How CHT™ Ceramic Hydroxyapatite Works

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CHT ceramic hydroxyapatite is a versatile chromatography support used for separation of biological molecules as diverse as polyclonal and monoclonal antibodies, antibody fragments, enzymes, nucleic acids, and membrane proteins. The interactions that occur between CHT and the molecules it binds are complex; this article aims to clarify the most significant features of these interactions.

CHT, $Ca_{10}(PO_4)_6(OH)_2$, is a mixed-mode support with functional groups consisting of pairs of positively charged crystal calcium ions (C-sites) and clusters of six negatively charged oxygen atoms associated with triplets of crystal phosphates (P-sites) (Figure 1). The C-sites, P-sites, and hydroxyl groups are distributed in a fixed pattern on the CHT crystal structure, as presented in classic studies by Kawasaki (1978a, 1978b) and Kawasaki et al. (1985).

In theory, CHT can retain solutes by weak anion exchange or calcium metal affinity with C-sites, by cation exchange with P-sites, and by hydrogen bonding with hydroxyl groups (Gorbunoff 1984a, 1984b, Gorbunoff and Timasheff 1984). Experimental evidence, however, suggests that most proteins bind CHT by a combination of metal affinity and phosphoryl cation exchange (Figure 2), with little contribution by hydrogen bonding. The affinity interaction of protein carboxyl clusters with CHT C-sites represents a classic metal chelating mechanism in which protein carboxyl groups approximate the carboxyl configuration of chelating agents such as EDTA.

Fig. 1. Crystal structure of CHT ceramic hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$. Each molecule consists of five positively charged calcium pairs (C-sites); two phosphate triplets (P-sites), each with six negatively charged oxygen atoms; and two hydroxyl residues.

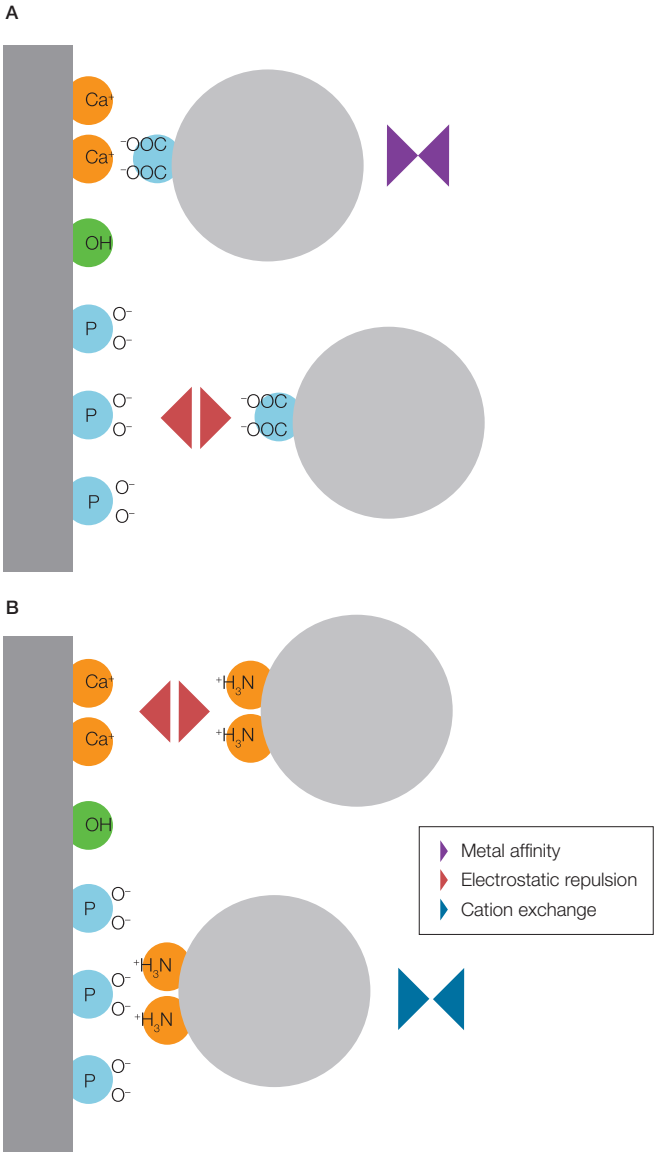

Fig. 2. Interaction of proteins with CHT. A, interaction of carboxyl groups and CHT. Note the metal affinity interaction between CHT C-sites and carboxyl groups, and the repulsion of carboxyl groups from P-sites. **B**, interaction of amino groups with CHT. Note the phosphoryl cation exchange interaction between CHT P-sites and amino groups, and the repulsion of amino groups from C-sites.

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Stronger than electrostatic interactions, these metal affinity interactions withstand the presence of even saturated sodium chloride since chloride ions do not form a complex with Ca^{2+}. This indicates that any anion exchange between CHT C-sites and protein carboxyl groups does not contribute significantly to protein binding (Gorbunoff 1984a, 1984b, Gorbunoff and Timasheff 1984, Gagnon 1998). Further evidence of this comes from the demonstration that acidic proteins are retained more weakly with ascending pH (Ogawa and Hiraide 1995). In addition, binding experiments with urea, which disrupts hydrogen bonds (Tanford 1968, Timasheff and Fasman 1969), indicate that the contribution of hydrogen bonding is likewise negligible.

