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Bio-Scale Ceramic Hydroxyapatite, Type I Columns

Instruction Manual

Catalog Numbers
751-0021, 751-0023,
751-0025, 751-0027

BIO-RAD

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Section 1
Characteristics of the Bio-Scale CHT2, CHT5, CHT10, CHT20 Ceramic Hydroxyapatite, Type I Columns

1.1 Introduction

Bio-Scale prepacked hydroxyapatite columns meet the needs of the bio-chromatographer for rapid and reproducible high resolution separations of biomolecules including proteins, peptides and polynucleotides. The availability of four column sizes (bed volumes of 2, 5, 10, and 20 ml) provides unrivaled flexibility for the economical and predictable scale up of separation and purification protocols without sacrificing resolution due to overloading.

1.2 The CHT-I Separation Medium

Each column contains a newly developed, chemically pure form of hydroxyapatite. The spherical, ceramic nature of the CHT-I support overcomes the physical and chemical instability that limited the use of traditional crystalline forms of hydroxyapatite for high resolution chromatography. The 10 µm particle size and narrow particle size distribution produce excellent resolution of biomolecules at high flow rates and with very low back-pressures.

The CHT-I support demonstrates high affinity for basic proteins of relatively high pI and lower affinity for proteins of relatively low pI. The hydroxyapatite structure (Ca^{2+} ions at the positively-charged centers and PO_4^{3-} ions at the negatively-charged centers) results in essentially a mixed-mode ion exchange separation. Separation of biomolecules using hydroxyapatite is a very useful complement to other separation techniques such as ion-exchange and hydrophobic interaction chromatography particularly in the later stages of a purification scheme. It will often resolve components that other techniques fail to separate. The support binds proteins under mild conditions using neutral phosphate buffer systems. Selective elution is accomplished with higher concentrations of phosphate and/or a pH gradient. Recovery of protein/enzymatic activity is normally high due to the gentle elution conditions employed.

Stability of the CHT-I Support

The columns are stable over the pH range 5.5-14, allowing for easy cleaning and regeneration. The support is compatible with aqueous solutions of 6 M guanidine-HCl and 8 M urea. Detergents such as Triton® (reduced form), CHAPS, CTAB and 1% SDS and organic solvents such as methanol, ethanol and acetonitrile may also be used.

Buffers containing calcium chelators such as EDTA and EGTA should be avoided. If chelating agents must be present in the buffers, the addition of some calcium chloride may be necessary.

1.3 Connection to FPLC and HPLC Systems

Each Bio-Scale column is supplied with 0.02 inch ID (1/16 inch OD) Tefzel tubing, 1/4x28 flangeless fittings and end caps for connection to the BioLogic system. For connection to HPLC systems, 10-32 fingertight fittings and M6fittings for FPLC systems.

Instructions for assembling the fittings are included with the column.

Table 1. Column Characteristics CHT2-I

	CHT2-I	CHT5-I	CHT10-I	CHT20-I
Column volume (ml)	2	5	10	20
Recommended max. protein loading (mg)	20	50	100	200
Recommended flow arates (ml/min)	0.5 to 3.0	0.5 to 5.0	0.5 to 7.0	0.5 to 10.0
Dynamic protein binding capacity (mg Lysozyme)/ column	30	75	150	300
Static DNA binding capacity (mg calf thymus DNA)	0.20	0.5	1.0	2.0
Average particle size (µm)	10 ± 3.0	10 ± 3.0	10 ± 3.0	10 ± 3.0
Column dimensions (mm)	7 x 52	10 x 64	12 x 88	15 x 113
Maximum operating pressure (psi/bar)	1,000/67	750/50	600/40	500/34

Note: Bio-Rad Laboratories does not recommend nor warranty the use of the Bio-Scale columns with solvent delivery systems containing stainless steel parts and used with corrosive eluents containing e.g. halide salts. We recommend the use of inert, biocompatible (ceramic, PEEK, titanium) solvent delivery systems for maximum column life and recovery of sample biological activity.

Section 2
Use of the Bio-Scale CHT-I Columns

2.1 Preparation for Initial Use

The columns are supplied in a storage solution of 20% ethanol in 5 mM phosphate buffer, pH 6.8. Prior to initial use and after extended storage periods, each column should be conditioned as described below (steps 1-4). Always use HPLC grade reagents, and filter and degas buffers). During this operation do not exceed more than **25%** of the recommended maximum flow rates (see Table 1).

