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Notes & Tips

Hydroxyapatite chromatography: altering the phosphate-dependent elution profile of protein as a function of pH

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Hydroxyapatite is a form of calcium phosphate that has long been used in the chromatographic separation of proteins and DNA [1]. Hydroxyapatite is best known as a crystalline material but is now available in a range of ceramic derivatives that are vastly superior in terms of flow rate, stability, and reproducibility over many cycles of use. These developments have led to a renewed interest in the use of this media with unique separation properties. This report aims to further extend the usefulness of hydroxyapatite for the purification of proteins.

The adsorption of proteins to hydroxyapatite is complicated because it involves both anionic and cationic exchange. The Ca^{2+} functional groups can interact with carboxylate residues at the protein surface while the PO_4^{2-} functional groups can interact with basic protein residues [2]. Proteins are most commonly adsorbed in a low concentration (10–25 mM) of phosphate buffer, although some acidic proteins are adsorbed only if loaded in water, saline, or a nonphosphate buffer [3]. Proteins are usually eluted by an increasing phosphate gradient, although gradients of Ca^{2+}, Mg^{2+}, or Cl^- ions are also useful, especially for the selective elution of basic proteins [2]. When using phosphate, acidic proteins are more readily eluted than basic proteins, although the phosphate concentration required to elute any protein can be reduced by raising the pH [4]. A mixture of proteins bound to hydroxyapatite can be fractionated by a series of phosphate wash steps of increasing pH [5]. However, phosphate is a weak buffer outside the 6.0–7.5 pH range, yet strong buffering is desirable when using hydroxyapatite to avoid the local pH changes that occur in the highly polarized environment at the surface [6]. Analysis of the effect of altering the pH upon protein elution would therefore benefit from improved buffering because the buffering capacity of the phosphate rapidly decreases away from neutrality. In the present paper, a simple procedure is described that extends the phosphate-mediated hydroxyapatite elution of proteins over a wider pH range while maintaining a properly buffered environment. Others have not previously reported the approach of using a second buffer system to broaden the pH range of separation with hydroxyapatite media.

Materials and methods

Hydroxyapatite media. Hydroxyapatite media was obtained from Bio-Rad Laboratories (Hercules, CA) as Macro-Prep ceramic hydroxapatite type II (CHT II), which is a spherical, macroporous form of crystalline calcium phosphate with particles 40 μm in diameter (Catalogue No. 158–4200). The CHT II instruction manual is available on-line at http://www.biorad.com/cmc_upload/0/000/039/227/Lit-611d.pdf. A mutant of alkyl hydroperoxide reductase component C (AhpC), referred to as AhpC* [7], was chosen as the test protein and was prepared and purified using methods described for the wild-type enzyme [8]. Fifty milliliters of CHT II were packed into a column (10 cm in height and 2.6 cm in diameter) according to the manufacturer's instructions and attached to an ÄKTA Explorer automated chromatography system (Amersham Biosciences, Piscataway, NJ).

Buffer preparation. Equilibration buffers were prepared with 25 mM Mes[1] or Hepes and adjusted to the desired pH at room temperature using HCl or NaOH, respectively. The equilibration buffers were entirely phosphate free. Elution buffers were prepared at 100 mM with the respective equilibration buffer and at

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[1] *Abbreviation used:* Mes, 4-morpholinepropanesulfonic acid.

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400 mM with potassium phosphate, followed by adjustment to the desired pH at room temperature. The potassium phosphate was treated as an additive that promotes the elution of protein, rather than as a buffer that maintains the pH. Mes buffers were prepared at pH 5.5 and pH 6.0 with and without phosphate, respectively. The Mes buffers also contained 7.5 μM $CaCl_2$ to counter the loss of calcium from the hydroxyapatite media within the pH range 5.5–6.5, as recommended by manufacturer's instructions. Hepes buffers were prepared at pH 7.0 and pH 8.0, with and without phosphate, respectively. All buffers were chilled, filtered, and degassed before use. The term "mixed-phosphate buffer" is used to describe the addition of phosphate to a second buffer system.

Column chromatography. Chromatographic runs were performed at 4 °C using the respective equilibration and elution buffers at pH 8.0, pH 7.0, pH 6.0 and pH 5.5. Following injection of 4 mg of AhpC* in 25 mM Hepes, pH 7.0, unbound protein was removed by washing with equilibration buffer and loosely bound protein was removed by washing with 25 mM potassium phosphate. Finally, more tightly bound protein was eluted with a linear concentration gradient of potassium phosphate in the presence of the second buffer system.

As a control, protein was eluted with phosphate as the only buffer present. For each control run, 4 mg of AhpC* was injected onto the column with 10 mM potassium phosphate, pH 7.0 (Fig. 1A) or with 25 mM Hepes, pH 7.0 (Fig. 1B). Unbound protein was removed by washing with 10 mM potassium phosphate, pH 7.0 (Fig. 1A) or with 25 mM Hepes, pH 7.0 (Fig. 1B). In each case, loosely bound protein was removed by washing with 25 mM potassium phosphate, pH 7.0, and bound protein was eluted with an increasing concentration of potassium phosphate as before but without the presence of a second buffer system. Refer to the figure legends for more details.