The contributions of metal affinity and phosphoryl cation exchange are distinctive for every protein and can be investigated by eluting with various neutral salt and phosphate concentrations. Elution of proteins bound by metal affinity interactions requires phosphate, which outcompetes CHT-protein metal interactions with its own strong affinity for calcium. In contrast, elution of proteins bound by cation exchange requires either neutral salts, such as sodium chloride, or buffering salts, such as phosphates. Therefore, elution of proteins by phosphate-mediated buffer can result in distinct separation, depending upon the mechanism by which the protein is bound.

Some proteins, such as lysozyme, bind CHT exclusively by cation exchange between their amino groups and CHT P-sites (Figure 2B), while others, such as bovine serum albumin (BSA), bind almost exclusively by metal affinity interactions (Figure 2A). Rich in carboxyl groups and with a strong affinity for CHT C-sites, BSA elutes in a linear phosphate gradient at pH 6.5 in 110 mM sodium phosphate (Gagnon 1998). However, when the same linear phosphate gradient is run in the presence of 1.0 M sodium chloride, the phosphate concentration required for elution of BSA drops only to 100 mM (Gagnon et al. 2005a), indicating that ion exchange is a minor contributor to the binding energy while calcium affinity dominates retention.

Still other proteins bind CHT by a combination of interaction mechanisms. Monoclonal antibodies (IgG) elute in 100–200 mM sodium phosphate (Josic et al. 1991, Bukovsky and Kennett 1987, Brooks and Stevens 1985), and unlike with BSA, even modest levels of sodium chloride sharply reduce the retention times and dynamic capacity for IgG by CHT (Gagnon 1998). This demonstrates that phosphoryl cation exchange is a major contributor to IgG binding. However, metal affinity is also a factor, albeit less so than with BSA. Though as little as 5 mM phosphate weakens binding to the point where sodium chloride can elute IgG, even this relatively weak calcium affinity must be overcome to achieve elution. Unless a threshold concentration of phosphate is present, most IgGs remain bound to CHT even in saturated sodium chloride. As shown in Figure 3, retention on CHT is progressively reduced with increased phosphate concentration.

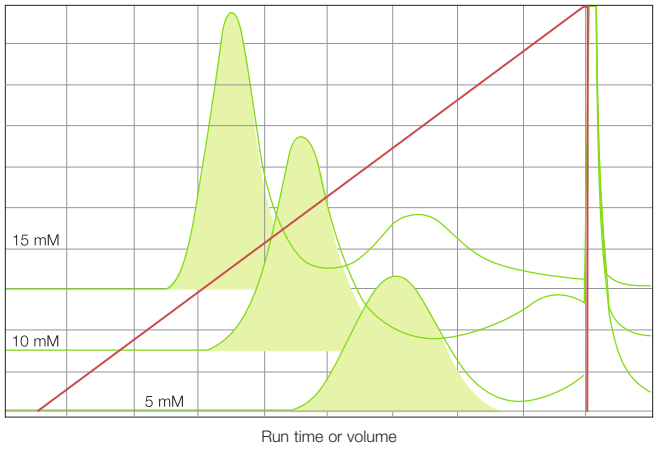

Fig. 3. Elution of protein A-purified IgG1 from CHT ceramic hydroxyapatite. A 40 column volume linear gradient to 1.0 M NaCl (—) at three constant phosphate concentrations is shown. Data indicate a trend of decreasing aggregate resolution with increasing phosphate concentration.

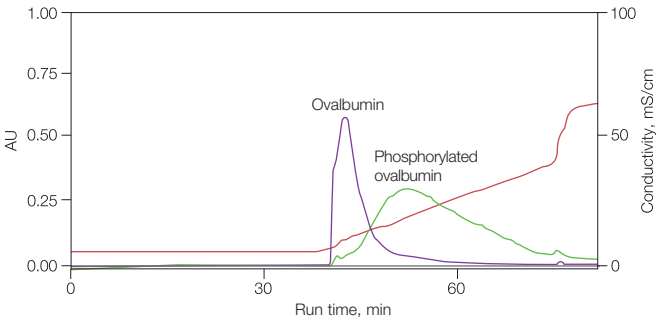

Fig. 4. Resolution of ovalbumin from its phosphorylated counterpart using CHT ceramic hydroxyapatite. Note the longer retention time for phosphorylated ovalbumin. Increasing the phosphate content of ovalbumin by conjugation of phosphoserine enhanced retention on CHT. Red trace shows conductivity (phosphate gradient).

The ability of phosphate to effect elution implies that phosphorylated solutes bind strongly to CHT. In fact, phosphoryl groups on proteins and other solutes interact even more strongly with C-sites than do carboxyls (Kawasaki 1991), and phosphoproteins bind more strongly than their unphosphorylated counterparts (Ng et al. 2005a) (Figure 4). DNA, which is highly phosphorylated, binds strongly and with an apparent correlation between its size and retention time: Small fragments elute at about 0.1 M phosphate, and chromosomal DNA at 0.2–0.3 M phosphate (Kawasaki 1991, Ng et al. 2005b).

