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Full Length Article

Fluorescent activity-based probe for the selective detection of Factor VII activating protease (FSAP) in human plasma

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ABSTRACT

The zymogen form of circulating Factor VII activating protease (FSAP) is activated by histones that are released as a consequence of tissue damage or excessive inflammation. This is likely to have consequences in a number of disease conditions such as stroke, atherosclerosis, liver fibrosis, thrombosis and cancer. To investigate the existence, as well as the concentration of active FSAP (FSAPa) in complex biological systems an active site probe is needed. We used Hybrid Combinatorial Substrate Library (HyCoSuL) to screen for natural and unnatural amino acids that specifically bind to P4-P2 pockets of FSAPa. This information was used to designing a fluorogenic substrate (Ac-Pro-*D*Tyr-Lys-Arg-ACC) as well as an irreversible, fluorogenic activity-based probe Cy5-6-Ahx-Pro-*D*Tyr-Lys-Arg^{P}$(OPh)_2$. In normal human plasma the probe showed very low non-specific reactivity with some plasma proteins but upon activation of pro-FSAP with histones, strong labelling of FSAPa was observed. This labelling could be inhibited by aprotinin and was not found in the plasma of a subject that was homozygous for a polymorphism, which leads to loss of activity, or in plasma that was depleted of FSAP by antibodies. This 2nd generation substrate exhibited 6-fold higher catalytic efficiency than the 1st generation substrate and a much higher selectivity for FSAPa over other plasma proteases. This substrate and probe can be useful to detect and localize FSAPa in normal and pathological tissue and plasma to gain more insight into its functions.

1. Introduction

FSAP is a circulating serine protease that is secreted by the liver as an inactive zymogen [1]. Human genetic data, that is based on the study of the proteolytically inactive Marburg I polymorphism, as well as studies from FSAP knock-out mice indicate that FSAP may play an important role in many patho-physiological conditions such as carotid stenosis [2], stroke [3,4], liver fibrosis [5,6] and thyroid cancer [7,8]. Zymogen activation in whole blood or plasma is mediated by factors released from damaged cells such as histones [9] as well as by positively charged surfaces [10]. In traumatic injury or sepsis nucleosome concentrations in plasma correlate strongly with FSAP activation [11,12]. Recently it was shown that nucleosomes *per se* do not activate pro-FSAP but that the release of histones upon DNA degradation with DNase can release histones which activate FSAP [13,14]. Activated two-chain FSAP (FSAPa), in turn, degrades histones which may be a key function in the context of inflammation, thrombosis and vascular biology [9]. DNases are naturally present in blood and can also be released by microbes as part of their survival strategy and can indirectly activate FSAP by freeing histones [15]. FSAPa can be rapidly inhibited by serine protease inhibitors such as α1-proteinase inhibitor, α2-plasmin inhibitor, antithrombin, C1 inhibitor [16–19], as well as plasminogen activator inhibitor-1 (PAI-1) [20] and protease nexin-1 [21]. Thus reliable methods are needed to specifically and directly measure FSAPa in the plasma as well as to localize it *in situ*.

Recently, we developed a specific 1st generation substrate for FSAPa using a phage display and a natural amino acid peptide library and have also established that FSAPa has a broad substrate specificity with a predilection of clusters of positively charged amino acids [22]. However, a substrate and probe with a higher selectivity for FSAPa are

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needed. The greater variety of chemical structures of unnatural amino acids has allowed the development of selective compounds for the target proteases (caspases [23], legumain [24], human neutrophil serine proteases [25,26]). Thus, we have used a library approach consisting of tetrapeptides of natural and unnatural amino acids such as the Hybrid Combinatorial Substrate Library (HyCoSuL) to develop specific 2nd generation substrate, inhibitor and activity-based probe for FSAPa.

2. Material and methods

2.1. Recombinant serine protease domain (SPD) of FSAP

Protease domain (amino acids 292–560) of wild type (WT) FSAP as well as MI-FSAP (G534E mutation) was cloned into the pASK-IBA33plus vector (IBA-Lifesciences, Goettingen, Germany) including a C-terminal 6xHis tag. Expression was in BL21(DE3) cells (Agilent, La Jolla, CA) and inclusion bodies were prepared by sonification and centrifugation. SPD was purified over Ni-Agarose column (Qiagen, Hilden, Germany). The purified protein was refolded over 48 h and then characterized by SDS-PAGE and activity assays with chromogenic substrate S-2288 (H_2N-*D*Ile-Pro-Arg-pNA) (Haemochrome Diagnostica, Molndal, Sweden). Further details will be described in a separate manuscript.

2.2. HyCoSuL library screening

To investigate FSAPa substrate specificity at the S4-S2 subsites, the previously synthesized HyCoSuL P1-Arg library was used [27]. The library contains 19 natural (omitting cysteine and substituting norleucine for methionine) and 108 unnatural amino acids in each position of three sub-libraries (P4, P3 and P2). FSAP was assayed in the following buffer: 20 mM Tris, 150 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$ and 0.1% *w/v* Tween 20, pH 7.5. The final library concentration was 100 μM and the final enzyme concentration was 100 nM, reaction volume was 100 μL. The enzyme was preincubated in assay buffer for 30 min at 37 °C prior to addition to each well. Library screening was carried out using 96-well plates and an fMax spectrofluorimeter (Molecular Device SpectraMax Gemini XPS). The excitation and emission wavelength were 355 nm and 460 nm respectively. Substrate hydrolysis was recorded in kinetic mode at 37 °C for 30 min. From each single experiment, the linear portion of the progress curve was used to calculate RFU/s (relative fluorescence unit per second). All experiments were repeated three times. Specificity was calculated by normalizing RFU/s to the highest value (100%).

