl_2$, pH 7.0
Inj: 500 µL papain-digested $hIgG_1$ Mab
Sample 1: 50 mM NaCl, 2.5 mM $CaCl_2$
Sample 2: 1.0 M NaCl, 2.5 mM $CaCl_2$
Wash with equilibration buffer.
Restore native HA by washing with 10
mM $NaPO_4$, pH 7.0
Elute: 20 CV LG to 500 mM $NaPO_4$
pH 7.0

These results show that although Fab is retarded, it mostly fails to bind, while Fc and IgG bind strongly. The more concentrated flow-through peak at 1.0 M NaCl indicates that retardation is diminished at high salt concentration. This is consistent with previous results showing that NaCl weakens calcium affinity as well as cation exchange. About 5% of the Fab binds to the column regardless of NaCl concentration.

Fab purification with Ca-HA

Concentrate Fab by binding to native HA, then elute by conversion to Ca-HA

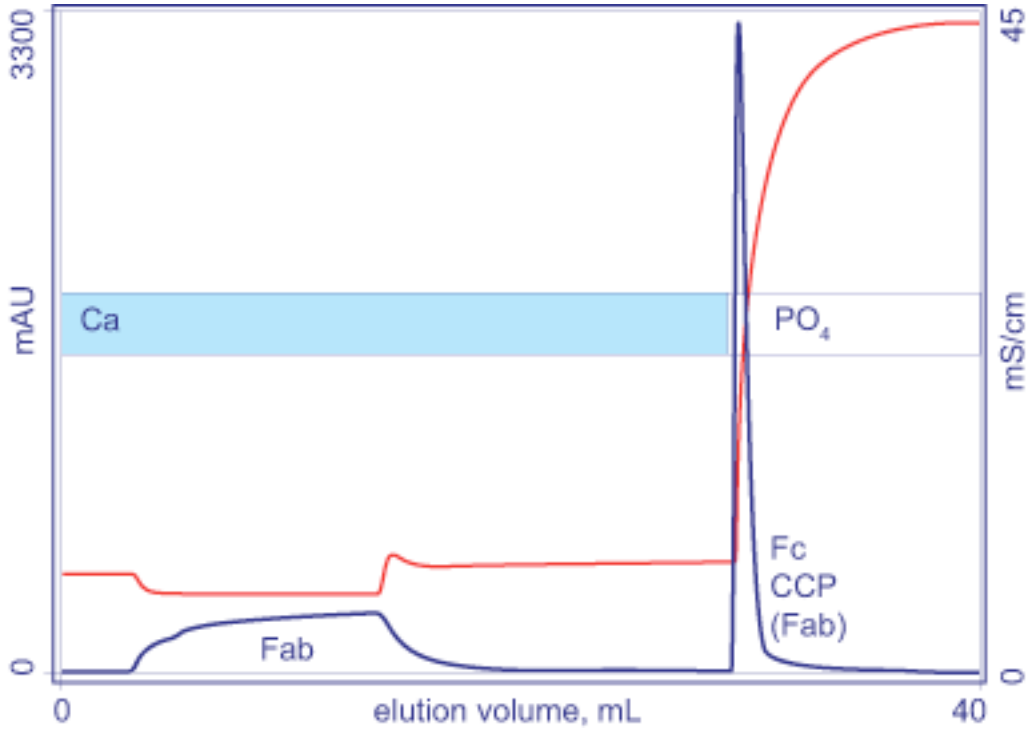


CHT type I, 20 µm, 2.5 mL
MediaScout 8 x 50 mm,
2.5 mL/min (300 cm/hr)
Equilibrate native HA with 5 mM $NaPO_4$, pH 7.0
Inj: 500 µL papain-digested $hIgG_1$ Mab
Convert to Ca-HA and elute Fab with 50 mM Hepes, 2.5 mM $CaCl_2$, pH 7.0
Restore native HA by washing with 10 mM $NaPO_4$, pH 7.0
Elute: 20 CV LG to 500 mM $NaPO_4$ pH 7.0

This examples shows that Fab can be concentrated from a dilute source on native HA prior to selective elution by conversion to Ca-HA. Note the transient reduction of pH at the down-arrow and the transient increase at the up-arrow. These transients provide a convenient means of determining when native HA has been converted completely to Ca-HA or when Ca-HA has been restored to native HA.

