Development of a Robust Affinity Adsorbent for Primary Capture and Purification of a tPA-Urokinase Fusion Protein

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ABSTRACT

Amediplase™ is a tissue plasminogen activator-urokinase fusion protein containing the catalytic domain of urokinase linked to the kringle 2 fibrin binding motif of tPA and produced by mammalian cell culture. Traditional methods of purifying such proteins (e.g. lysine affinity chromatography) fail to provide the desired capacity, recovery and purity in a primary capture mode. Whilst affinity chromatography is desirable as a capture step, binding must be unaffected by the presence of culture media components and the adsorbent must be sufficiently robust to withstand the increased level of column cleaning and sanitisation required. Consequently, the development of a synthetic triazine-based affinity ligand was investigated.

Triazine ligand libraries were prepared which incorporated a wide variety of substituted alkyl or phenyl groups attached to a central triazine core. These libraries were screened in 250 µl micro-columns against clarified Amediplase containing cell culture media and binding and recovery assessed by performance of a specific enzyme activity assay. Rather unexpectedly, ligand structures which contained positively charged groups (e.g. lysine or benzamidine analogues) did not provide particularly good binders. Instead, very specific ligands were identified which contained strongly negatively charged groups.

On scale-up, the selected ligand provided binding capacities in excess of 10 mg/ml adsorbent with a recovery of 90% and a purity in excess of 99%. This adsorbent also withstood continuous incubation in 0.5 M NaOH for 120 hours at 40°C.

OBJECTIVES

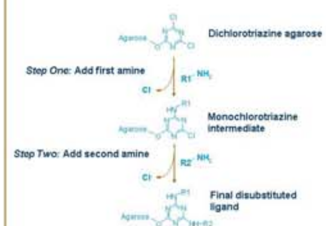
Proteases such as urokinase and tissue plasminogen activator (tPA) are commonly isolated using immobilised cationic affinity ligands such as lysine and aminobenzamidine [1,2]. Such ligands can also be used to capture Amediplase from cell culture media, though in the case of immobilised lysine, the low capacity (approx. 1 mg/ml), purity (< 80%) and number of cycles achievable before media replacement (< 50) mean these adsorbents are not viable for use in commercial manufacture of Amediplase. Instead, ligands which are more robust and have a higher selectivity are required.

DESIGN SPECIFICATIONS

- Direct capture from clarified culture media
- Column Capacity ≥ 10 mg/mL adsorbent
- Flow rate (loading) ≥ 300cm/hr (15cm bed length)
- Recovery ≥ 80%
- Purity ≥ 80%
- Alkali stable (0.5M NaOH sanitization)

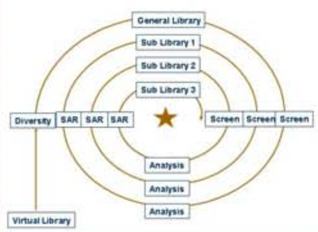
TRIAZINE LIGAND LIBRARIES

Diverse libraries of affinity ligands can be obtained by stepwise reaction of primary amine-containing compounds with immobilised dichlorotriazine groups [3]. Such Mimetic™ ligands have proven utility for downstream process applications and are inherently inert due to the lack of fissile bonds.



SCREENING STRATEGY

Combinatorial libraries were constructed which contained 2-dimensional arrays of triazine ligands derived from primary amine compounds with chemically diverse "R" groups. Two libraries (#23 & #85) were prepared each containing 64 ligands/library using combinations of 24 different primary amines. These "General Libraries" contained a wide spectrum of ligand functionalities including combinations of anionic, neutral, cationic, hydrophilic and hydrophobic groups. Included within these libraries were lysine and aminobenzamidine groups and analogues thereof. The libraries were screened in 96 column blocks (each column containing 250 µl of adsorbent) using a Tecan Genesis robotic liquid handling system. Since Amediplase is unstable at alkaline pH, the preferred range for process buffers is pH 8.5 – 3.0. Consequently buffers in this pH range were selected for ligand screening.



SCREENING CONDITIONS

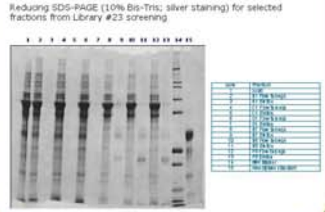
- Equilibration: 3mL 25mM sodium phosphate pH 7.5, 100 mM NaCl
- Load: 1mL cell culture supernatant containing Amediplase, followed by 0.25mL equilibration buffer
- Wash: 1.5mL of equilibration buffer
- Elution: 1mL 10mM sodium citrate, 20% ethylene glycol, 250mM NaCl, pH 3.5
- Sanitisation: 1mL 30% isopropanol / 0.2M NaOH
- Amediplase micro-well activity assays were performed using S-2444 substrate (Chromogenix). Protein concentration was determined by Bradford assay.