2.3. Synthesis of fluorogenic substrates

Designed substrates were synthesized using ACC as a reporter group and Fmoc-protected amino acids on a solid support according to literature protocols [23,28]. In the first step, Fmoc-ACC was coupled to the Rink Amide AM Resin using HOBt and DICI in DMF as coupling reagents. After Fmoc-group removal (20% piperidine in DMF), Fmoc-Arg(Pbf)-OH was attached to the H_2N-ACC-resin with HATU and collidine. The Fmoc-group was removed and Fmoc-P2-OH was coupled with HOBt and DICI. Elongation was continued until H_2N-P4-P3-P2-Arg(Pbf)-ACC-resin was obtained. The free amino group of the P4 residue was acetylated with AcOH/HBTU/DIPEA. The peptide substrates were cleaved from the resin with TFA/TIPS/H_2O (95%,2.5%,2.5%, v,v,v) and purified by HPLC. Products were lyophilized, dissolved in DMSO and stored at −20 °C until use. Compounds were analyzed by analytical HPLC and HRMS.

2.4. Kinetics for individual substrates

The kinetic parameters (k_{cat}, K_M, k_{cat}/K_M) of selected FSAP substrates were determined by measuring the increase of fluorescence over time. FSAP was preincubated in the assay buffer (20 mM Tris, 150 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 0.1% *w/v* Tween 20, pH 7.5) for 30 min at 37 °C. The concentration of substrates ranged from 18 to 500 μM, the enzyme concentration was 10–20 nM and reaction volume was 100 μL. Substrate cleavage was monitored for 30 min. The linear part of each progress curve was used to calculate the kinetic parameters with the Michaelis-Menten equation. Results from three different experiments were analyzed in GraphPad Prism software. The average value and standard deviation were calculated.

2.5. Synthesis of phosphonate activity-based probe and determination of inhibition kinetics (k_{obs}/I) for probe

The best recognized substrate was transformed into an activity-based probe. In the first step, the peptide motif (Boc-Ahx-Pro-*D*Tyr(tBu)-Lys(Boc)-OH) containing 6-aminohexanoic acid as a linker was synthesized on solid support – 2-chlorotrityl chloride resin according to the method described in literature [25]. As an electrophilic warhead, diphenyl phosphonate was utilized and synthesized according to previous methodology [29]. In the second step Boc-Ahx-Pro-*D*Tyr(tBu)-Lys(Boc)-OH was attached to H_2N-Arg(Boc)$_2{}^{P}$(OPh)$_2$ using HATU/collidine in DMF as coupling reagents. After removal of the protecting group, the crude product was purified by HPLC and lyophilized. In the last step Cy5 NHS ester was attached to obtained compound H_2N-Ahx-Pro-*D*Tyr-Lys-Arg^{P}(OPh)$_2$ with DIPEA in DMF. The final product was purified by HPLC and lyophilized. The purity of probe was confirmed by analytical HPLC and molecular weight was confirmed by high-resolution mass spectrometry on a High Resolution Mass Spectrometer WATERS LCT premier XE with Electrospray Ionization (ESI) and Time of Flight (TOF) module. ABP with biotin tag was obtained following procedures similar to those used for fluorescent activity-based probe, but Biotin-Ahx-Pro-*D*Tyr(tBu)-Lys(Boc)-OH was synthesized instead of Boc-Ahx-Pro-*D*Tyr(tBu)-Lys(Boc)-OH. For Biotin-Ahx-Pro-*D*Tyr-Lys-Arg^{P}(OPh)$_2$ probe k_{obs}/I value was determined. FSAP was preincubated in the assay buffer (20 mM Tris, 150 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 0.1% *w/v* Tween 20, pH 7.5) for 30 min at 37 °C. The probe was diluted in a 96-well plate and mixed with the substrate (Ac-Hyp(Bzl)-*D*Tyr-Lys-Arg-ACC); the final substrate concentration was 100 μM, the final enzyme concentration was 5 nM, the final probe concentration was ranging from 100 nM to 26 nM. After 30 min FSAP was added and fluorescence was monitored for 30 min. The measurement was repeated at least 3 times and presented as an average value with SD. The k_{obs}/I value was calculated using GraphPad Prism software as described [25].

2.6. Labelling of FSAPa and other proteases by the probe

The following enzymes were used; recombinant human WT-SPD of FSAP, MI-SPD, human plasma-purified FSAP, Human neutrophil elastase (Merck-Millipore, Oslo, Norway), human thrombin (Sigma Aldrich, Oslo, Norway), Low molecular weight urokinase (uPA) (Sekisui, Pfungstadt, Germany), tissue plasminogen activator (tPA), (Actilyse, Boehringer Ingelheim, Germany), Factor XIIa (Haemochrome Diagnostica, Essen, Germany), bovine Factor Xa (NEB, Ipswich, MA, USA), Plasmin (in-house purified human plasma plasminogen activated with LMW-uPA in a ratio of 100:1). First, 500 nM of the recombinant WT-SPD or MI-SPD was incubated with varying concentrations of probe and time points to establish the optimal labelling conditions. Other plasma serine proteases (100 nM) were incubated with the probe at 50 or 200 nM for 30 or 90 min in Tris (50 mM, pH 7.4), NaCl (100 mM), T-80 (0.1% *w/v*) and $CaCl_2$ (2 mM). The reaction was stopped with SDS sample buffer and the samples boiled under reducing or non-reducing conditions and run on a 12% (*w/v*) SDS-PAGE. The gels were washed in water and an image at 700 nm wavelength was obtained using a Licor Odyssey imaging system.

2.7. Labelling of active FSAP in human plasma by the probe

Human citrated-plasma was used for these studies and activation was performed with variable concentration of histones (Calf thymus histones, Sigma Aldrich). Where indicated, aprotinin, a known inhibitor of FSAP, was included to block the activity of FSAP. In some experiments plasma was depleted of FSAP by passing over an anti-FSAP-antibody column or control column. Plasma of a subject homozygous for the Marburg I single nucleotide polymorphism was also used as described before [22]. The plasma was diluted 1:5 in same buffer as used above for purified proteases. Concurrently, the probe was added to the plasma sample and incubated for 60 min at 37 °C and analyzed by gel electrophoresis as described above. The samples were centrifuged prior to loading and an equivalent of 1 μL plasma was loaded per lane.