1. Wash with 5 column volumes of water.
2. Wash with 5 column volumes of low ionic strength equilibration buffer. Typically 5-10 mM sodium phosphate pH 6.8
3. Wash with 5 column volumes of high ionic strength limit buffer. Typically 500 mM sodium phosphate pH 6.8.
4. Wash with 5 column volumes of low ionic strength equilibration buffer.

The column may now be further equilibrated in the starting buffer at the desired flow rate.

Always use buffer components of the highest available purity as UV absorbing impurities may cause baseline disturbances that interfere with the detection of protein peaks.

2.2 Sample Preparation

Proper adjustment of the sample pH and ionic strength is critical for consistent and reproducible chromatography. For best results, the sample should be exchanged into the start buffer

or diluted to the start buffer's concentration. Buffer exchange can be accomplished using Bio-Spin® 6 or Bio-Spin 30 columns, Econo-Pac® 10DG desalting columns, Bio-Gel® P-6DG size exclusion gel or the Econo-Pac P6 cartridge. The choice of product depends on sample volume. Always centrifuge or filter the sample (0.2-0.45 mm filter) to remove particulates. Application of turbid or lipid-containing samples may reduce the column lifetime. See Ref. 1 for further information about sample preparation.

Sample Load

The recommended sample load for each column is shown in Table 1. This amount may vary somewhat depending on the actual sample composition. We do not recommend overloading the column as both resolution and column lifetime will decrease. For larger loads, either change to a larger column or perform several chromatographic runs with a reduced loading. Ideally, samples should be bound in a concentrated zone at the top of the column. Higher sample loads produce a broad application zone in which components with less affinity for the support are displaced by more tightly bound components. This may result in a shift of certain peaks to an earlier elution position in the gradient.

2.3 Elution Conditions

Separations using hydroxyapatite are typically accomplished by increasing the phosphate concentration of the eluent either as a "step" or as a "continuous" gradient. For certain separations, varying the pH of the elution buffer in addition to its phosphate concentration may be advantageous. Generally, it is best to choose initial pH and ionic strength conditions such that the protein of interest elutes early in the gradient. This is especially true for labile proteins or where a higher phosphate concentration is undesirable. **If Tris buffer must be used, do not use at a concentration ≥ 10 millimolar.**

Gradient Volumes & Phosphate Concentrations

As a starting point for developing a separation, we recommend using the Bio-Scale CHT2-I column with a simple gradient profile over 48 ml.

Protocol: Use a flow-rate of 2.0 ml/min. Following sample application, wash unbound proteins from the column with 6 ml (3 bed volumes) of Start buffer A. For elution, use a gradient volume of 24 ml (12 bed volumes) to a phosphate concentration of 0.5 M (100% B). Hold at 0.5 M for 6 ml before re-equilibrating the column with 12 ml of start buffer A. This gradient is shown schematically in Fig. 1. Once an initial separation has been performed and the elution position of the protein of interest determined, the gradient composition and volume is adjusted to achieve maximum resolution. Normally, a gradient volume of 10 to 20 ml per ml of column bed volume is sufficient. The slope of the gradient will affect resolution. A steep gradient will result in relatively small peak volumes but short peak-to-peak distances. A shallower gradient normally gives greater resolution but peak volumes are larger.

Where binding and elution conditions are well established for a particular biomolecule, the sample concentration effect seen with hydroxyapatite means that this form of chromatography is ideal for the recovery of low abundance proteins from a large sample volume.

References 2 and 3 provides useful background information on the theory and applications of hydroxyapatite chromatography.

