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

ARTICLE *in* FEMS YEAST RESEARCH · DECEMBER 2005

Impact Factor: 2.82 · DOI: 10.1016/j.femsyr.2005.03.005 · Source: PubMed

6 AUTHORS, INCLUDING:

Eric C de Bruin
Sanquin Blood Supply Foundation
70 PUBLICATIONS **1,070** CITATIONS
SEE PROFILE

Dorina Roem
Sanquin Blood Supply Foundation
21 PUBLICATIONS **656** CITATIONS
SEE PROFILE

Miranda C Dieker
Sanquin Blood Supply Foundation
7 PUBLICATIONS **98** CITATIONS
SEE PROFILE

ELSEVIER

FEMS Yeast Research 5 (2005) 1069–1077

FEMS Yeast Research

www.fems-microbiology.org

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*

Received 9 February 2005; received in revised form 22 March 2005; accepted 29 March 2005

First published online 27 April 2005

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).

1567-1356/$22.00
doi:10.1016/j.femsyr.2005.03.005

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

using the "soft spike" method as described by Potter et al. [19]. During the fermentation, dissolved oxygen (DO) was maintained at 20% and pH was controlled at 5.0 using pure oxygen and 30% (w/v) of ammonium hydroxide, respectively. After the fermentation, cells were separated from the culture medium by centrifugation and rFahsin was purified from the fermentation broth by using differential acetone precipitation [20]. After dialysis overnight against 20 mM Tris-buffer, pH 8.0, rFahsin was separated by anion-exchange chromatography on a HiLoadQ-column using an Äkta explorer (Amersham Pharmacia Biotech, Little Chalfont, UK). Elution was started with a linear salt gradient up to 1 M NaCl in 20 mM Tris-buffer, pH 8.0. Purified fractions of rFahsin were pooled, dialysed against distilled water and subsequently freeze–dried. Lyophilised rFahsin was reconstituted in phosphate-buffered saline, pH 7.4, and analysed by SDS–PAGE for integrity.

2.5. Protein determination

Protein concentrations were determined with the bicinchoninic acid (BCA) protein assay according to the manufacturer's instructions (Pierce, Rockford, IL, USA), with bovine serum albumin as a standard.

2.6. SDS–PAGE and N-terminal amino acid sequencing

SDS–PAGE was performed according to manufacturer's instructions in MES buffer (50 mM (*N*-morpholino)ethanesulfonic acid (MES), 50 mM Tris, 1 mM EDTA, 0.1% (w/v) SDS) using a Novex-system with 12% pre-cast gels (Invitrogen). Samples were diluted in NuPage sample buffer (Invitrogen) with or without the addition of DTT as a reducing agent. Gels were stained using a silver staining kit (Bexel, Union City, CA, USA) or according to standard Coomassie brilliant blue staining protocols.

For N-terminal protein sequencing, protein was blotted onto Immobilon P^{SQ} (Millipore, Bedford, MA, USA) by applying 100 V for 1 h in a Novex Blot Module using NuPage transfer buffer (25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, 1 mM chlorobutanol). Blots were stained with Coomassie brilliant blue and selected bands were cut out. N-terminal sequencing using Edman-degradation was performed by Sequencing Centre, University of Utrecht (CBLE), Utrecht, The Netherlands.

2.7. Mass spectrometry

Electrospray mass spectrometry (ESMS) was performed on a Micromass Q-TOF mass spectrometer at the Department of Molecular and Cellular Neurobiology, Faculty of Earth and Life Sciences, Vrije Universiteit, Amsterdam, The Netherlands.

2.8. Chemical modification of rFahsin

For the identification of cysteine sulfenic acid in rFahsin a chemical modification with dimedone (5,5-dimethyl-1,3-cyclohexanedione) was used [21]. This nucleophilic reagent attacks the sulfenate sulfur, displacing hydroxide and forming a thiol adduct, which can be monitored by increasing mass in ESMS. Also a modification with electrophilic reagent NBD-Cl was performed, which reacts with both the cysteine sulfenic acid and free SH-groups, forming thiol adducts in the protein.

S-alkylation was performed by incubating 20 μg of protein with 5 mM of DTT for 15 min at 60 °C. Subsequently, 10 mM of IAA was added and incubated for 25 min at RT in the dark. Finally, 0.5 mM of DTT was added and samples were again incubated for 25 min at room temperature (RT) in the dark.

AAT and rFahsin were oxidised with 2.5 mM of NCS using the method of Boudier and Bieth [22].

