Removal and purification of trypsin-like serine proteases

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Removal and purification of trypsin-like serine proteases

Johan Färenmark, Thomas Pless, Jill Sigrell, Anna Heijbel, Jan Gustavsson, Inger Lagerlund, Helena Westergren, and Fredrik Calais
GE Healthcare Bio-Sciences AB, Björkgatan 30, SE-751 84 Uppsala, Sweden

Introduction

In the living organism, proteolytic enzymes (proteases) are produced to degrade and modify proteins. Serine proteases form the most common group of the proteolytic enzymes, and they are extremely widespread and diverse. The trypsin family is the largest and most extensively studied family in the group and includes, among others, trypsin, chymotrypsin, and thrombin.

The affinity medium, Benzamidine Sepharose™ 4 Fast Flow (high sub), has high selectivity for trypsin and trypsin-like serine proteases. It can be used, as shown here, to remove proteolytic activity from a protein preparation or for purification of specific proteases.

Description of the medium

The ligand, p-aminobenzamidine, is firmly attached to the highly cross-linked agarose matrix, Sepharose 4 Fast Flow, via a long spacer arm (Fig 1).

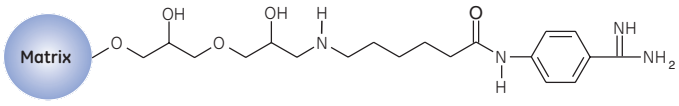

Fig 1. Partial structure of Benzamidine Sepharose 4 Fast Flow (high sub)

Average particle size:	90 µm
Bead structure:	Macroporous cross-linked 4% agarose
Ligand density:	> 12 µmol/ml medium
Binding capacity:	> 35 mg trypsin/ml medium
Flow:	150–250 cm/h, 25 cm bed height, 0.1 MPa, 25°C, distilled water in an XK 50 column

Conclusions

- Benzamidine Sepharose 4 Fast Flow (high sub) can be used for either rapid removal of proteolytic activity or for purifying trypsin-like serine proteases.
- Trypsin-like serine proteases have a high and specific binding to Benzamidine Sepharose 4 Fast Flow (high sub), as shown by the activity measurements.
- Benzamidine Sepharose 4 Fast Flow (high sub) is a medium with high capacity and good stability at pH 2–8 as well as in 8 M urea or 8 M guanidine hydrochloride.

HiTrap

Stability studies

The most sensitive part of Benzamidine Sepharose 4 Fast Flow (high sub) is the amidine group of the ligand. p-Aminobenzamidine is hydrolyzed to p-aminobenzamide and then to p-aminobenzoic acid, the two latter forms having no affinity for trypsin.

As can be seen in Figure 2, the trypsin capacity is largely unaffected by storage at pH 2–8 for 28 days at ambient conditions. At higher or lower pH the hydrolysis reaction of p-benzamidine is faster. This causes a decrease in trypsin capacity but there is still more than 50% of the original trypsin capacity left on the adsorbent after 1 week (168 h) contact time at pH 9.

Because of the limited stability at high pH, alternative cleaning methods are necessary. We have tested the stability of the medium by exposing it to both 8 M urea and 8 M guanidine hydrochloride for one week without loss of trypsin capacity (Fig 3).

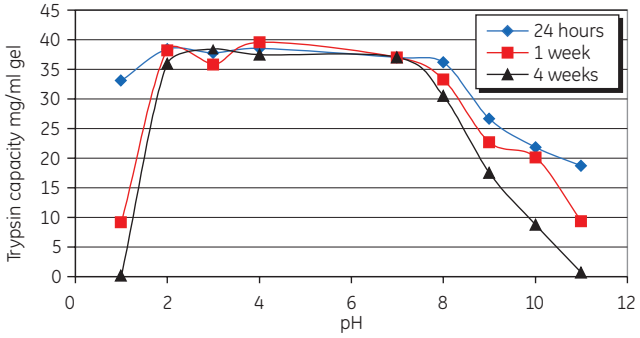

Fig 2. Trypsin capacity test results for Benzamidine Sepharose 4 Fast Flow (high sub) after storage at ambient conditions at pH 1–11 for 24 h–4 weeks.

