

## APPLICATION NOTE

# Separation of Serine Proteases by para-Aminobenzamidine Affinity Chromatography

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### Introduction

A diverse variety of chromatographic media can be employed for commercial manufacture of therapeutic biologicals, all of which interact in one way or another with the unique set of characteristics inherent to a protein of interest.

Serine proteases constitute a diverse range of enzymes, defined by the presence of serine residues in their active site and the ability to hydrolyse peptide bonds. They play an important physiological role in blood clotting, inflammation and digestion. Their activity is prone to inhibition by Aminobenzamidine, and immobilisation of this molecule on a solid matrix provides a potent method by which to purify such proteins.

In the manufacture of proteinaceous bio-pharmaceuticals, capture of proteases can be of benefit because such products are prone to proteolytic degradation and therefore early removal of proteases can improve the overall process yield.

In this application note the use of immobilised para-Aminobenzamidine is demonstrated for capture and partitioning of two proteases, chymotrypsin and trypsin, on the basis of their differing affinity for the Aminobenzamidine ligand.

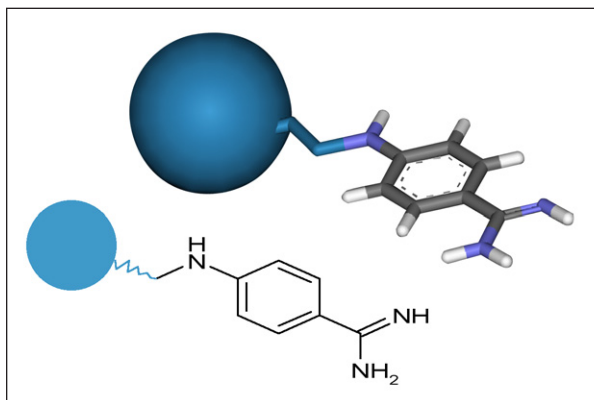


Figure 1: Chemical structure and schematic of para-Aminobenzamidine ligand attached to an agarose bead

**Table 1: Examples of Affinity Chromatography Using Aminobenzamidine**

Enzyme	Source	Reference
Acetyl cholinesterase	Electric eel electrical organ	Taylor, R.F. & Marenchic, I.G. (1984) J. Chromatogr. 317, 193-200
Aldehyde oxidase*	Rabbit liver	Stell, J.G.P., Warne, A.J. & Lee-Woolley, C. (1989) J.Chromatogr. 475, 363-372
Alkaline phosphatase	Bovine intestinal mucosa	Taylor, R.F. & Marenchic, I.G. (1984), J.Chromatogr. 317. 193-200
Collagenase/ Clostripain	Clostridium histolyticum	Emod, I. & Kell, B. (1977) FEBS Lett. 77, 51-56
Enterokinase	Human duodenal fluid	Grant, D.A.W., Magee, A.I., & Herman-Taylor, J. (1978) Eur. J. Biochem. 88, 183-189
Kallikrein	Human plasma	Sampiao, C., Wong, S.-C. & Shaw, W. (1974) Arch Biochem. Biophys. 165, 133-139
Kallikrein	Human urine	Kanamori, A., Seno, N. & Matsumoto, I. (1986) J. Chromatogr. 363, 231-242
Papain**	Papaya latex	Taylor, R.F. & Marenchic, I.G. (1984), J.Chromatogr. 317, 193-200
Plasminogen (subspecies)	Human plasma	Ito, N., Noguchi, K., Kazama, M., Shimura, K. & Kasai, K.-I. (1987). J.Chromatogr. 386, 51-56
Prekallikrein	Bovine plasma	Heimark, R.L., & Davie, E.W. (1979) Biochemistry, 18, 97-108
Thrombin	Bovine crude thrombin preparation	Khamlichi, S., Muller, D., Fuks, R. & Jozefonvicz, J. (1990), J. Chromatogr. 510, 123-132
Trypsin	Bovine pancreas	Hixson, H.F. & Nishikawa, A. (1973). Arch. Biochem. Biophys. 154, 501-509
Trypsin	Streptomyces griseus	Kanamori, A., Seno, N. & Matsumoto, I. (1986), J.Chromatogr. 363, 231-242
Urokinase	Human urine	Male, K.B., Nguyen, A.L. & Luong, J.H.T., (1989) Biotechnol. & Bioeng. 35, 87-93

\* Aminobenzamidine is an unusual competitive inhibitor of aldehyde oxidase.