2.9. Chromogenic assays

To test the inhibitory activity of rFahsin, a chromogenic assay with NE and the chromogenic substrate MeO-SAAPVpNA was used. Samples (25 μl) were diluted in 20 mM Tris–HCl, pH 7.5, containing 0.2% (v/v) Tween 20, and pre-incubated with 34 nmol of NE (in 25 μl) for 30 min at 37 °C. Subsequently, MeO-SAAPVpNA was added (at a final concentration of 1 mM, final volume 100 μl) and incubated for 1 h at 37 °C. In control experiments without inhibitor it was established that the conversion of the substrate in time was linear. The absorbance was measured at dual wavelength 405 nm with 540 nm as a reference, using a Labsystems Multiscan plate-reader (Thermo Electron, Vantaa, Finland). Similarly, chromogenic assays were performed with cathepsin G, proteinase 3, chymotrypsin, trypsin, C1s, kallikrein, plasmin, trombin and coagulation factors VIIa, Xa, XIa and XIIa, using their respective chromogenic substrates. The residual protease activity (in percentage) was calculated by $[A_i/A_0]\times 100$, where A_i and A_0 are the absorbance with and without inhibitor, respectively.

To determine the inhibition constant (K_i) of rFahsin for NE, the method of Dixon [23] was used. The reactions were initiated by the addition of chromogenic substrate. The analysis was done with three different concentrations of SAAApNA. A405/540 in time was measured in a Multiscan Spectrum (Thermo Electron) containing an incubator, which was preset at 37 °C.

2.10. Functional activity of rFahsin in the presence of AAT

To compare the inhibition kinetics of AAT and rFahsin towards NE, a competitive ELISA was conducted.

In this assay known molar concentrations of AAT and rFahsin are incubated with a known lower concentration of NE. In case of equal functional activity and equimolar amounts of AAT and rFahsin, half the amount of NE-AAT complexes is expected compared to levels with AAT only. If rFahsin is a faster inhibitor of NE than AAT, less than an equimolar amount of rFahsin is required to get a 50% reduction in the total amount of NE-AAT complexes. To investigate this, 100 nmol of AAT was mixed with rFashin at concentrations of 10, 25, 50, 75 or 100 nmol and incubated with 10 nmol of NE. Reactions were incubated for 30 min at 37 °C and NE-AAT complexes were subsequently measured with an ELISA adapted from the radioimmunoassay as described by Nuijens et al. [24].

Briefly, rabbit IgG antibodies directed to NE (1.5 μg^{-1}; 100 μl/well) were coated on Maxisorp-plates (NUNC, Roskilde, Denmark) by overnight incubation at 4 °C in 0.1 M $NaHCO_3$, pH 9.6. After washing, the plate was subsequently blocked for 30 min at RT with 150 μl/well 2% (v/v) semi-skimmed milk in PBS. After washing, plates were incubated for 1 h at 37 °C with 100 μl/well of serial dilutions of samples and references diluted in PTG (PBS with 0.2% (w/v) gelatine and 0.02% (w/v) Tween 20) in the presence of 1% (v/v) bovine/rabbit serum. Bound NE-AAT complexes were detected using 100 l/well biotinylated mAb AT 15, which binds to a neodeterminant on complexed AAT. After 1 h at 37 °C, the plate was subsequently incubated with 100 μl streptavidine–horse radish peroxidase (AP Biotech) diluted 1:1000 in PTG. After 30 min at RT, the plate was incubated with substrate buffer (0.11 M sodium acetate, pH 5.5, 0.01% (v/v) TMB (dissolved in DMSO), 0.003% (v/v) H_2O_2). Colour development was stopped by addition of 100 μl of 2 M H_2SO_4. Absorbance was measured at dual wavelength 450 nm/540 nm.

2.11. Purification and stimulation of human PMN

Human PMN were isolated from citrated venous blood by centrifugation through Lymphoprep (Nycomed, Oslo, Norway) followed by hypotonic lysis of erythrocytes and suspended at 1.67×10^6 per cells in Hepes-buffer (120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 25 mM Hepes (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid)). In 96-well plates, 150 μl of the cell-suspension (0.25×10^6 cells) was treated with cytochalasin B (10 μM) for 5 min at 37 °C and subsequently stimulated with f-MLP (1 μM) for 20 min at 37 °C in the presence of different amounts of rFahsin and AAT. After removal of cells by centrifugation, the supernatants were analysed for NE-activity using the chromogenic substrate MeO-SAAPVpNA as described above.

To prevent biological oxidation of the inhibitors by reactive oxygen species (ROS) generated by PMN, superoxide dismutase (SOD) (5 U) plus catalase (10 U) were added. To check for superoxide production, PMN were mixed with cytochrome *c* (1 mg^{-1}) and incubated for 10 min at 37 °C in the presence or absence of AAT and rFahsin. After removal of cells by centrifugation, absorbance at 550 nm was read.