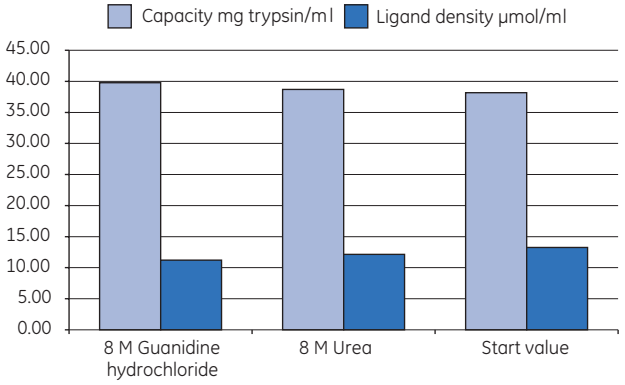

Fig 3. Trypsin capacity and ligand density test results for Benzamidine Sepharose 4 Fast Flow (high sub) after storage in 8 M guanidine hydrochloride and 8 M urea for 1 week at ambient conditions.

Purification of trypsin-like serine proteases from human plasma

A HiTrap™ Benzamidine FF (high sub) column was used to purify trypsin-like serine proteases from human plasma (Fig 4). Non-binding components were washed out using binding buffer and bound proteins were eluted by decreasing the pH. An arginine-specific assay was used to determine trypsin-like serine protease activity in the eluted fractions. Specific protease activity was much higher in the eluted fractions than in the flow through. The whole procedure was completed in less than half an hour.

Column:	HiTrap Benzamidine FF (high sub), 1 ml
Sample:	1 ml human plasma filtered through a 0.45 µm filter
Binding buffer:	20 mM Tris-HCl, 0.5 M NaCl, pH 7.4
Elution buffer:	50 mM glycine, pH 3.0 0–100% elution buffer in one step
Flow rate:	1.0 ml/min
System:	ÄKTAexplorer™ 10
Protease activity:	A_{405} measurement, S-2288 from Chromogenix, Heamochrom Diagnostica AB.
	The activity is presented as the proteolytic activity/mg protein

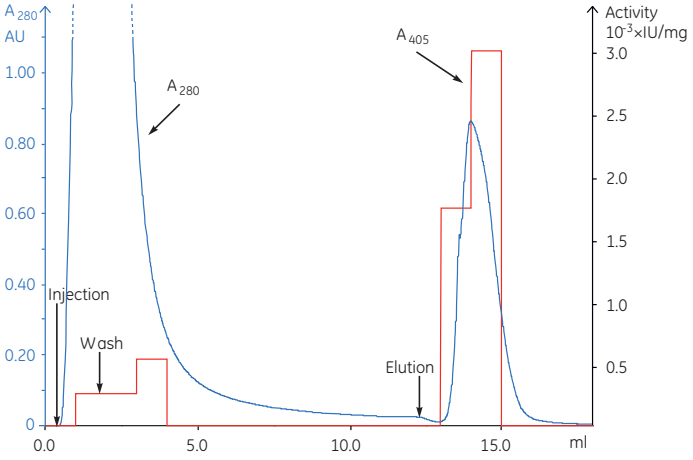

Fig 4. Purification of trypin-like serine proteases from human plasma using HiTrap Benzamidine FF (high sub), 1 ml.

Removal of thrombin after on-column cleavage of a GST-tagged protein

Material and methods

Columns:	GSTrap™ FF, 1 ml HiTrap Benzamidine FF (high sub), 1 ml
Sample:	2 ml clarified *E. coli* homogenate expressing a M_r 37 000 SH2-GST-tagged protein with a thrombin protease site
Binding buffer:	20 mM Na phosphate, 0.15 M NaCl, pH 7.5
High salt wash buffer:	20 mM Na phosphate, 1.0 M NaCl, pH 7.5
Benzamidine elution buffer:	20 mM p-aminobenzamidine in binding buffer
GST elution buffer:	20 mM reduced glutathione, 50 mM Tris, pH 8.0
Flow rate:	0.5 ml/min
Instrument:	ÄKTAprime
Protease treatment:	20 U/ml thrombin protease (GE Healthcare) for 2 h at room temperature
Thrombin activity:	S-2238 was used as a substrate and its absorbance at 405 nm was measured