2.8. Selectivity of the substrate and the probe against various plasma enzymes

Cleavage of the substrate was measured using a synergy HI plate reader (BioTek Instruments, Winooski, USA) with excitation at 320 nm and emission at 460 nm (37 °C for 60 min). The initial velocity was calculated from the initial, linear part of the progress curve. The standard assay system consisted of 25 mM Tris-HCl, pH 7.5, and 150 mM NaCl with $CaCl_2$ (2 mM) and Tween-20 (0.1% *w/v*). Enzyme concentrations are indicated in the figure legends and the concentration of peptide substrates was from 0 to 600 μM. K_M, k_{cat} and k_{cat}/K_M was determined as described above.

For inhibition assays the enzymes (20 nM) were incubated with the biotinylated probe (0–10 μM) for 30 min at 37 °C and then the respective chromogenic substrate was added to determine residual enzymatic activity by determining absorbance at 405 nm for 60 min at 37 °C. Activity of Factor XIIa and plasma kallikrein was measured with the substrate CS31 (H_2N-*D*Pro-Phe-Arg-pNA, 1000 μM), thrombin with CS01 (H_2N-*D*Phe-Pip-Arg-pNA, 1000 μM), plasmin with CS41 (PyroGlu-Phe-Lys-pNA, 1000 μM), LMW-uPA with PNAPEP 1344 (PyroGlu-Gly-Arg-pNA, 250 μM), Factor Xa with S-2765 (N-a-*Z*-*D*Arg-Gly-Arg-pNA, 250 μM), tPA and SPD-FSAP with S2288 (H_2N-*D*Ile-Pro-Arg-pNA, 250 μM). All chromogenic substrates were from Hyphen Biomed (Neuville Sur Oise, France). IC_{50} values were calculated using the GraphPad Prism software.

2.9. Use of the substrate to detect FSAPa in human plasma

Human plasma was used for these studies and activation was performed with either dextran sulphate (DS) or histones. Activation was also performed in the presence of inhibitors or antibodies as indicated in the figure legends. Thereafter, the enzymatic activity was determined by direct addition of fluorogenic substrate to the plasma for 60 min at 37 °C. Turnover of the fluorescent substrates was determined as described above.

3. Results

3.1. FSAP substrate specificity based on the HyCoSuL library

HyCoSuL library was employed to determine WT-SPD-FSAP substrate preferences. The library comprised of arginine in P1 position and 19 natural and 108 unnatural amino acids in P2-P4 positions. The utilization of unnatural amino acids significantly increases the possibility of designing selective substrates, inhibitors and activity-based probes [30]. Kinetic analysis with this substrate library revealed that WT-SPD-FSAP exhibits a narrow specificity in S4-S2 subsites (Fig. 1). The most preferred amino acids in the S2 pocket are Lys(2-ClZ) (100%), Nle (58%), hLeu (55%), 2-Aoc (51%), hArg (45%). Suprisingly, D-amino acids such as *D*-Tyr, *D*-Trp, *D*-Phe were the best recognized amino acids in the S3 pocket. Other amino acids were also recognized but with a significantly lower affinity. S4 pocket preferred hydrophobic amino acids, where the best were Lys(2-ClZ), Hyp(Bzl) and Dab(Z).

3.2. Kinetic constants for the cleavage of the fluorogenic substrate by WT-SPD-FSAP

To validate the results of the library screening, as well as to design an optimal substrate, we synthesized ACC-labelled substrates containing optimal natural and unnatural amino acids in the P4-P2 positions. The results from previous studies that incorporated phage display and PS-SCL methods revealed that lysine in P2 position determined selectivity toward FSAP [22]. *D*-amino acids were the best recognized in P3 position thus we selected *D*-Tyr. In P4 position Lys(2-ClZ) and Hyp(Bzl) were the preferred amino acids. However, substrates containing large hydrophobic residues like Lys(2-ClZ) and Hyp(Bzl) in P4 position or Lys(2-ClZ) in P2 position were not efficiently cleaved by WT-SPD-FSAP. This was probably due to steric hindrance and substrate cooperativity (Supplementary Fig. S1) as seen in several other proteases earlier [31]. Substrates containing the best natural amino acids in P4-P2 positions were synthesized and kinetic parameters were determined (Table 1). Incorporation of unnatural amino acids, significantly, increased activity toward the substrates and the best substrate, Ac-Pro-*D*Tyr-Lys-Arg-ACC (k_{cat}/K_M = 55,045 M^{-1} s^{-1}), was around 3.6-fold better comparing to optimal sequence with natural amino acids only (Ac-Pro-Lys-Nle-Arg-ACC) (Table 1). Furthermore, Ac-Pro-*D*Tyr-Lys-Arg-ACC was above 6 times better recognized than the 1st generation substrate (Ac-Ala-Lys-Nle-Arg-ACC) (Table 1, Supplementary Fig. S2).

The selectivity of this 2nd generation substrate was tested against purified enzymes from the coagulation and fibrinolysis pathway. The selectivity of this substrate for FSAP was 4-fold higher than for tPA, 11-fold higher than for plasmin and 22-fold higher than for kallikrein (Table 2). Thus, we obtained the 2nd generation substrate that was not only better recognized by FSAP, but exhibited much better substrate selectivity compared to the 1st generation substrate.

We then compared the 1st and 2nd generation substrates in a FSAPa-generation assays in plasma. Histones are the only known physiological activators of pro-FSAP in plasma and *in vivo* [9]. These are released from injured or inflamed tissue and alter FSAP activity in the plasma significantly. The cleavage of the new substrate was stimulated in dose-dependent manner by histones and the turnover of this substrate was around 5-fold higher than the 1st generation substrate (Fig. 2A). The substrate was equally well cleaved in citrate plasma or hirudin plasma but there was no turnover in plasma immuno-depleted of FSAP over an FSAP-antibody column or in homozygous MI plasma that is devoid of enzymatically active FSAP (Fig. 2B). Plasma circulated over a control antibody column showed normal activation whereas heterozygous MI plasma showed about 50% of the activation seen in normal plasma (Fig. 2B).