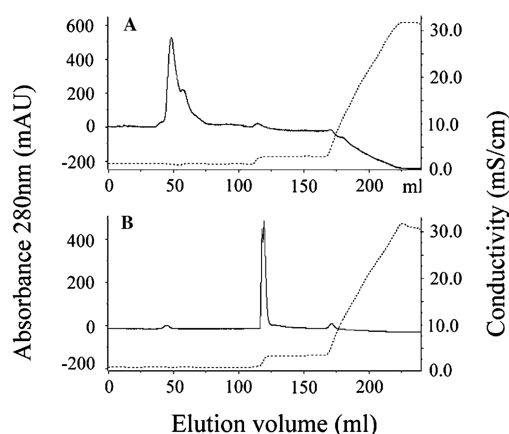

Fig. 1. Elution profile of AhpC* from hydroxyapatite CHT II media using potassium phosphate buffer only. The protein (4 mg of AhpC* in 25 mM Hepes, pH 7.0) was injected onto the column either with 10 mM phosphate, pH 7.0 (A) or with 10 mM Hepes, pH 7.0 (B). The absorbance at 280 nm (solid line) and the conductance of the eluent (mS/cm) (dashed line) were monitored continuously. Unbound protein was removed by washing with 60 ml of 10 mM phosphate, pH 7.0 (A) or with 60 ml of 25 mM Hepes, pH 7.0 (B). Loosely bound protein was removed with a wash of 60 ml of 25 mM phosphate, pH 7.0 (A) or with 60 ml of 25 mM phosphate, pH 7.0 (B). Finally, an increasing phosphate gradient (25–400 mM) at pH 7.0 was applied to the column over 60 ml. The flow rate was maintained at 2 ml/min.

Results and discussion

Effect of pH upon the phosphate-dependent elution of AhpC from hydroxyapatite*. AhpC* is a mildly acidic protein with an isoelectric point of 5.0 [9]. Originally,

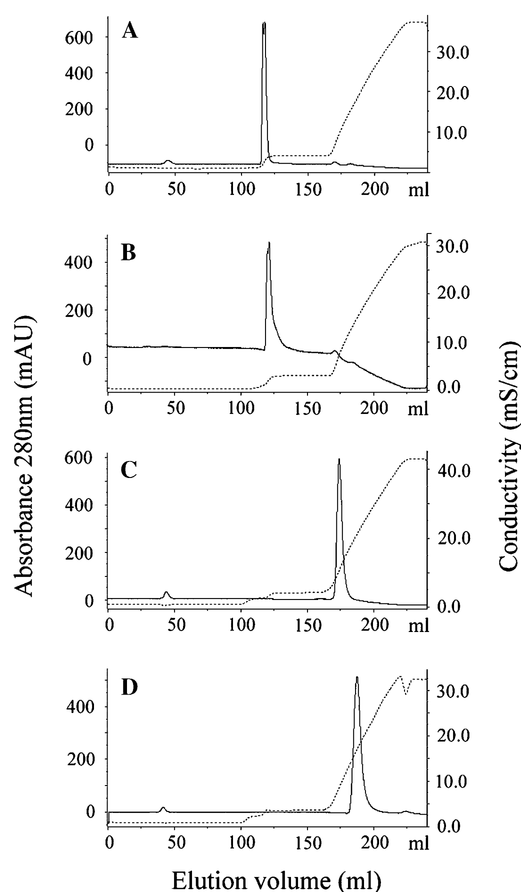

Fig. 2. Elution profiles of AhpC* from hydroxyapatite CHT II media using a potassium phosphate gradient with an additional buffer. The elution buffer had been adjusted to an overall pH of 8.0 with Hepes buffer (A), to pH 7.0 with Hepes buffer (B), to pH 6.0 with Mes buffer (C), and to pH 5.5 with Mes buffer (D). The absorbance at 280 nm (solid line) and the conductance of the eluent (mS/cm) (dashed line) were monitored continuously. After the protein (4 mg in 25 mM Hepes, pH 7.0) was injected with the respective (nonphosphate) equilibration buffer, the column was washed with 60 ml of the equilibration buffer, followed by a wash with 60 ml of 25 mM phosphate in equilibration buffer. Finally, an increasing phosphate gradient (25–400 mM) was applied to the column over 60 ml in the presence of the additional buffer at the respective pH. The flow rate was maintained at 2 ml/min.

AhpC* did not bind the CHT II medium in 10–25 mM phosphate buffer, pH 7.0 (Fig. 1A). Binding to hydroxyapatite could be achieved only by loading the AhpC* in a nonphosphate buffer (Hepes at pH 7.0; Fig. 1B) and this observation prompted a comparison of the phosphate elution profile over the 5.5–8.0 pH range (Fig. 2). Mixed-phosphate buffers were introduced to preserve the buffering capacity outside the effective pH buffering range for phosphate. At pH 8.0 (Fig. 2A) and at pH 7.0 (Fig. 2B), AhpC* was eluted by as little as 25 mM phosphate. At pH 6.0 (Fig. 2C), elution required 130 mM phosphate and at pH 5.5 (Fig. 2D) (the lowest recommended operating pH according to the manufacturer's instructions), the phosphate concentration required for elution increased to approximately 200 mM. The effect of lowering the pH from 7.0 to 5.5 by the incorporation of a nonphosphate buffer was therefore to increase the phosphate concentration required for elution of the AhpC* by up to 10-fold, while maintaining the protein in a properly buffered environment.

Use of a mixed-buffer system with hydroxyapatite medium. Traditionally, protein is bound to hydroxyapaptite with a low concentration of phosphate buffer followed by elution with a phosphate gradient (Fig. 1A). This work clearly shows that once an acidic protein (such as AhpC*) has been induced to bind to hydroxyapatite (Fig. 1B), its phosphate-dependent elution can be delayed by decreasing the pH with the addition of a second buffer system (Fig. 2). The use of a mixed phosphate buffer system allowed optimization of the phosphate-mediated elution of the test protein as a function of pH and under properly buffered conditions. This procedure could therefore be used to improve the phosphate-dependent elution from hydroxyapatite media of a wide range of proteins.

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