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Production, purification and characterisation of recombinant Fahsin, a novel antistasin-type proteinase inhibitor

Eric C. de Bruin [a,b,*], Dorina Roem [a,b], Ingrid Bulder [a,b], Miranda Dieker [a,b], Gerard Voerman [c], C. Erik Hack [a,b,d]

[a] *Department of Immunopathology, Sanquin Research at CLB, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands*
[b] *Landsteiner Laboratory at AMC, University of Amsterdam, 1066 CX, Amsterdam, The Netherlands*
[c] *VitaLeech Bioscience N.V., 3032 AC, Rotterdam, The Netherlands*
[d] *Department of Clinical Chemistry, VU University Medical Center, 1081 HV, Amsterdam, The Netherlands*

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Abstract

Serine proteinases from inflammatory cells, including polymorphonuclear neutrophils, are involved in various inflammatory disorders, like pulmonary emphysema and rheumatoid arthritis. Inhibitors of these serine proteinases are potential drug candidates for the treatment of these disorders, since they prevent the unrestricted proteolysis. This study describes a novel specific antistasin-type inhibitor of neutrophil serine proteinases, we called Fahsin. This inhibitor was purified from the Nile leech *Limnatis nilotica*, sequenced and heterologously expressed using a synthetic gene in the methylotrophic yeast *Pichia pastoris*, yielding 0.5 g^{-1} of the protein in the culture medium. Recombinant Fahsin was purified to homogeneity and characterised by N-terminal amino acid sequencing and mass spectrometry. Inhibition-kinetic analysis showed that recombinant Fahsin is a fast, tight-binding inhibitor of human neutrophil elastase with inhibition constant in the nanomolar range. Furthermore, recombinant Fahsin was, in contrast to various other neutrophil elastase inhibitors, insensitive to chemical oxidation and biological oxidation via myeloperoxidase-generated free oxygen radicals.

Thus, Fahsin constitutes a novel member of a still expanding family of naturally occurring inhibitors of serine proteinases with potential therapeutic use for treatment of human diseases.

Keywords: Fahsin; Human neutrophil elastase; Inflammation; Leech; *Pichia pastoris*

1. Introduction

Leeches, as parasites living from blood, have evolved potent inhibitors to interfere with proteinases present in the blood of their hosts. These inhibitors constitute attractive leads for drugs to treat human diseases in which proteinase–proteinase inhibitor balances are impaired. Among these leech-derived inhibitors is the family of antistasin-type serine proteinases inhibitors, which includes the neutrophil elastase inhibitor guamerin [1], the trypsin inhibitor piguamerin [2], both derived from *Hirudo nipponia*, and the tissue kallekrein inhibitor hirustasin [3] and the plasmin inhibitor bdellastasin [4], which are both derived from *Hirudo medicinalis*. Antistasin-type serine proteinases inhibitors are characterised by 10 similarly spaced cysteine residues forming five disulfide bonds, which are important for the compact structure of this inhibitor family [5]. In this study, we describe the novel antistasin-type serine proteinase inhibitor from the leech *Limnatis nilotica*, called

* Corresponding author. Tel.: +31 20 5123223; fax: +31 20 5123170.
E-mail address: e.debruin@sanquin.nl (E.C. de Bruin).

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Fahsin, which specifically inhibits the neutrophil-derived serine proteinases, elastase, cathepsin G and proteinase 3. The uncontrolled activity of the latter proteinases may cause significant tissue destruction at sites of inflammation, which plays a role in diseases such as emphysema, cystic fibrosis, arthritis, psoriasis and periodontitis [6–10].

The reactive site amino acid residue at the P1-position of a serine proteinase inhibitor not only has a major effect on the specificity of the inhibitor for the target proteinase [11], but in some instances may also regulate the activity of the inhibitor. For example, α_1-antitrypsin (AAT), the archetype of the serpin-family of proteinase inhibitors, has a methionine residue at its P1-position [12] and is responsible for the inhibition of serine proteinases released from stimulated polymorphonuclear neutrophils (PMN), thereby protecting the invaded host tissue from proteolytic damage. However, the antiproteinase activity of AAT is inhibited in the direct environment of activated neutrophils due to oxidation of the reactive-site methionine by the neutrophil-derived reactive oxygen species (ROS), O_2^-, H_2O_2 and HOCl [13,14]. As a consequence AAT will only inhibit elastase at some distance from activated neutrophils. Also guamerin contains a methionine residue at the P1-position [1], making it also susceptible to oxidation and to loss of function at sites of chronic inflammation. Remarkably, Fahsin is the first antistasin-type inhibitor which possesses a leucine as its P1-residue and is free of any methionine residue.