Dextran sulphate can activate the contact pathway in plasma and further activation studies were performed to evaluate the selectivity of this substrate over the enzymes of the contact pathway. Dextran sulphate induced a moderate increase in FSAP activity and this could be reduced completely by corn trypsin inhibitor (CTI), which is an inhibitor of FXIIa. PKSI527, a kallikrein inhibitor, only partially inhibited this activation whereas a FSAP-blocking antibody, Mab570, had no effect suggesting that dextran sulphate activates the contact pathway that shows some reactivity toward the substrate. On the other hand, the effect of histone on FSAP activation was inhibited by Mab570 but not by CTI or PKSI527 indicating an exclusive activation of FSAP (Fig. 2C). Thus, the 2nd generation substrate shows a high selectivity and processivity to FSAP and can be used to measure fluorogenic activity in plasma in a sensitive manner.

3.3. Development of activity-based probe for FSAPa

Next we used the optimal sequence obtained in the previous step to

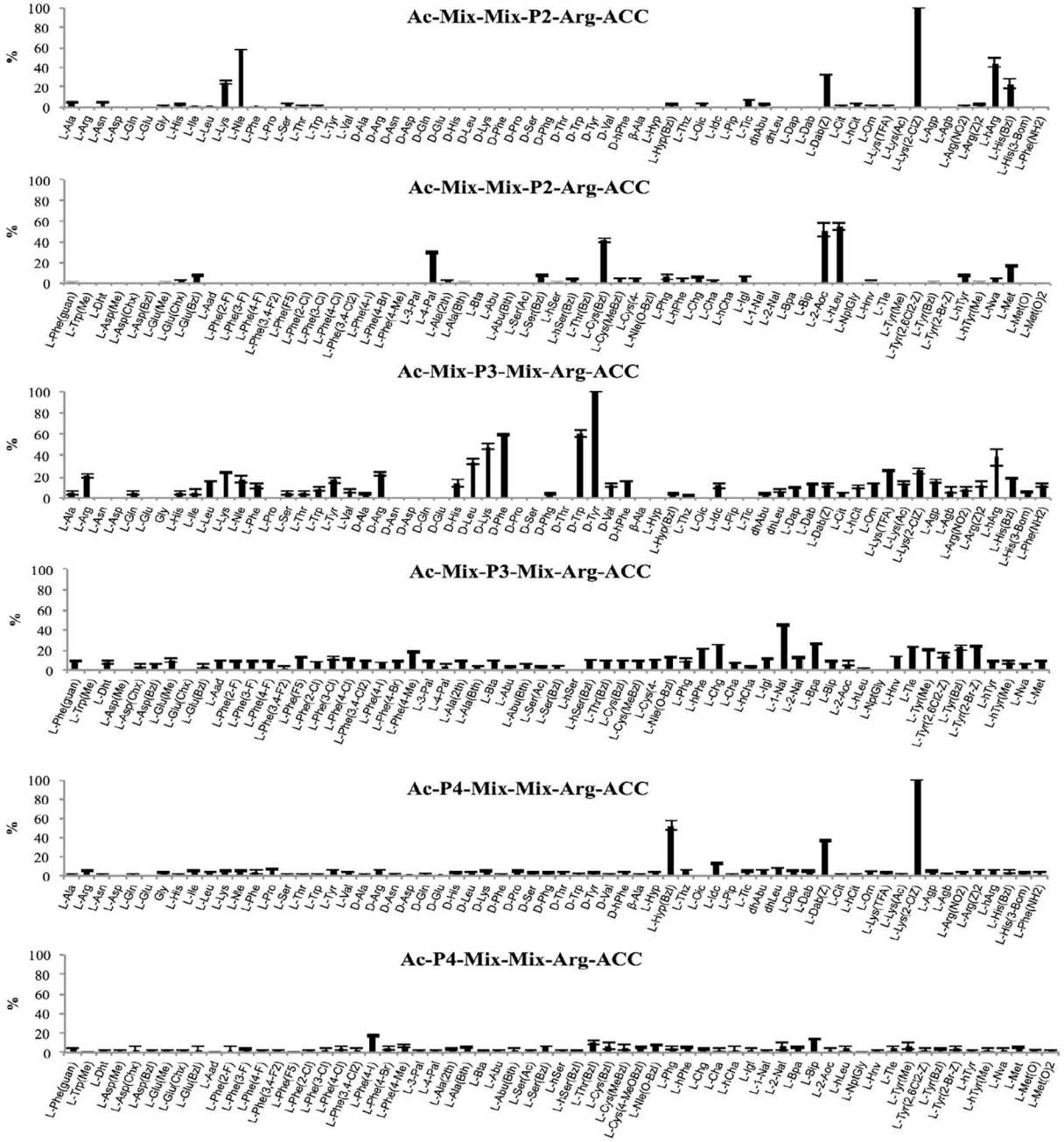

Fig. 1. WT-SPD-FSAP substrate specificity profile. X-axis represents 19 natural and 108 unnatural amino acids in P2, P3 and P4 positions (Mix – equimolar mixture of 18 natural amino acids and norleucine, cysteine and methionine were omitted due to susceptibility to oxidation); Y-axis represents the average relative activity adjusted to a percentage of the best amino acid.

Table 1
Kinetic parameters for WT-SPD-FSAP substrates. The results are presented as mean value with SD of at least three independent experiments.

Substrate sequence	K_M, μM	k_{cat}, s^{-1}	k_{cat}/K_M, $M^{-1} s^{-1}$
Ac-Hyp(Bzl)-*D*Tyr-Lys-Arg-ACC	28.71 ± 0.66	1.081 ± 0.042	37,652 ± 2259
Ac-Pro-*D*Tyr-Lys-Arg-ACC	22.11 ± 1.09	1.217 ± 0.041	55,045 ± 896
Ac-Pro-Lys-Nle-Arg-ACC	71.46 ± 0.63	1.079 ± 0.058	15,106 ± 714
Ac-Ala-Lys-Nle-Arg-ACC	117.47 ± 5.71	1.033 ± 0.041	8806 ± 331
Ac-Ala-Leu-Lys-Arg-ACC	93.28 ± 1.97	0.054 ± 0.001	576 ± 19

Table 2
Selectivity of Ac-Pro-*D*Tyr-Lys-Arg-ACC toward selected enzymes from the coagulation and fibrinolysis pathway and plasma FSAP. The results are presented as mean value with SD of at least three independent experiments.