\*\* Papain is a sulfhydryl endopeptidase

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### Method

Chromatography Conditions for Serine Protease Capture	
Column Height	2.8 cm
Column Volume	5.0 mL
Linear Flow Rate	up to 300 cm hr <sup>-1</sup>
Equilibration Buffer	100 mM Na Acetate, pH 5.5, 10 mM CaCl <sub>2</sub>
Wash Buffer	100 mM Na Acetate, pH 5.5, 10 mM CaCl <sub>2</sub> + 0.9M KCl
Elution	50 mM glycine-HCl, pH 2.1
Sanitisation	See note below

### Notes on Sanitization of para-Aminobenzamide

Sodium hydroxide is the bio-pharmaceutical industry's sanitant of choice for chromatographic applications, since it offers relatively inexpensive, robust clearance of microbial contamination from substrates, particularly those that are impenetrable to steam.

Para-Aminobenzamide is however degraded by prolonged exposure to extremes of pH, and this includes the application of sodium hydroxide.

To ensure the maintenance of an aseptic media without the use of NaOH, ProMetic BioSciences is able to recommend a demonstrably effective clean-in-place regime using 20% ethanol and 1M acetic acid which has a pH of 2.6 – sufficiently high to ensure the integrity of the ligand. Application of this mixture for 3 hours has been shown to neutralize a range of bacterial contaminants, thereby ensuring the sterilization of the media. Further details are available in the regulatory support package provided with this media. Additionally, the media can be autoclaved.

### Results

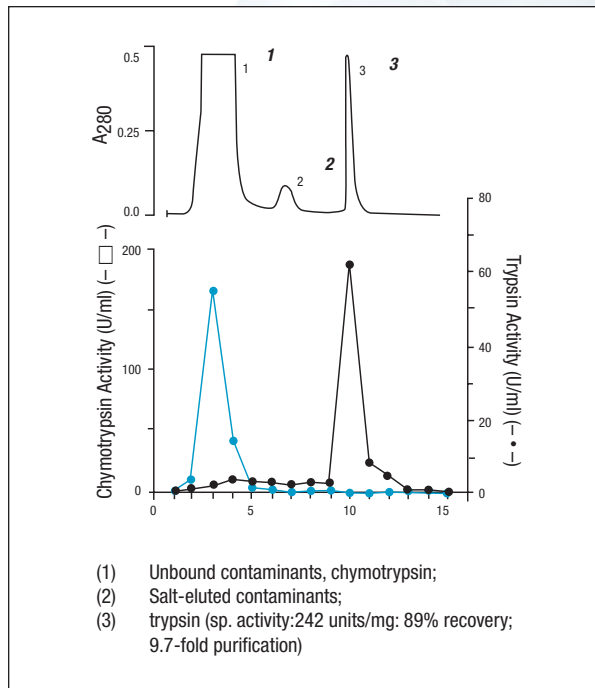


Figure 2: Chromatographic trace and protease activities illustrating separation of chymotrypsin from the serine protease, trypsin by para-aminobenzamide

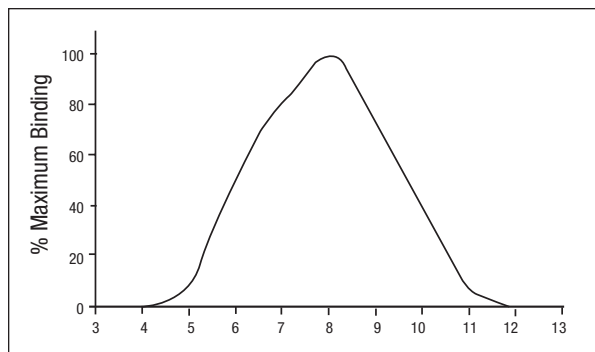


Figure 3: pH Dependence of Trypsin adsorption by p-Aminobenzamide Agarose

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### Summary

The use of immobilised para-Aminobenzamidine can be applied for the capture of a diverse range of serine proteases irrespective of source (native microbial, mammalian or recombinant). Although serine proteases dominate the type of enzymes that can be captured by para-Aminobenzamidine, other endopeptidases, such as papain, can also be captured by this ligand.

This type of purification can be applied to avoid proteolysis of proteins of interest (thereby enabling higher yields in a manufacturing process) or to remove proteases that have been used in the manufacturing process itself (e.g. for cleavage of antibodies, or release of confluent adherent cells which have been grown in roller bottles or on microcarriers).

The experimental data shown here illustrate the application of para-Aminobenzamidine:

- for capture of trypsin and its separation from contaminating proteins
- the use of salt and pH change to promote removal of contaminants and de-sorption of trypsin from the matrix

Attention is also drawn to the importance of using a well considered cleaning regime, given the sensitivity of para-Aminobenzamidine to extremes of pH.

In terms of commercial-scale manufacturing, para-Aminobenzamidine agarose can be applied to enable purification of endopeptidases or in the removal of proteolytic activity which might deleteriously impact on the overall yield and economy of a downstream process.

This chromatographic adsorbent is well suited to large-scale application and is supported by the necessary regulatory documentation for cGMP of bio-therapeutics.

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