3. Results

3.1. Production and purification of rFahsin

From lysates of the leech *L. nilotica* a novel antistasin-type proteinase inhibitor, that we called Fahsin (Fig. 1), was isolated using regular chromatographic procedures and chromogenic assays [15]. Amino acid sequence comparison revealed that Fahsin shows high identity with the neutrophil elastase inhibitor guamerin (66% [1]) and the trypsin inhibitor piguamerin (56% [2]) (both derived from *H. nipponia*) and with hirustasin (51% [3]) and bdellastasin (42% [4]) (both derived from *H. medicinalis*), which are inhibitors of tissue kallekrein and plasmin, respectively. Also similarity was found with the N-terminal domain of the factor Xa inhibitors, antistasin [25], ghilanten [26] and therostasin [27], which are all antistasin-type inhibitors comprising two domains (data not shown). Furthermore, Fahsin contains, like guamerin, piguamerin, hirustasin and bdellastasin, 10 similarly spaced cysteine residues forming five disulfide bonds, which characterise the antistasin-type serine proteinase inhibitor family [5].

Based on the complete amino acid sequence of Fahsin, a synthetic gene was designed encoding the inhibitor. After cloning the gene into the expression vector, subsequent transformation of *P. pastoris* GS115 and

```
                                                P1
Fahsin          --------DDNCGGKVCSKGQLCHDGHCECTPIRCLIFCPNGFAVDENGCELPCSCKHQ-- 51
Guamerin        VDENAEDTHGLCGEKTCSPAQVCLNNECACTAIRCMIFCPNGFKVDENGCEYPCTCA---- 57
Piguamerin      ---------TDCGGKTCSEAQVCKDGKCVCVIGQCRKYCPNGFKKDENGCTFPCTCA---- 48
Hirustasin      ------TQGNTCGGETCSAAQVCLKGKCVCNEVHCRIRCKYGLKKDENGCEYPCSCAKASQ 55
Bdellastasin    --FDVNSHTTPCGPVTCSGAQMCEVDKCVCSDLHCKVKCEHGFKKDDNGCEYACICADAPQ 59
                          **  .** .*:*  ..* *   :*   *  *:  *:***  .* *
```

Fig. 1. Alignment of the primary amino acid sequence of five different antistasin-type proteinase inhibitors including Fahsin. The similarly spaced cysteine residues in the proteins are indicated in bold. The reactive site (P1) amino acid residue, reflecting the specificity of the inhibitor, is underlined. The selected sequences were aligned using the *ClustalW* algorithm (http://clustalw.genome.jp).

screening of rFahsin-producing strains in shake flasks, fermentations in mineral medium were performed as described in Section 2. After fermentation and removal of cells, the supernatant was subjected to acetone fractionation. Endogenous extracellular *P. pastoris* proteins were precipitated at 40% (v/v) of acetone. Subsequently, rFahsin could be precipitated by increasing the acetone concentration to 70% (v/v). This precipitation did not affect the functional activity of Fahsin as was assessed in a chromogenic assay with NE (data not shown). After evaporation of the acetone, the pellet was resuspended in distilled water, dialysed against 20 mM Tris, pH 8.0, and applied onto a HiloadQ column. During elution of the column, three different Fahsin peaks appeared (Fig. 2(a)), indicated as F1, F2 and F3. The three different forms also behaved differently in SDS–PAGE under non-reducing conditions (Fig. 2(b)). Only Fahsin F1 shows the expected migration rate (see Fig. 2(b)) of about 6 kDa. The migration rate in SDS–PAGE of the F2 and the F3 form of rFahsin was lower than expected. Glycosylation of F2 and F3 as a possible reason for their lower mobility was ruled out, because Concanavalin A (Con A) staining of the proteins on blot was negative (data not shown).

The chromogenic assay with NE revealed that the F1- and F2-form were biologically active, whereas the F3-form was not. N-terminal amino acid sequencing showed that of all three molecules the α-mating factor signal sequences were properly processed by the *P. pastoris* homolog of the kex-2p protease of *Saccharomyces cerevisiae*.

3.2. Characterisation of the different forms of Fahsin produced by P. pastoris

ESMS-data showed that the F1-form of rFahsin had a molecular mass of 5477.8 Da, about 10 Da lower than the theoretical (average) molecular weight of 5487 Da. This difference reflected the five sulfur bridges which are formed in the rFahsin F1 molecule.

The molecular mass of the Fahsin F2-form was only 18 Da higher than that of the F1. An explanation for this could be that F2 contains one single oxidised sulfhydryl group, which cannot participate in a disulfide bond, giving an increase in mass of 18 Da. To prove this, a chemical modification was performed with the reagents dimedone and NBD-Cl, which attack the sulfenate sulfur forming a thiol adduct. However, incubating the abberant forms of rec. Fahsin with these agents did not result in conjugates with higher mass in ESMS. Instead, the molecular weights of F1, F2 and F3 were still the same, indicating that neither free SH-groups nor cysteine sulfenic acids were present in the molecules.