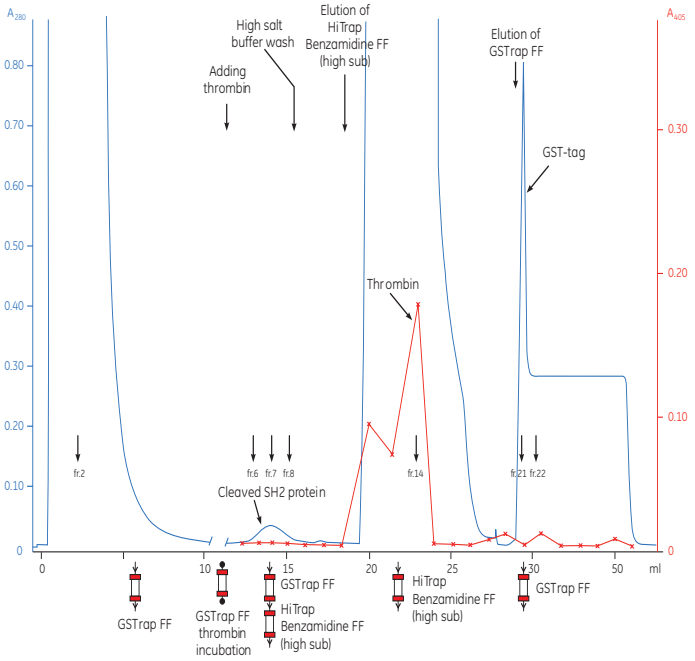

Fig 5. Chromatogram and activity curve demonstrating all steps in the cleavage and purification of the SH2-GST protein.

Gel: ExcelGel™ SDS Gradient 8–18, Coomassie™ staining

Lane 1: Low Molecular Markers

Lane 2: Sample

Lane 3: Flow-through fraction (Fraction 2)

Lane 4: SH2 domain, washed off with binding buffer through both columns (Fraction 6)

Lane 5: Same as lane 4, but Fraction 7

Lane 6: Same as lane 4, but Fraction 8

Lane 7: Eluted fraction from HiTrap Benzamidine FF (high sub) with the highest thrombin activity (Fraction 14)

Lane 8: Eluted fraction from GSTrap FF containing cleaved off GST-tag and uncleaved fusion protein, SH2-GST (Fraction 21)

Lane 9: Same as lane 8, but Fraction 22

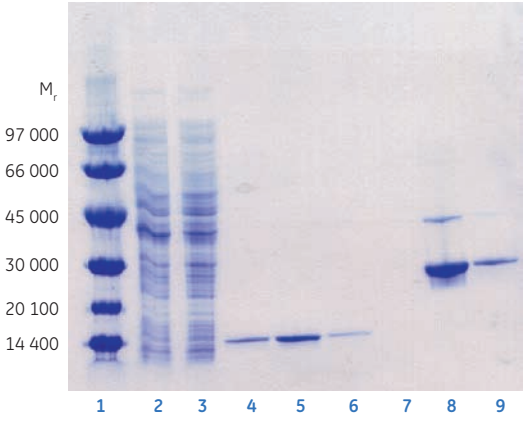

Fig 6. SDS-PAGE analysis of thrombin activity.

www.gelifesciences.com/protein-purification
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GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden

GE Healthcare Europe GmbH
Munzinger Strasse 5, D-79111 Freiburg, Germany

GE Healthcare UK Ltd
Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK

GE Healthcare Bio-Sciences Corp
800 Centennial Avenue, P.O. Box 1327, Piscataway,
NJ 08855-1327, USA

GE Healthcare Bio-Sciences KK
Sanken Bldg. 3-25-1, Hyakunincho, Shinjuku-ku,
Tokyo 169-0073, Japan

Asia Pacific T +85 65 62751830 F +85 65 62751829 • Australasia T +61 2 8820 8299 F +61 2 8820 8200 • Austria T 01 /57606 1613 F 01 /57606 1614 • Belgium T 0800 73 890 F 02 416 8206 • Canada T 1 800 463 5800 F 1 800 567 1008 • Central & East Europe T +43 1 972 720 F +43 1 972 722 750 • Denmark T +45 70 25 24 50 F +45 45 16 2424 • Eire T 1 800 709992 F +44 1494 542010 • Finland & Baltics T +358 9 512 3940 F +358 9 512 39439 • France T 01 69 35 67 00 F 01 69 41 98 77 Germany T 0800 9080 711 F 0800 9080 712 • Greater China T +852 2100 6300 F +852 2100 6338 • Italy T 02 26001 320 F 02 26001 399 • Japan T 81 3 5331 9336 F 81 3 5331 9370 • Korea T 82 2 6201 3700 F 82 2 6201 3803 • Latin America T +55 11 3933 7300 F +55 11 3933 7304 • Middle East & Africa T +30 210 96 00 687 F +30 210 96 00 693 • Netherlands T 0800-82 82 82 1 F 0800-82 82 82 4 • Norway T +47 815 65 777 F +47 815 65 666 • Portugal T 21 417 7035 F 21 417 3184 • Russia, CIS & NIS T +7 495 956 5177 F +7 495 956 5176 • Spain T 902 11 72 65 F 935 94 49 65 • Sweden T 018 612 1900 F 018 612 1910 • Switzerland T 0848 8028 10 F 0848 8028 11 • UK T 0800 515 313 F 0800 616 927 • USA T +1 800 526 3593 F +1 877 295 8102

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