Though phosphate concentrations of 0.5 M are recommended for elution of all size classes of DNA, even higher concentrations are required if sodium chloride is present (Figure 5). This has been attributed to sodium chloride–mediated suppression of the charge repulsion between the phosphate groups on DNA and those on CHT (Figure 6). The higher conductivity obtained by adding sodium chloride may also make DNA less rigid, allowing it to conform to the geometry of available CHT C-sites (Ng et al. 2005b).

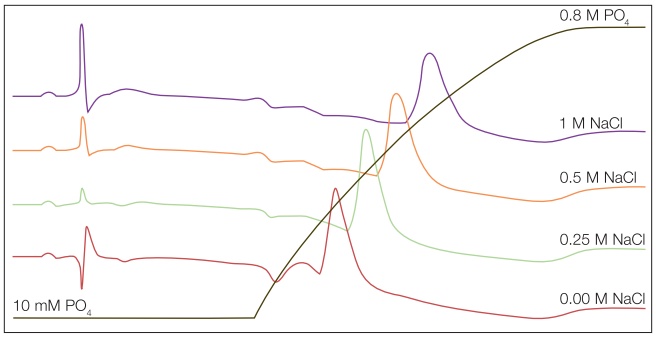

Fig. 5. Elution of sheared salmon sperm DNA from CHT ceramic hydroxyapatite. Data compare the behavior of DNA on CHT as a function of sodium chloride concentration. Increased NaCl concentration improved DNA retention.

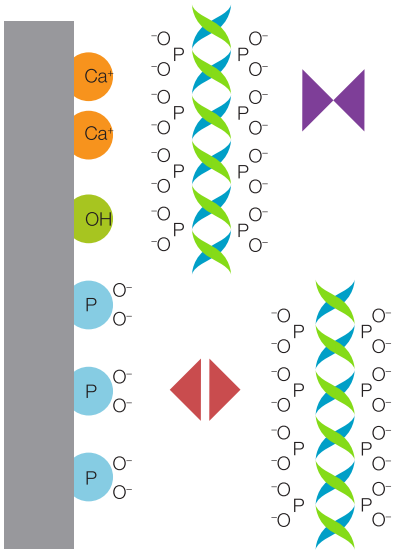

Fig. 6. Interaction of DNA with CHT ceramic hydroxyapatite. Note the electrostatic repulsion between CHT P-sites and the phosphate backbone of DNA, and the interaction of that backbone with the C-sites and hydroxyl groups. (Symbols same as in Figure 2.)

Endotoxins, which are also phosphorylated, may require up to 1.0 M phosphate for complete removal; subpopulations can elute over the entire range of 0–0.5 M potassium phosphate, but reductions in retention are apparent when phosphate gradient elution is carried out at high sodium chloride concentrations, indicating that binding involves a cation exchange component (Gagnon et al. 2005b).

The mechanism by which proteins interact with CHT is multifaceted. Its unique resolution property makes it a powerful tool for process developers. The ability of CHT to purify a variety of proteins — including monoclonal antibodies, which are leading licensed products or therapeutic candidates in many drug companies — strengthens its versatility. It is anticipated that CHT will enjoy increasing attention in the years to come.

For more information, request bulletin 2156 or visit **www.bio-rad.com/process/**.

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Ordering Information

Catalog #	Description*
CHT Ceramic Hydroxyapatite, Type I	
158-2000	20 µm particle size, 10 g
157-0020	20 µm particle size, 100 g
157-0021	20 µm particle size, 1 kg
157-0025	20 µm particle size, 5 kg
158-4000	40 µm particle size, 10 g
157-0040	40 µm particle size, 100 g
157-0041	40 µm particle size, 1 kg
157-0045	40 µm particle size, 5 kg
158-8000	80 µm particle size, 10 g
157-0080	80 µm particle size, 100 g
157-0081	80 µm particle size, 1 kg
157-0085	80 µm particle size, 5 kg
732-4322	Bio-Scale™ Mini CHT-I cartridge, 40 µm, 1 x 5 ml
732-4324	Bio-Scale Mini CHT-I cartridge, 40 µm, 5 x 5 ml

Catalog #	Description*
CHT Ceramic Hydroxyapatite, Type II	
158-2200	20 µm particle size, 10 g
157-2000	20 µm particle size, 100 g
157-2100	20 µm particle size, 1 kg
157-2500	20 µm particle size, 5 kg
158-4200	40 µm particle size, 10 g
157-4000	40 µm particle size, 100 g
157-4100	40 µm particle size, 1 kg
157-4500	40 µm particle size, 5 kg
158-8200	80 µm particle size, 10 g
157-8000	80 µm particle size, 100 g
157-8100	80 µm particle size, 1 kg
157-8500	80 µm particle size, 5 kg
732-4332	Bio-Scale Mini CHT-II cartridge, 40 µm, 1 x 5 ml
732-4334	Bio-Scale Mini CHT-II cartridge, 40 µm, 5 x 5 ml

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