Another explanation for the increased mass of 18 Da could be that Fahsin F2 contained one single hydrolysed peptide-bond, without affecting its biological activity. Protein-fragments of F2 are thought to be kept together by the intra-molecular sulfur bridges of the molecule. To investigate this idea, rFahsin F1 and F2 were reduced by DTT and subsequently S-alkylated by treatment with IAA as described in Section 2 and analysed by SDS–PAGE. Upon reduction and S-alkylation of F2, two protein fragments of about 2.5 and 3 kDa appeared on SDS–PAGE gel (Fig. 3, lane 2), while the alkylated F1-sample remained still intact (Fig. 3, lane 1). Reduction alone of F2 was not sufficient to show this effect (not shown). Possibly, protein fragments re-associate during migration in the gel via their many sulfur bridges.

ESMS-data of reduced and S-alkylated F2 showed two masses, one of 2739 Da and one of 3333 Da. Based on the two peptide masses we could determine the cleavagesite within the rFahsin F2 molecule. This site appeared to be exactly between the leucine and the isoleucine amino acid residue at the active site positions P1 and P1′, respectively.

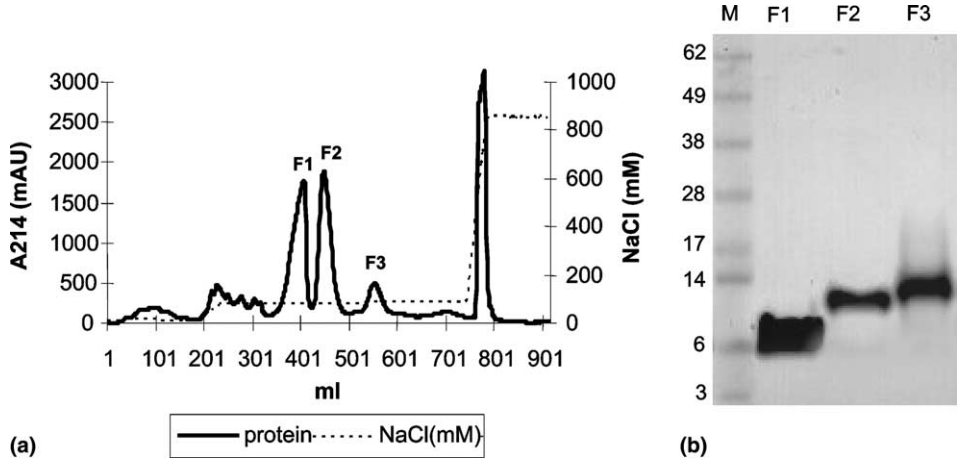

Fig. 2. (a) Elution profile at 214 nm during the purification of rFahsin by anion-exchange chromatography (HiLoad Q sepharose, AP Biotech). During elution using a linear concentration gradient of 4–7% (v/v) of 1 M NaCl, three different Fahsin peaks appeared, indicated as F1, F2 and F3. (b) SDS–PAGE analysis of different pooled fractions of rFahsin; F1, F2 and F3. Lane M, SeeBlue® Plus 2 pre-stained marker (Invitrogen).

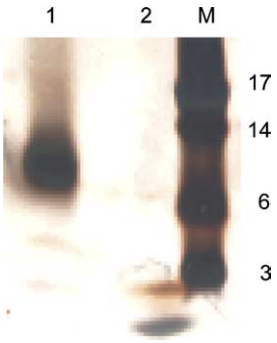

Fig. 3. SDS–PAGE analysis of rFashin after reduction and S-alkylation, using silver staining. Lane 1, Fahsin F1 reduced and S-alkylated. Lane 2, Fahsin F2 reduced and S-alkylated. Lane M, SeeBlue® Plus 2 pre-stained markers (Invitrogen).

Like F2, also the F3-form of rFahsin appeared to be internally hydrolysed. The molecular masses of rFahsin F3 fragments after reduction and S-alkylation appeared to be 2626 and 3333 Da. These masses revealed that the isoleucine amino acid residue at the active site position P1′ was removed from the molecule. This fact explained the lack of biological activity of the F3 form in the chromogenic assay with HNE.

Thus, the rFahsin F1 reflected the properly folded form of rFahsin, free of any modifications, whereas rFahsin F2 and F3 represented subsequent degradation products of the F1-form due to the action of one or more endogenous proteases of *P. pastoris*. Pooled fractions of F1 were dialysed against distilled water, lyophilised and used for further analysis.