In this paper, we describe the production, purification and characterisation of a novel antistatin-type proteinase inhibitor, which specifically inhibits serine proteinases released from PMNs. Furthermore, we show that the recombinant product is insensitive to both chemical and biological oxidation. These features render rFahsin an attractive candidate for treatment of chronic inflammatory diseases.

2. Materials and methods

2.1. Materials

Chromatographically purified human α1-antitrypsin (AAT) and cathepsin G were obtained from Sigma (St. Louis, MO, USA). Human neutrophil elastase (NE) and proteinase 3 were obtained from the Elastin Products Company (Owensville, MI, USA). Stock solutions of cytochalasin B and *N*-formyl-methionyl-leucyl-phenylalanine (f-MLP) and of the chromogenic substrates *N*-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (MeO-SAAPVpNA) and *N*-succinyl-Ala-Ala-Ala-p-nitroanilide (SAAApNA) (all purchased from Sigma) were prepared in dimethylsulfoxide (DMSO) (J.T. Baker, Deventer, The Netherlands). Dithiothreitol (DTT), iodoacetamide (IAA), 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), *N*-chlorosuccinimide (NCS), dimedone (all from Sigma) were used for chemical modification of proteins. Superoxide dismutase (SOD) and catalase (both derived from bovine erythrocytes) were obtained from Sigma.

2.2. Synthetic gene design and vector construction

From lysates of the leech *Limnatis nilotica* a novel antistasin-type proteinase inhibitor, called Fahsin, was isolated using regular chromatographic procedures and chromogenic assays [15]. Based on the amino acid sequence, a synthetic gene for Fahsin was constructed by overlap extension PCR [16] of four long oligonucleotides, codon usage optimised for the host *Pichia pastoris*;

FA-1 5′-GGGGTATCTCTCGAGAAAAGAGACGACAACTGTGGTGGTAAGGTTTGTTCTAAGGGTCAA-3′,
FA-2 5′-AATCAAACATCTAATTGAGTACACTCACAGTGACCGGTCGTGACACAATTGACCCTTAGAACAAAC-3′,
FA-3 5′-CCAATTAGATGTTTGATTTTCTGTCCAAACGGTTTCGCTGTTGACGAGAACGGTTGTGAG-3′, and
FA-4 5′-GCTGGCGGCCGCTCATTGGTGCTTACAAGAACATGGCAACTCACAACCGTTCTCGTC-3′.

After cloning of the PCR-product using the pGEMT-easy cloning kit (Promega, Madison, WI, USA) and subsequent DNA-sequencing, the proper gene was cloned into the *Pichia* vector pPIC9, using the *Xho*I and *Not*I restriction endonucleases (Invitrogen, Carlsbad, CA, USA).

2.3. Transformation of P. pastoris strain GS115

P. pastoris GS115 (*his4*, see [17]) was transformed by electroporation according to Becker and Guarente [18], using a GenePulser (Bio-Rad, Richmond, CA, USA). Prior to transformation, plasmid pPIC9Fahsin was linearised with *Sal*I (Invitrogen). After growth for 3 days on selective plates at 30 °C, several colonies were selected for PCR-conformation using the vector primers 5′AOX1 and 3′AOX1 (Invitrogen).

2.4. Fermentative production and purification of rFahsin

After selection of rFahsin-producing *P. pastoris* transformants in shake flasks, fermentations were conducted in a 5-l BioFlo 3000 fermentor (New Brunswick Scientific, Edison, NJ, USA) in minimal basal-salt medium supplemented with 0.2% (v/v) PTM_1-trace salts (Invitrogen). Methanol fed-batch fermentations were performed