Enzyme	K_M, μM	k_{cat}, s^{-1}	k_{cat}/K_M, $M^{-1} s^{-1}$
FSAP	55 ± 3	1 ± 0.08	17,833 ± 533
tPA	208 ± 15	0.93 ± 0.08	4471 ± 117
FXIIa	–	–	–
Plasmin	1087 ± 135	1.63 ± 0.17	1500 ± 117
FXa	–	–	–
Kallikrein	682 ± 33	0.55 ± 0.05	806 ± 117
Thrombin	–	–	–
uPA	–	–	–

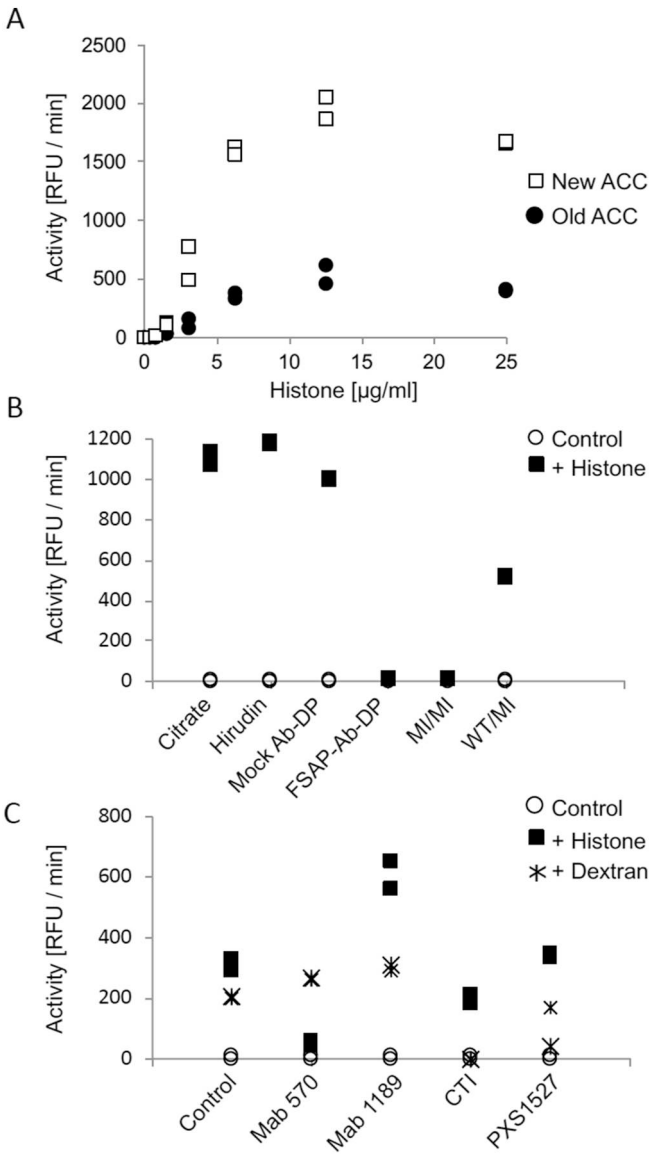

Fig. 2. Use of the substrate to characterize pro-FSAP activation in plasma: (A) Citrate plasma was used at 1:5 dilution and histones (20 µg/mL) were added followed by the 1st generation substrate (Ac-Ala-Lys-Nle-Arg-ACC, 20 µM) and the 2nd generation substrate (Ac-Pro-*D*Tyr-Lys-Arg-ACC, 20 µM). Fluorescence was measured for every min for 60 min at 37 °C and maximal velocity was calculated as RFU/min. (B) Conditions were as above in (A) except that citrate plasma was compared to hirudin plasma. WT-FSAP-deficient citrate plasma (FSAP-Ab-DP) was prepared by adsorption over an FSAP-Mab column and compared to the same plasma passed over a control Mab column (Mock-Ab-DP). These were compared to citrate plasma from a donor with WT/MI or MI/MI genotype. (C) Activation of pro-FSAP in hirudin plasma by histone (25 µg/mL) and dextran sulphate (20 µg/mL) was determined in the presence of a blocking monoclonal antibody against FSAP (Mab 570), non blocking anti-FSAP Mab (Mab 1189) (20 µg/mL each), CTI (50 µg/mL) or PKSI-527 (5 µM). Results are shown as maximal velocity (RFU/min). Experiments were performed in duplicate and each individual value is shown and similar results were obtained in three independent experiments.

design activity-based probes for FSAPa. As a warhead we used diphenyl phosphonate, which is selective toward serine proteases and forms a stable covalent complex with the active enzyme and inhibits it. As detection tags we have used Cy5 and Biotin, which allow for enzyme-probe detection in the assay. In order to eliminate the influence of the Cy5/Biotin group on the tetrapeptide recognition sequence, we have introduced 6-aminohexanoic acid as a spacer. Activity-based probes

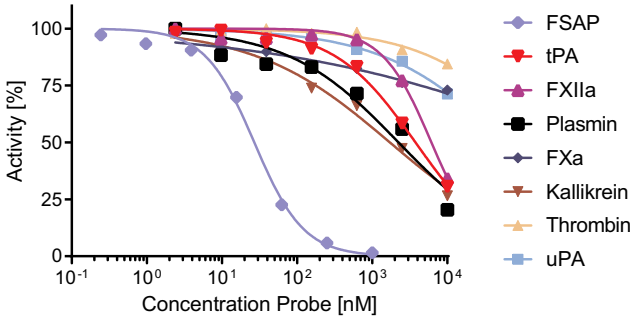

Fig. 3. Determination of IC_{50} value for biotinylated FSAP probe against purified enzymes from the coagulation and fibrinolysis pathway and their comparison to plasma FSAP. The enzymes (20 nM) were incubated with the biotinylated probe (0–10 µM) for 30 min at 37 °C and then their respective chromogenic substrate was added to determine residual enzymatic activity. The enzymes showed robust activity against their respective substrate.