Fab purification with Ca-HA

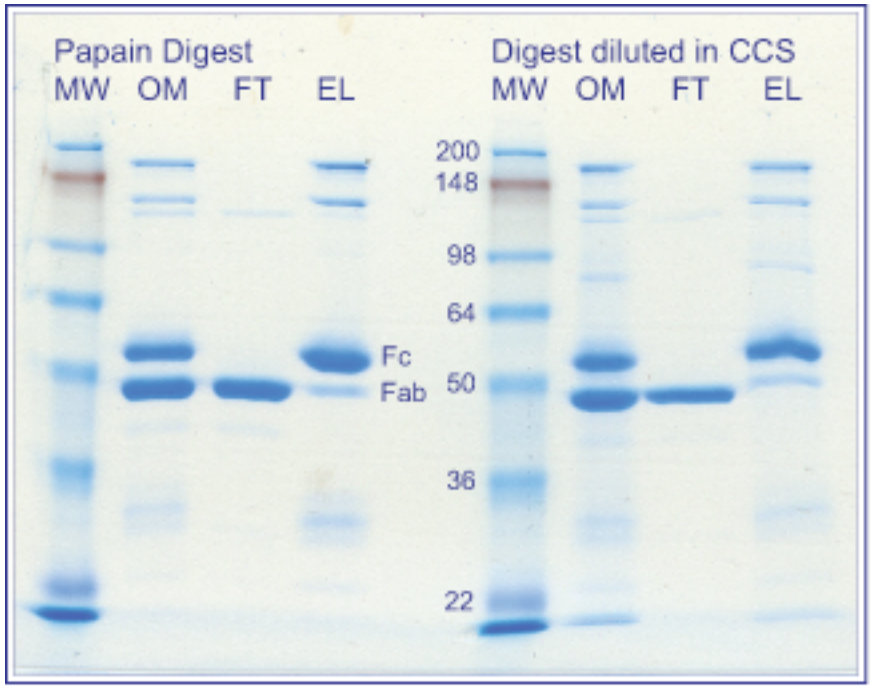
Purification of a papain digest diluted in cell culture supernatant



CHT type I, 20 µm, 2.5 mL
MediaScout 8 x 50 mm,
2.5 mL/min (300 cm/hr)
Convert to Ca-HA and equilibrate with
50 mM Hepes, 2.5 mM $CaCl_2$, pH 7.0
Inj: 10 mL (1 mL papain-digested
$hIgG_1$ Mab diluted in 9 mL CCS, plus
2.5 mM $CaCl_2$).
Wash with equilibration buffer.
Restore native HA and elute by
washing with 500 mM $NaPO_4$, pH 7.0
CCP indicates cell culture proteins.

Fab purification with Ca-HA

Non-reduced SDS-PAGE of Fab purification on Ca-HA



MW: molecular weight stds (Kd)
OM: original material
FT: flow-through fraction
EL: elution fraction

Conclusions

HA offers a variety of effective options for purification of Fab.

- *Bind/elute with phosphate gradients on native HA*
- *Bind/elute with chloride gradients on native HA*
- *Flow-through on Ca-HA*
- *Bind native HA, elute Fab by conversion to Ca-HA*

Flow through on Ca-HA is simplest from a process development perspective, requiring only two buffers. Only the contaminant-binding capacity needs to be determined.

This approach can also conserve low sample conductivity to facilitate a subsequent ion exchange step, or tolerate the high conductivity of a sample following (or leading to) a hydrophobic interaction chromatography step.

Conclusions

Ca-HA is similar to protein A for removal of Fc, in that both selectively remove Fc-containing species.

Protein A is simpler because it does not require sample preparation. Ca-HA requires that the sample contain calcium and that it not contain phosphate.

HA is more economical, it does not leach immunotoxins, and it is stable for thousands of hours in 1 M NaOH. Thus it supports rigorous sanitization and storage conditions that protein A cannot.

Most important, Ca-HA removes other CCS contaminants along with Fc species. Protein A does not.

Conclusions

How well Ca-HA will accommodate other fragment constructs, or Fab from other antibodies, remains to be seen.

In cases where Ca-HA fails to meet the needs of a given purification challenge, phosphate or chloride gradients on native HA may provide useful alternatives.

This flexible suite of capabilities suggests that HA should be a valuable addition to the process development tool box in this field.

Acknowledgements

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Copies of this presentation can be downloaded at www.validated.com