3.3. Proteinase specificity and kinetic analysis towards HNE of rFahsin

rFahsin (F1) was reconstituted in distilled water and used to determine the protease specificity for the molecule. Chromogenic assays were performed with several serine proteases using their respective chromogenic substrates (see Section 2). Besides NE also the neutrophil-derived serine proteases cathepsin G and proteinase 3 and the pancreatic protease chymotrypsin were inhibited by rFahsin (data not shown). Other serine proteases like trypsin, C1s, kallekrein, plasmin, trombin, tPA, uPA and coagulation factors VIIa, Xa, XIa and XIIa were not inhibited.

The K_i of rFahsin for NE was determined with the substrate according to the method of Dixon [23], and was found to be 1.5 nM. In addition, when NE and Fahsin were pre-incubated for varying time intervals prior to adding the substrate, no differences in the inhibitory activities were found, suggesting that Fahsin is a fast- and tight-binding inhibitor of NE.

The inhibitory capacity of rFahsin was compared with that of AAT with respect to NE inhibition by two methods. The chromogenic assay was performed as described in Section 2 using an end-point measurement in which a fixed amount of NE was incubated with varying concentrations of rFahsin and AAT. Residual activity of NE was then measured with the chromogenic substrate. From Fig. 4(a) it is clear that equimolar amounts of Fahsin and AAT were required to inhibit NE activity.

As a serpin, AAT acts as suicide substrate and is cleaved by its target proteases to form essentially irreversible 1:1 complexes. To assess the relative inhibitory activities of rFahsin and AAT, we mixed known molar concentrations of either inhibitor and incubated these with NE (at lower concentration than that of each inhibitor). Subsequently, we measured NE-AAT complexes in the mixtures with ELISA. We found that only 50 nM of rFahsin was required to reduce the total

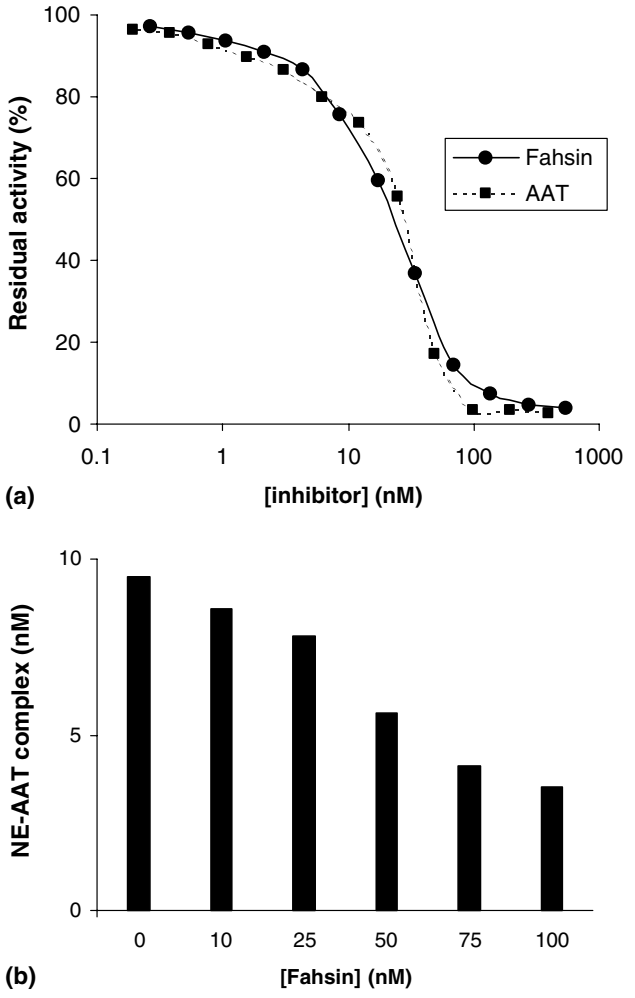

Fig. 4. (a) Effect of increasing concentrations of rFahsin and AAT on the activity of a fixed amount of NE (34 nM). (b) Competition of rFahsin and AAT for complex formation with NE. 10 nmol NE was incubated with 100 nmol AAT and varying doses of rFahsin, as indicated. Subsequently, total amount of NE-AAT complexes was measured in the ELISA.

amount of NE-AAT complexes formed in the presence of 100 nM AAT by about 50% (Fig. 4(b)).

3.4. Chemical and biological oxidation of rFahsin and AAT

rFahsin and AAT were chemically oxidised by treatment with NCS. Subsequently, a chromogenic assay with NE was performed (Fig. 5(a)).

To investigate oxidation of rFahsin and AAT by ROS, PMN were stimulated with f-MLP in the presence of the proteinase inhibitors and radical scavengers (as described in Section 2). Cells were then separated by centrifugation after which NE activity in the supernatant was measured with the chromogenic assay. In Fig. 5(b) it is shown that the inhibitory activity of AAT decreases in the presences of stimulated neutrophils. The latter effect was partly prevented by the presence of SOD plus catalase during stimulation of PMN, indicating that ROS generated are responsible for the decrease of the inhibitory capacity of AAT (data not shown). In contrast, the inhibitory capacity of Fahsin remained unaffected upon PMN-stimulation. ELISA data showed that NE release during stimulation was not influenced in the presence of rFahsin or AAT.