Cy5-6-Ahx-Pro-*D*Tyr-Lys-$Arg^P(OPh)_2$ and Biotin-6-Ahx-Pro-*D*Tyr-Lys-$Arg^P(OPh)_2$ were synthesized using mixed synthetic protocol by solid and solution phase approach. For biotinylated probe we determined the k_{obs}(app)/I value under pseudo first-order conditions and calculated substrate-independent value k_{obs}/I ($k_{obs}/I = k_{obs}(app)/I_* (1 + ([S]/K_M))$, where [S] was substrate concentration used in the inhibition assay and K_M was the Michaelis-Menten constant of that substrate). The value is equal to 297,465 ± 16,328 $M^{-1} s^{-1}$.

To determine probe selectivity we measured inhibitory potencies of biotinylated FSAP probe toward purified enzymes from the coagulation and fibrinolysis pathway (Fig. 3). The kinetic analysis revealed very good potency and selectivity of biotinylated probe toward inhibition of FSAP (Table 3).

3.4. FSAPa labelling with Cy5-6-Ahx-Pro-DTyr-Lys-$Arg^P(OPh)_2$

Since the probe was developed with recombinant WT-SPD-FSAP as a target protease we first compared the reactivity of the probe to this protein as well as the inactive MI-isoform. Time course analysis showed that the probe reacted quite rapidly within 2 min but there was a sustained increase in fluorescence over 60 min (Fig. 4A). WT-SPD was tested in a concentration-dependent manner for 30 min (Fig. 4B). The active WT-SPD could be detected with the probe in the low nM range. The probe was also tested against MI-SPD, which showed no binding indicating that the active site of this isoform is incorrectly folded as suggested earlier [32] (Fig. 4C).

The cross-reactivity of the probe with other relevant serine proteases from plasma was analyzed by SDS-PAGE. At different probe concentrations (50 and 200 nM) as well as 30 and 90 min time points, there was strong labelling of plasma FSAP but little detectable cross reactivity with any other protease (Fig. 5). At longer incubation times

Table 3
Calculated IC_{50} value of biotinylated probe toward enzymes from the coagulation and fibrinolysis pathway. FSAP refers to plasma FSAP and along with plasmin was prepared in house whereas other enzymes were obtained from commercial sources.

Enzyme	Average IC_{50} (nM) ± SD	Residual activity (%) at 10 µM probe ± SD
FSAP	38 ± 15	0
uPA	> 3000	81 ± 8
Thrombin	> 3000	86 ± 2
Kallikrein	> 3000	44 ± 11
FXa	> 3000	74 ± 2
Plasmin	> 3000	34 ± 9
FXIIa	> 3000	33 ± 1
tPA	> 3000	38 ± 7

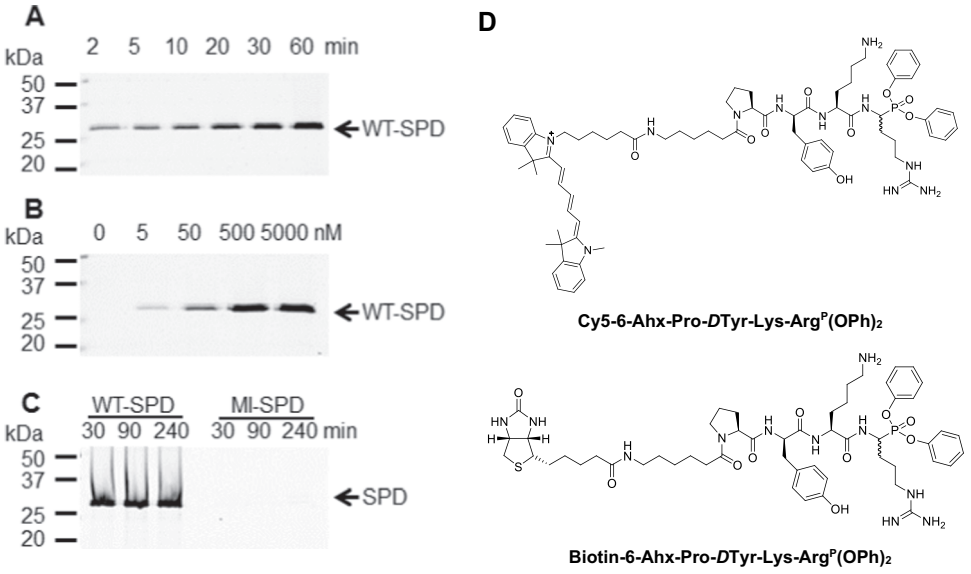

Fig. 4. Characterization of fluorescence labelling of recombinant WT- and MI-SPD-FSAP: (A) The probe Cy5–6-Ahx-Pro-*D*Tyr-Lys-$Arg^{P}(OPh)_2$ (500 nM) was incubated with WT-SPD 500 nM for various time points between 2 and 60 min. (B) WT-SPD at 500 nM was incubated with the probe Cy5–6-Ahx-Pro-*D*Tyr-Lys-$Arg^{P}(OPh)_2$ (0–5000 nM) for 60 min. (C) WT- and MI-SPD of FSAP was labelled from 30 to 240 min with the probe Cy5–6-Ahx-Pro-*D*Tyr-Lys-$Arg^{P}(OPh)_2$. After SDS PAGE under non-reducing conditions the gels were washed and an image at (700 nm) wavelength was obtained using a Licor Odyssey imaging system. Similar results were obtained in 3 independent replicates of the experiment. (D) Structure of the activity-based probes.

and higher protease concentrations, some cross reactivity with kallikrein, plasmin and FXIIa was observed (Fig. 5). This result confirms that the probe labels FSAP with high specificity in line with the inhibition results shown earlier.