PRIMARY SCREENING

General libraries (#23 & #85) were screened for Amediplase binding and elution. A number of ligands could be identified which captured Amediplase from the applied feedstock. A proportion of these ligands also allowed the release of bound enzyme under the elution buffer conditions employed. On the basis of these findings, a 64 ligand sub-library (library #86) was constructed using analogues of the primary amine compounds from libraries #23 and #85 which demonstrated the best Amediplase binding properties. The most promising ligands from libraries #23, #85 and #86 were selected for secondary screening at the 1 ml column scale.

Amediplase recovery (µg/fraction)

Reducing SDS-PAGE (10% Bis-Tris; silver staining) for selected fractions from Library #23 screening



SECONDARY SCREENING

- Scale: 1mL of adsorbent (1 cm bed height)
- Flow Rate: 1.2 mL/min
- Equilibration: 5 column volumes of 25mM sodium phosphate, pH 7.5, 100 mM NaCl
- Load: Cell culture containing approximately 5mg Amediplase
- Wash: To baseline (5-10 column volumes of equilibration buffer)
- Elution 1 (E1): 10mM sodium citrate, 20% ethylene glycol, 250mM NaCl, pH 3.5
- Elution 2 (E2): 25mM sodium phosphate, 40% ethylene glycol, 500mM NaCl, pH 7.5
- Sanitisation: 30% isopropanol / 0.2M NaOH

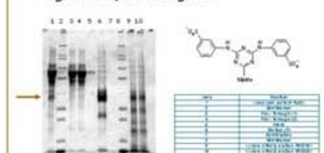
Ligand	E1 Purity (%)	E2 Purity (%)	µg Rec E1	µg Rec E2	Total Eluted	Bind Cap	% Recovery
#23/B2	n/a	72	0	4247	4247	>4247	>90
#23/B8	n/a	n/a	0	0	0	1833	0
#85/H8	77	52	64	193	257	421	61
#86/F1	100	n/a	261	n/a	261	609	43
#85/H7	88	n/a	274	n/a	274	336	82
#86/F2	100	27	284	29	313	885	35
#85/G4	100	85	255	208	463	969	48
#85/H5	91	13	286	18	304	501	63
#85/A3	100	2	217	17	234	364	64
#23/H8	25	1	313	22	335	335	>90
#85/B2	52	5	248	27	275	275	>90
#85/A7	45	1	167	16	183	413	44
#86/C1	100	9	1281	62	1343	1961	68
#86/H1	100	11	474	22	496	808	61

Ligand B2 from library #23 exhibited the highest capacity with reasonable purity. A further 2 additional ligands (near-neighbour compounds of ligand #23/B2) were made and tested:

Ligand	E1 Purity (%)	E2 Purity (%)	E1 Recovery (µg)	E2 Recovery (µg)	Total eluted (µg)	Recovery (%)
Analogue 1	n/a	91	0	4370	4370	92
Analogue 2	n/a	91	0	3720	3720	78

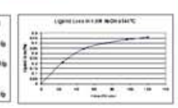
LEAD LIGAND SELECTION

Ligand #23/B2 Analogue 1



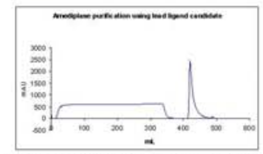
STABILITY

Ligand loss (24h; 20 – 25°C)	
1M NaOH	<0.002%
30% propan-2-ol/ 0.2M NaOH	<0.002%
20% Ethanol	<0.002%

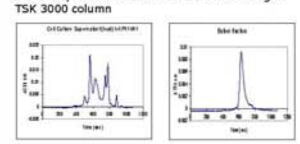


PERFORMANCE VERIFICATION

- Column dimensions: 10mL column - 4.5cm bed height, 1.6cm diameter
- Adsorbent: Ligand #23/B2 Analogue 1 attached to Purabead™ 6HF; ligand density 17 µmol/g.
- Flow rate: 300cm/hr
- Equilibration / wash buffer: 25mM sodium phosphate, pH 6.5, 100mM NaCl
- Loading with concentrated cell culture supernatant, pH 6.5
- Elution buffer: 25mM sodium phosphate, pH 6.5, 0.5M NaCl, 40% v/v ethylene glycol
- Sanitization: 0.5M NaOH



GPC analysis of load and elution fraction using a TSK 3000 column



- Capacity: 11.4mg/mL
- Purity: ≥ 99%
- Recovery: 92%
- Loading flow rate: 300cm/hr
- Alkali Stable (0.5M NaOH)

SUMMARY

By screening combinatorial libraries of triazine based affinity ligands, compounds were identified which provide improved purification performance compared to established chromatography methods. The identification of ligands with a strongly anionic character was unexpected given the cationic nature of ligands normally associated with urokinase and tPA binding. This work demonstrates the benefits of a combinatorial approach to ligand discovery and the utility of triazine ligands in downstream process applications.

REFERENCES

1) Male, K.B., Nguyen, A.L. & Luong, J.H.T. Biotechnology & Bioengineering, 35, 87-93 (1990).

2) Mori, K., Dwek, R.A., Downing, A.K., Opdenakker, G. & Rudd PM. J. Biol. Chem., 270, 3261-3267 (1995).

3) International patent application PCT/DK96/00399

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