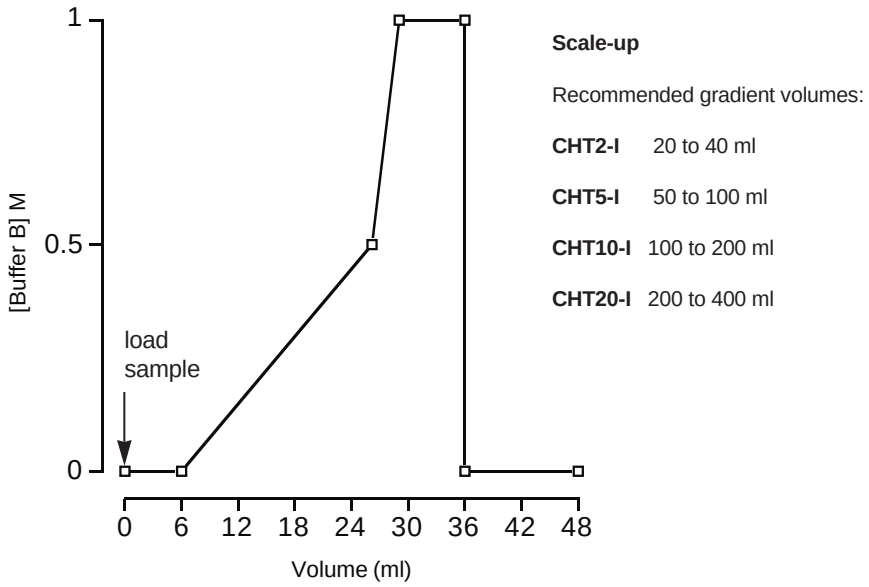


Fig. 1. Schematic gradient for separation on Bio-Scale CHT2-I column.

2.4 Use of Detergents

Most of the common detergents used for protein isolation may be used with the Bio-Scale CHT-I support. We recommend the use of the reduced form of Triton® X-100 to minimize UV absorption artifacts. It is essential to thoroughly equilibrate the column with the detergent-containing buffer prior to sample application. Pay particular attention to pH, which influences the solubility of the various classes of detergents. Problems may arise during gradient elution due to the formation of micelles. This may cause a sudden increase in the UV baseline as the micelles themselves scatter light. We recommend using a concentration of detergent above the critical micelle concentration (CMC) during gradient elution. If subsequent chromatographic steps (e.g. hydrophobic interaction) or other biochemical manipulations will be affected by the presence of the detergent, then the initial choice of detergent should be influenced by the availability of a protocol for its removal. See Refs. 1, 4, and 5 for a more detailed explanation of the characteristics and use of detergents in biology and biochemistry and their removal from biological samples.

2.5 Effect of Calcium Chloride

$CaCl_2$ may be added to the buffers to increase the binding of acidic proteins. The buffers should contain calcium and phosphate at the level of the solubility product for calcium phosphate. For example, at pH 7.0, the solubility product is $3x10^{-6}$ M. Hence, if a 10 mM to 500 mM phosphate gradient is used, the 10 mM phosphate buffer should be 0.3 mM in calcium and the 500 mM phosphate buffer should be 0.006 mM in calcium.

Note: At pH 6.0, the solubility product is for calcium phosphate is 3×10^{-5} M and at pH 8.0 the solubility product is 3×10^{-7}M.

2.6 Chromatography Pre-Runs

Prior to loading the sample, we recommend running a blank gradient to check that the column is clean. In addition, detergents and other buffer components may themselves elute as

sharp, UV-absorbing peaks during the gradient, complicating subsequent analysis of the chromatogram.

Section 3
Care of the Bio-Scale CHT-I Columns

3.1 Column Hygiene

Careful preparation (especially filtration) of the sample and the buffers will maintain the column performance and lifetime. Normally, washing with 0.5 M phosphate will remove most bound components. However, if there is a significant decrease in column performance (ie increasing backpressures or a significant drop in resolution) then a more extensive cleaning protocol as described below should be used. **Always reverse the flow during this procedure so tightly bound substances at the top of the column are quickly removed.**

During this operation, do not exceed more than **25%** of the maximum recommended flow rates (see Table 1).

1. Wash with 2 column volumes of 2.0 M NaCl or KCl followed by 3 column volumes of water or low salt buffer.
2. Wash with 1 column volume of 2.0 M NaOH followed by 3 column volumes of water or low salt buffer.
3. Change the top frit, readjust the bed height to eliminate any voids and wash with 3 column volumes of equilibration buffer.

If lipid contamination is a problem, wash with 1 column volume of methanol followed by 3 column volumes of water followed by at least 2 column volumes of 0.5 M phosphate. Change the top frit, readjust the bed height to eliminate any voids and wash with 3 column volumes of equilibration buffer.

3.2 Bed Height Adjustment

Under certain conditions of buffer composition, high flow rates or long-term use, the resin bed may compress creating a void between the frit and the top of the bed. Normally, the void can be eliminated by turning the lock nut clockwise until the frit just touches the top of the bed. If the bed compresses at high flow rates, stop the pump and loosen the top fitting. Then adjust the lock nut to remove the void, retighten the top fitting and resume pumping buffer. If the bed has compressed from long-term use, then as a precaution the top frit should be replaced.

3.3 Frit Removal

The top frit may need to be replaced after extensive column use or if increasing backpressures are noticed. Each column is supplied with a Frit Removal Tool, Polyethylene Frit and Distribution Screen.