3.5. Labelling of active FSAP in plasma using in-gel fluorescence

Addition of increasing amounts of histones to plasma led to the labelling of a 64 kDa protein which corresponds to the MW of intact two-chain FSAPa (Fig. 6A). On the reducing gels only the protease domain at 28kDa was detected as the heavy chain is separated (Fig. 6A, complete gels are shown in Supplementary Fig. S3). Western blotting and Coomassie staining of gels showed the equal presence of proteins and immunoreactive FSAP in all samples (Supplementary Fig. S3). Western blotting also confirmed the activation of FSAP in the plasma with histones which led to an increase in a 150 kDa MW band that presumably represents FSAP-inhibitor complexes. A subject homozygous for the MI genotype showed no increase in labelling in the presence of histones (Fig. 6A). The same was the case when normal plasma was depleted of FSAP on an FSAP-antibody column (Fig. 6B). Addition of aprotinin, which inhibits FSAPa, also prevented the labelling of FSAPa by the probe. Some non-specific staining of higher and lower MW bands was also observed under non-reducing conditions but this pattern did not alter in the presence of histones, or in the presence of aprotinin or upon depletion of plasma FSAP. Taken together these results indicate that the probe can bind to FSAPa in plasma in a selective manner.

4. Discussion

The proteolytic activity of circulating plasma serine proteases is mainly regulated through the zymogen activation mechanism as well as through their inhibition by the abundance of inhibitors. Thus, the mere presence of immunoreactive protease is not indicative of its function. The ability to measure enzyme activity in a selective and sensitive manner is important for the delineation of protease function *in vivo*. Recent studies on FSAP point to a unique mechanism of activation by histones [9] and positively charged surfaces [10]. However, detection of active FSAP *in situ* has not yet been attempted because of the lack of appropriate tools. In the present study we have used HyCoSuL approach to determine optimal natural and unnatural amino acids recognized in the S4-S2 pockets. In the first step we have determined individual preferences in each pocket. The data obtained for natural amino acids are well overlapping with these published recently using PS-SCL

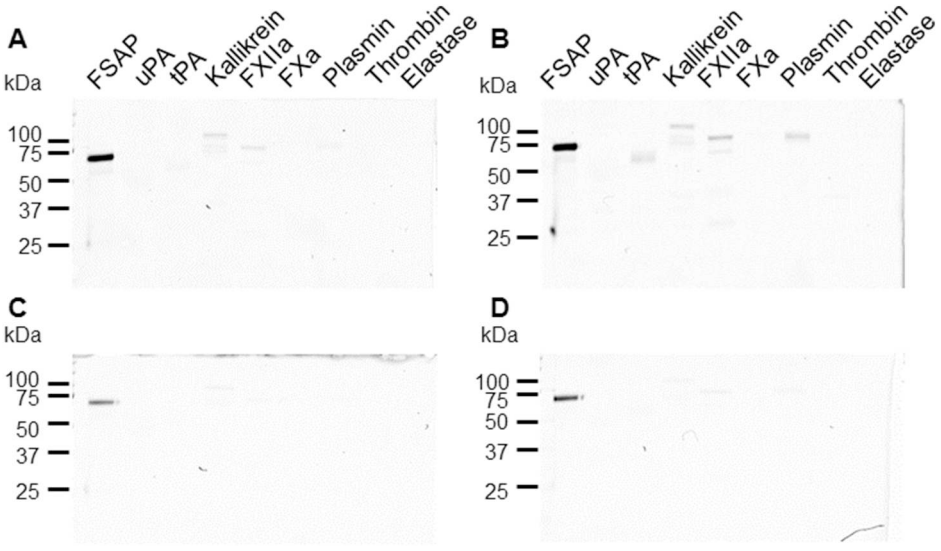

Fig. 5. Comparison of different plasma serine proteases: The probe was incubated with 100 nM each of plasma FSAP, uPA, tPA, kallikrein, FXIIa, FXa, plasmin, thrombin and elastase under 4 different sets of conditions. (A) 200 nM probe 30 min incubation; (B) 200 nM probe 90 min incubation; (C) 50 nM probe 30 min incubation; (D) 50 nM probe 90 min incubation. Similar results were obtained in 3 independent replicates of the experiment.