Thus, rFahsin is resistant to both chemical and biological oxidation.

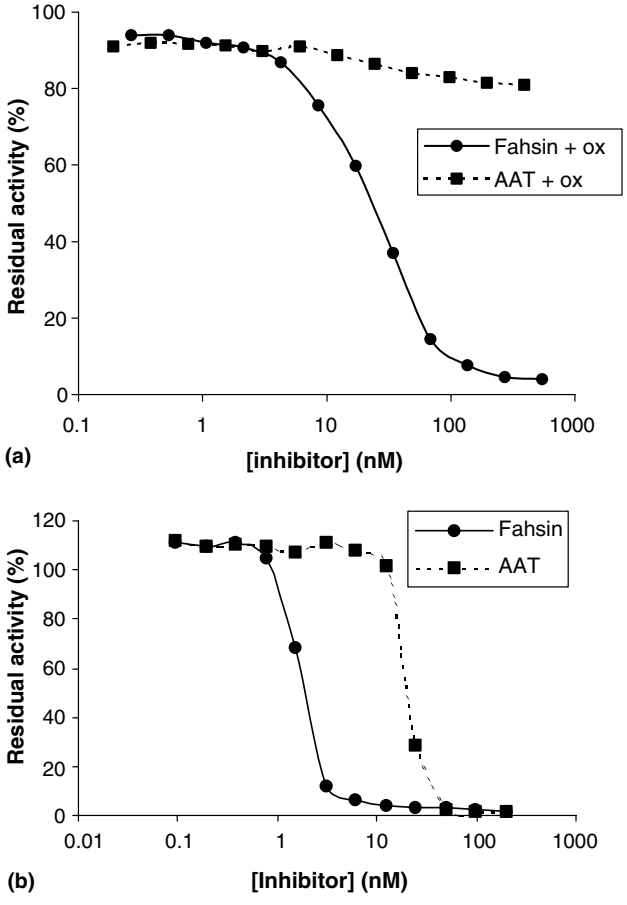

Fig. 5. (a) Residual activity of a fixed amount of NE (34 nM) using different concentrations of rFahsin and AAT after oxidation with NCS. (b) Residual activity of released NE after stimulation of PMN with cytochalasin B and fMLP in the presence of different concentrations of rFahsin and AAT.

4. Discussion

The biochemical characteristics and biological function of a variety of proteinase inhibitors from leeches have been described. In addition, several expression systems for the production of these inhibitors have been developed because of their therapeutic potential. Hirudin, a thrombin inhibitor from *H. medicinalis*, is most intensively studied [28] and was amongst others produced in the *P. pastoris* system, where expression levels of 1.5 g^{-1} were reached [29].

The antistasin-type serine proteinase inhibitors hirustasin [30], bdellastasin [4] and guamerin [31], which all show high identity with Fahsin, are also produced in yeast systems with yields ranging from 0.03 to 0.69 g^{-1}. Expression levels of antistasin [32] and ghilanten [33], which are both antistasin-type inhibitors comprising two domains, are much lower (1 and 10 mg^{-1}, respectively).

This paper describes a novel member of the antistasin-type serine proteinase inhibitor family, namely Fahsin. Fahsin is the first antistasin-type inhibitor having a leucine residue at its P1-residue, resulting in a specific inhibitor of neutrophil-derived serine proteinases. During recombinant production in *P. pastoris*, levels of 0.5 g^{-1} culture medium were obtained, but three different forms of rFashin were found. S-alkylation and mass spectrometry revealed that the two aberrant forms of Fahsin were intra-molecularly cleaved products of the intact recombinant molecule, due to the action of one or more endogenous *P. pastoris* proteases. Werten et al. [20] also had found proteolytic cleavage at specific sites in recombinant gelatins produced in *P. pastoris*. These sites could successfully be abolished by site-directed mutagenesis resulting in an intact product. Whether the same strategy can be followed to enhance the integrity, without affecting the biological activity of rFahsin, is not yet known.

Despite the presence of degradation products, the F1 form of rFahsin, which contained five disulfide bonds and which was free of any proteolytic degradation, could be purified to homogeneity by acetone precipitation and anion-exchange chromatography.

The inhibitory constant of rFahsin for NE was calculated to be 1.5×10^{-9} M with the chromogenic substrate SAAApNa. This K_i-value is more than a factor 20 lower than that of recombinant guamerin, using the same substrate [3].