The screen assists in distributing the sample over the entire column surface and also acts as a pre-filter for the frit. It should be replaced every time the frit is changed. Figure 2 describes in detail how to remove and change the top frit.

3.4 Top-Off Resin

If the top of the resin bed becomes fouled and the above hygiene steps do not restore performance, then a few mm of the bed should be removed and replaced with fresh resin obtainable from Bio-Rad. See page 6 for ordering information.

3.5 Storage Conditions

Prior to long-term storage, the column should be cleaned as previously described and then washed with 3 column volumes of 20% ethanol. This will prevent microbial growth. Store the column in a safe place at room temperature. Never allow the column to freeze.

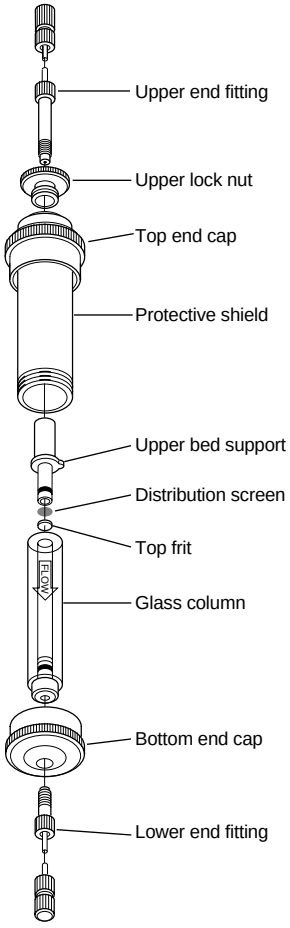


Fig. 2. Instructions for removal of top frit.

1. Remove the upper and lower end fittings from the column.
2. Remove the upper lock nut from the top end cap.
3. Unscrew the bottom end cap from the protective shield and slide out the glass column.
4. Pull out the upper bed support from the glass column.
5. Using the frit removal tool, remove the distribution screen and frit by pressing the hook into the frit in a sideways motion with slight downward pressure.
6. Add 5 ml of buffer to the top of the resin bed. Place a new frit and screen into the tube. Insert the upper bed support and use it to carefully push down the frit and screen until it just touches the top of the resin bed.
7. Insert the glass column through the protective shield, into the top end-cap and align the bed support prong with the top end-cap guide. Attach the bottom end-cap to the shield. Insert the lock nut into the top end-cap and twist down until the plunger is at the top of the bed.
8. Attach column to a pump and pump buffer at 2.0 ml/min for 5 to 10 minutes. Inspect the column for voids and adjust accordingly.

3.6 References

1. Guide to Sample Preparation. Bio-Rad Laboratories Technical Bulletin # 1322.
2. Hydroxylapatite. T. L. Brooks (Calbiochem Biochemicals).
3. HPLC of Proteins on an Hydroxyapatite Column. T. Kadoya et al, (1988), J. Liquid Chromatography, 11, No 14, 2951-2967.
4. Ceramic Hydroxyapatite HPLC of Complexes Membrane Protein and Sodium Dodecyl Sulfate. T. Horigome et. al., (1989), European Journal of Biochemistry, 186, 63-69.
5. A Guide to the Properties and Uses of Detergents in Biology and Biochemistry. J. Neugebauer (Calbiochem Biochemicals).

Section 4
Product Information

Catalog Number	Product Description
751-0021	**Bio-Scale CHT2-I Column**
751-0023	**Bio-Scale CHT5-I Column**
751-0025	**Bio-Scale CHT10-I Column**
751-0027	**Bio-Scale CHT20-I Column**
751-0029	**Top-Off Resin Kit CHT-I,** includes 1 ml of CHT-I support, 5 frits and 5 distribution screens for each column diameter
751-0091	**Bio-Scale 2 Replacement Part Kit,** includes 5 frits, 5 distribution screens, 2 o-rings, 1 frit remover
751-0093	**Bio-Scale 5 Replacement Part Kit,** includes 5 frits, 5 distribution screens, 2 o-rings, 1 frit remover
751-0095	**Bio-Scale 10 Replacement Part Kit,** includes 5 frits, 5 distribution screens, 2 o-rings, 1 frit remover
751-0097	**Bio-Scale 20 Replacement Part Kit,** includes 5 frits, 5 distribution screens, 2 o-rings, 1 frit remover
751-0099	**Bio-Scale Fittings Kit,** includes 2 Super Flangeless Nuts (1/4 x 28 threads) and 4 ferrules, 2 Flangeless M6 nuts, 4 ferrules and 2 caps, 2 fingertight II fittings (10-32 threads)

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