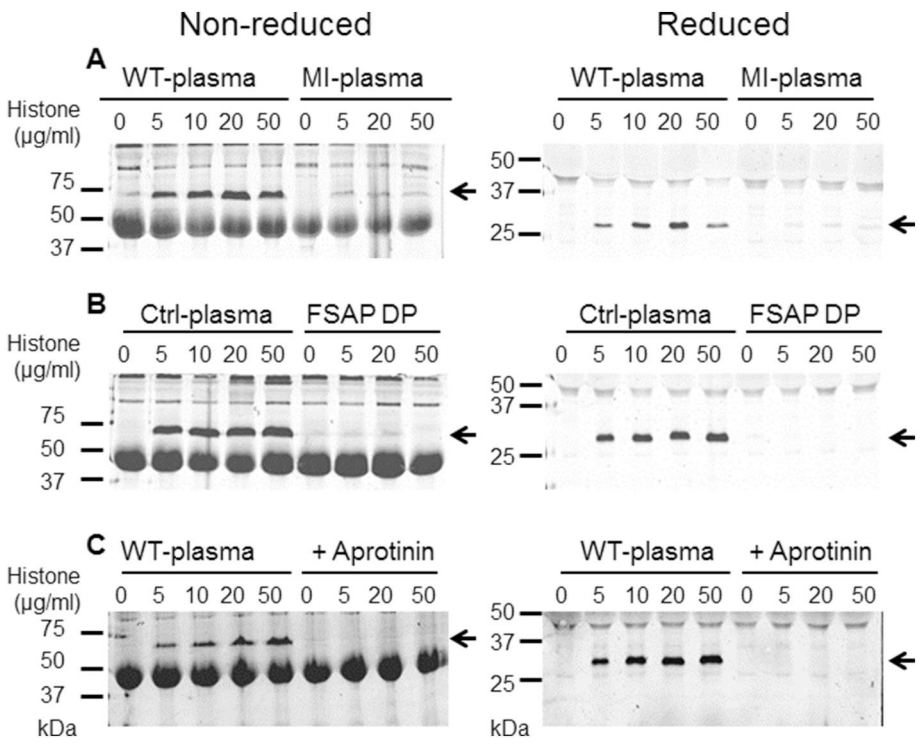

Fig. 6. Measurement of FSAP activation in the plasma: (A) Normal human plasma or plasma from a subject homozygous for the MI-SNP (1:5 dilution) was incubated with histones (0, 5, 10, 20, 50 μg/mL) and the probe (500 nM) was added for 1 h at 37 °C. SDS PAGE was run under non-reducing conditions (left panel) or reducing conditions (right panel). (B) The same conditions as above except that FSAP-deficient human plasma, which was prepared by adsorption over an FSAP-antibody column, and control plasma which was prepared in an identical manner but without any antibody. (C) Same as above except that normal human plasma was tested without and with aprotinin (50 μg/mL). Arrows indicate positions of two-chain FSAPa at 64 kDa under non-reducing conditions and the protease domain of FSAPa at 28 kDa under reducing conditions. Similar results were obtained in 3 independent experiments.

approach and phage display [22]. However, unnatural amino acids can potentially increase the activity and specificity of peptidic recognition epitopes. Especially interesting was the observation that selected D enantiomers of amino acids were recognized by FSAPa in the P3 pocket. *D*-Tyr in substrate sequence significantly increased activity of the fluorogenic substrate comparing to a sequence with natural amino acids only. This strategy was recently successfully used to design a selective legumain activity-based probe [24]. The 2nd generation substrate developed in this study has a 6-fold higher catalytic activity toward FSAP compared to the 1st generation substrate. This is a tremendous advantage when determining the activity of FSAP in plasma or cell supernatants. Moreover, the 2nd generation substrate exceeds the earlier one in its selectivity for FSAP. The 1st generation substrate had a disadvantage that it was cleaved quite strongly by the contact pathway proteases and this is not observed in the current substrate. The minimal substrate turnover in dextran sulphate plasma can be easily inhibited by CTI. This selectivity for FSAP was consolidated with studies on purified plasma enzymes as well as through the use of inhibitors for different plasma enzymes. For substrate Ac-Pro-*D*Tyr-Lys-Arg-ACC there was a 2-fold higher K_M for plasma purified FSAP compared to recombinant protease domain of FSAP for reasons which are not clear.

Based on the same core recognition sequence a fluorescent activity-based probe Cy5-6-Ahx-Pro-*D*Tyr-Lys-$Arg^P(OPh)_2$ for FSAP was designed. Specificity studies with recombinant SPD of FSAP showed that the probe only bound to WT-SPD but not the inactive MI-isoform. The lack of probe binding to MI-SPD and MI-FSAP in plasma indicates that the active site of the MI-isoform is not correctly formed as suggested earlier [32]. There was no labelling of FSAP in samples not activated with histones indicating that there is no active FSAP in normal human plasma. The fact that the probe binds to active FSAP leads to the presumption that there is free active FSAP that is not bound to plasma inhibitors under these conditions. Thus the probe could label recombinant protease domain of FSAP, plasma-purified FSAP and activated FSAP in human plasma. Of the other proteases tested only FXIIa, plasmin and plasma kallikrein showed some reactivity with the probe. The same pattern was observed when a biotinylated form of the probe was used. The pattern of FSAP activation in plasma and in the presence of inhibitors as well as plasma from MI-homozygous subject indicates that FSAP labelling by the probe in plasma is very specific.

Although natural inhibitors of FSAP have been described such as antithrombin [33], C1-inhibitor [12], plasminogen activator inhibitor-1 [20] and tissue factor pathway inhibitor [34] no systematic screens and structure function relationships have been determined. The probe developed here can be converted into a suicide substrate to inhibit FSAPa effectively as shown with the biotinylated form of the probe.

Taken together, in this study we have designed the first activity-based probe to detect FSAP activity *in situ*. Further modifications of the probe are needed to develop quenched activity-based probes which will allow a real time analysis of FSAP activity in live *in vitro* and *in vivo* imaging experiments. Recent studies have indicated that aberrant FSAP expression is found in lung cancer where it promotes development of metastasis [35] or in thyroid cancers where it promotes tumorigenic transformation of cells and promotes cell migration [8]. Immunoreactive FSAP is present in human coronary thrombi [13] and also in coronary atherosclerotic plaques [36]. In this context the measurement of the *in situ* activity of FSAP can be used to increase the understanding of the role of FSAP in cancer, thrombosis and atherosclerosis.

Abbreviations

ABP	activity-based probe
ACC	7-amino-4-carbamoylmethylcoumarin
AcOH	acetic acid
collidine	2,4,6-trimethylpyridine
CTI	corn trypsin inhibitor
DCM	dichloromethane
DICI	diisopropylcarbodiimide
DIPEA	N,N-diisopropylethylamine
DMF	*N,N′*-dimethylformamide
DS	dextran sulphate
HATU	2-(1-H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluranium hexafluorophosphate methanaminium
HBTU	O-benzotriazole-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate
HOBt	N-hydroxybenzotriazole
HyCoSuL	Hybrid Combinatorial Substrate Library

PS-SCL	Positional Scanning Substrate Combinatorial Library
RFU	relative fluorescence unit
TFA	trifluoroacetic acid
TIPS	triisopropylsilane

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Declaration of competing interest

The authors have no conflict of interest.

Author contributions

WR and JC carried out substrate specificity profiling using HyCoSuL, analyzed data, synthesized fluorogenic substrates and measured enzyme kinetics, synthesized and characterized activity-based probe. NVN expressed recombinant proteins and did all the labelling studies with proteins and plasma as well as all the kinetic studies with enzymes and plasma. MP contributed combinatorial library. MD and SMK designed the study acquired the funding, analyzed the data and wrote the MS. All authors have edited the MS.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2019.08.016.

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