Fahsin was found to be a faster inhibitor of NE than AAT. When incubated with AAT and NE, Fahsin at about half the concentration of AAT reduced HNE-AAT complexes twofold.

Besides this, AAT and guamerin both contain a methionine residue as their P1 residue. This makes these inhibitors susceptible to oxidation, resulting in the loss of their biological activity [12–14]. In contrast, Fahsin does not contain any methionine, making it theoretically insensible to oxidation. In experiments we showed that rFahsin is indeed resistant to both chemical and biological oxidation. These results suggest that rFahsin has potential as an anti-inflammatory drug that prevents tissue damage caused by release of serine proteinases from activated PMNs at sites of chronic inflammation. The therapeutic potential of rFahsin in the treatment of inflammatory diseases will be investigated further by using animal models.

5. Conclusion

Fahsin is the first antistasin-type inhibitor, having a leucine residue at its P1 position. This results in a specific inhibitor of neutrophil-derived serine proteinases, which is insensitive to chemical and biological oxidation. We showed here that we could combine the high specific efficacy of the leech component with the possibility of sufficient production in a recombinant system. This is essential for future development of rFahsin as a therapeutic agent for treatment of chronic inflammatory diseases.

Acknowledgement

We gratefully acknowledgeing Roel van der Schors, Department of Molecular and Cellular Neurobiology, Faculty of Earth and Life Sciences, Vrije Universiteit, Amsterdam, The Netherlands, for ESMS-analysis.

References

[1] Jung, H.I., Kim, S.I., Ha, K.S., Joe, C.O. and Kang, K.W. (1995) Isolation and characterization of guamerin, a new human leukocyte elastase inhibitor from *Hirudo nipponia*. J. Biol. Chem. 270, 13879–13884.

[2] Kim, D.R. and Kang, K.W. (1998) Amino acid sequence of piguamerin, an antistasin-type protease inhibitor from the blood-sucking leech *Hirudo nipponia*. Eur. J. Biochem. 254, 692–697.

[3] Sollner, C., Mentele, R., Eckerskorn, C., Fritz, H. and Sommerhoff, C.P. (1994) Isolation and characterization of hirustasin, an antistasin-type serine-proteinase inhibitor from the medical leech *Hirudo medicinalis*. Eur. J. Biochem. 219, 937–943.

[4] Moser, M., Auerswald, E., Mentele, R., Eckerskorn, C., Fritz, H. and Fink, E. (1998) Bdellastasin, a serine protease inhibitor of the antistasin family from the medical leech (*Hirudo medicinalis*) – primary structure, expression in yeast, and characterisation of native and recombinant inhibitor. Eur. J. Biochem. 253, 212–220.

[5] Mittl, P.R., Di Marco, S., Fendrich, G., Pohlig, G., Heim, J., Sommerhoff, C., Fritz, H., Priestle, J.P. and Grutter, M.G. (1997) A new structural class of serine protease inhibitors revealed by the structure of the hirustasin–kallekrein complex. Structure 5, 253–264.

[6] Moraes, T.J., Chow, C.W. and Downey, G.P. (2003) Proteases and lung injury. Crit. Care Med. 31, S189–S194.

[7] Fricker, J. (1998) Elastase inhibitor for detection and treatment of inflammation and infection. Mol. Med. Today 4, 5.

[8] Hiemstra, P.S. (2002) Novel roles of protease inhibitors in infection and inflammation. Biochem. Soc. Trans. 30, 116–120.

[9] Shapiro, S.D. (2002) Neutrophil elastase: path clearer, pathogen killer, or just pathologic. Am. J. Respir. Cell. Mol. Biol. 26, 266–268.

[10] Uitto, V.J., Overall, C.M. and McCulloch, C. (2003) Proteolytic host cell enzymes in gingival crevice fluid. Periodontology 31, 77–104.

[11] Salzet, M., Vieau, D. and Stefano, G.B. (1999) Serpins: an evolutionarily conserved survival strategy. Immunol. Today 20, 541–544.

[12] Johnson, D. and Travis, J. (1979) The oxidative inactivation of human α-1-proteinase inhibitor. Further evidence for methionine at the reactive center. J. Biol. Chem. 254, 4022–4026.

[13] Padrines, M., Schneider-Pozzer, M. and Bieth, J.G. (1989) Inhibition of neutrophil elastase by α-1-proteinase inhibitor oxidized by activated neutrophils. Am. Rev. Respir. Dis. 139, 783–790.

[14] Duranton, J. and Bieth, J.G. (2003) Inhibition of proteinase 3 by α1-antitrypsin in vitro predicts very fast inhibition in vivo. Am. J. Respir. Cell. Mol. Biol. 29, 57–61.

[15] Voerman, G. (1996) A novel family of protease inhibitors and other biologically active substances. Pat. Appl. WO 9613585.

[16] Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. and Pease, L.R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77, 51–59.

[17] Cregg, J.M., Barringer, K.J., Hessler, A.Y. and Madden, K.R. (1985) *Pichia pastoris* as a host system for transformations. Mol. Cell. Biol. 5, 3376–3385.

[18] Becker, D.M. and Guarente, L. (1991) High-efficiency transformation of yeast by electroporation. Methods Enzymol. 194, 182–187.

[19] Potter, K.J., Zhang, W., Smith, L.A. and Meagher, M.M. (2000) Production and purification of the heavy chain fragment C of botulinum neurotoxin, serotype A, expressed in the methylotrophic yeast *Pichia pastoris*. Protein Expr. Purif. 19, 393–402.

[20] Werten, M.W.T., van den Bosch, T.J., Wind, R.D., Mooibroek, H. and de Wolf, F.A. (1999) High-yield secretion of recombinant gelatins by *Pichia pastoris*. Yeast 15, 1087–1096.

[21] Ellis, H.R. and Poole, L.B. (1997) Novel application of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole to identify cysteine sulfenic acid in the *Ahp*C component of alkyl hydroperoxide reductase. Biochemistry 36, 15013–15018.

[22] Boudier, C. and Bieth, J.G. (1994) Oxidized mucus proteinase inhibitor: a fairly potent neutrophil elastase inhibitor. Biochem. J. 303, 61–68.

[23] Dixon, M. (1953) The determination of enzyme inhibitor constants. Biochem. J. 55, 170–171.

[24] Nuijens, J.H., Abbink, J.J., Wachtfogel, Y.T., Colman, R.W., Eerenberg, A.J., Dors, D., Kamp, A.J., Strack van Schijndel, R.J., Thijs, L.G. and Hack, C.E. (1992) Plasma elastase α1-antitrypsin and lactoferrin in sepsis: evidence for neutrophils as mediators in fatal sepsis. J. Lab. Clin. Med. 119, 159–168.

[25] Dunwiddie, C., Thornberry, N.A., Bull, H.G., Sardana, M., Friedman, P.A., Jacobs, J.W. and Simpson, E. (1989) Antistasin,

a leech-derived inhibitor of factor Xa. Kinetic analysis of enzyme inhibition and identification of the reactive site. J. Biol. Chem. 264, 16694–16699.

[26] Blankenship, D.T., Brankamp, R.G., Manley, G.D. and Cardin, A.D. (1990) Amino acid sequence of ghilanten: anticoagulant–antimetastatic principle of the South American leech, *Haementeria ghilianii*. Biochem. Biophys. Res. Commun. 166, 1384–1389.

[27] Chopin, V., Salzet, M., Baert, J., Vandenbulcke, F., Sautiere, P.E., Kerckaert, J.P. and Malecha, J. (2000) Therostasin, a novel clotting factor Xa inhibitor from the rhynchobdellid leech, *Theromyzon tessulatum*. J. Biol. Chem. 275, 32701–32707.

[28] Markwardt, F. (2002) Hirudin as alternative anticoagulant – a historical review. Semin. Thromb. Hemost. 28, 405–414.

[29] Rosenfeld, S.A., Nadeau, D., Tirado, J., Hollis, G.F., Knabb, R.M. and Jia, S. (1996) Production and purification of recombinant hirudin expressed in the methylotrophic yeast *Pichia pastoris*. Protein Expr. Purif. 8, 476–482.

[30] Di Marco, S., Fendrich, G., Knecht, R., Strauss, A., Pohlig, G., Heim, J., Priestle, J.P., Sommerhoff, C.P. and Grutter, M.G. (1997) Recombinant hirustasin: production in yeast, crystallization, and interaction with serine proteases. Protein Sci. 6, 109–118.

[31] Kim, K.Y., Lim, H.K., Lee, K.J., Park, D.H., Kang, K.W., Chung, S.I. and Jung, K.H. (2000) Production and characterization of recombinant guamerin, an elastase-specific inhibitor, in the methylotrophic yeast *Pichia pastoris*. Protein Expr. Purif. 20, 1–9.

[32] Przysiecki, C.T., Joyce, J.G., Keller, P.M., Markus, H.Z., Carty, C.E., Hagopian, A., Sardana, M.K., Dunwiddie, C.T., Ellis, R.W. and Miller, W.J. (1992) Characterization of recombinant antistasin secreted by *Saccharomyces cerevisiae*. Protein Expr. Purif. 3, 185–195.

[33] Brankamp, R.G., Sreekrishna, K., Smith, P.L., Blankenship, D.T. and Cardin, A.D. (1995) Expression of a synthetic gene encoding the anticoagulant–antimetastatic protein ghilanten by the methylotropic yeast *Pichia pastoris*. Protein Expr. Purif. 6, 813–820.