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The development and validation of a combined kinetic fluorometric activity assay for fibroblast activation protein alpha and prolyl oligopeptidase in plasma

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ABSTRACT

Background: Fibroblast activiation protein alpha (FAP) is considered a diagnostic and prognostic biomarker for various types of cancer. FAP shares substrate specificity with prolyl oligopeptidase (PREP), studied in (neuro) inflammation and neurodegeneration as well as cancer. Current assays inadequately discriminate between FAP and PREP and there is need for an assay that reliably quantitates the FAP/PREP activity ratio in plasma.
Methods: FAP and PREP activities were measured in human EDTA-plasma in presence of well characterized PREP and FAP inhibitors.
Results: A combined kinetic assay was developed in conditions to optimally measure FAP as well as PREP activity with *Z*-Gly-Pro-AMC as substrate. Limit of detection was 0.009 U/L and limit of quantitation was 0.027 U/L for the combined FAP-PREP assay. Within-run coefficient of variation was 3% and 4% and between-run precision was 7% and 12% for PREP and FAP, respectively. Accuracy was demonstrated by comparison with established end-point assays. Hemolysis interferes with the assay with 1.5 g/L hemoglobin as cut-off value. PREP (but not FAP) activity can increase upon lysis of platelets and red blood cells during sample preparation.
Conclusion: With this new assay, on average 67% of the *Z*-Gly-Pro-AMC converting activity in plasma can be attributed to FAP.

1. Introduction

Fibroblast activation protein alpha (FAP, EC 3.4.21.B28), is a proline-specific serine protease that exhibits endopeptidase activity as well as dipeptidyl peptidase activity. It is mostly found as a homodimeric transmembrane glycoprotein which is expressed by activated fibroblasts in the stroma of various epithelial cancers, mesenchymal tumors, as well as during wound repair [1]. In 2006 Lee et al. identified antiplasmin cleaving enzyme as a soluble form of FAP (sFAP), which circulates in the blood [2] and the melanoma-membrane-bound seprase was also identified as FAP [3].

From that moment on numerous studies investigated FAP as putative biomarker for several types of cancer and their corresponding prognosis [4–6], for arterial thrombosis [7–11] and liver fibrosis [12]. Low sFAP levels were found in plasma of cancer patients and sFAP was inversely correlated with survival in most types of cancer [6]. In a same way, low sFAP levels were associated with events of arterial thrombosis [8–11] and the occurrence of liver cirrhosis [7].

Prolyl oligopeptidase (PREP, EC 3.4.21.26) is a serine protease that cleaves relatively short peptides after a proline residue. Thus it exhibits, comparable to FAP, a proline-specific endopeptidase activity. PREP is present in all organs. In the brain, PREP is localized in specific cells and cell layers across the brain and peripheral tissues. PREP has been associated to several neurological processes. Additionally, there is evidence suggesting that PREP participates in an inflammatory response through the modulation of active peptides, reviewed in [13].

Abbreviations: AMC, 7-Amino-4-methylcoumarin; DMSO, Dimethylsulfoxide; DTT, dithiothreitol; ELISA, enzyme linked immunosorbent assay; EDTA, ethylenediaminetetraacetic acid; FAP, fibroblast activation prothein alpha; Hb, hemoglobin; LOD, platelet free plasma, limit of detection; LOQ, limit of quatitation; PFP, platelet free plasma; PPP, platelet poor plasma; PRP, platelet rich plasma; PREP, prolyl oligopeptidase; RBC, red blood cell(s); sFAP, soluble FAP; Tris, Tris(hydroxymethyl)aminomethane

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Because PREP, like FAP, appears as a circulating form in plasma [14], it has been repeatedly investigated as putative biomarker for several neurological and inflammatory diseases [15–23]. Low levels of circulating PREP were associated with depression [15], multiple sclerosis [18] and liver cirrhosis [22]. In contrast, PREP levels were found to be significantly higher in plasma of patients with colorectal cancer [21].

Most studies quantified the FAP antigen using enzyme linked immunosorbent assays (ELISA), others used an activity based method. PREP has mostly been quantified using activity measurements. However, among the activity based assays there is a large heterogeneity and some of them have disadvantages. Firstly, some of the FAP assays use an in-house developed selective substrate, which is not commercially available [24,25]. Secondly, most PREP assays are not enzyme specific as they measure total post-prolyl endopeptidase activity and do not take into account that a significant fraction of this activity must be attributed to FAP [16,17]. Moreover, a few reported PREP assays do not contain reducing agents like dithiothreitol (DTT) [18], which is known to be necessary for PREP to be active [26,27]. Finally, in some cases, FAP or PREP activity is measured using an endpoint method [9,14], which is more labor intensive than a kinetic method.

Since sFAP and PREP are both prolyl-specific endopeptidases circulating in plasma, it is not only advisable but also interesting from a functional point of view to study them together as putative biomarkers in future studies. Therefore we developed and validated a combined FAP-PREP kinetic fluorogenic activity assay.

2. Material and methods

2.1. Chemicals

Tris(hydroxymethyl)aminomethane ACS 99.8–100.1% (Tris) was from Alfa Aesar (Karlsruhe, Germany). Dimethylsulfoxide (DMSO) for molecular biology, ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate ACS 99–101% and NaF were from Sigma-Aldrich (St. Louis, MO, USA). DTT was from Thermo Scientific (Waltham, MA, USA), methanol 99.98% from Carl Roth (Karlsruhe, Germany) and salicylic acid from Merck (Darmstatt, Germany). The substrate *Z*-Gly-Pro-AMC and the 7-Amino-4-methylcoumarin (AMC) standard were obtained from Bachem Feinchemikalien (Bübendorf, Switzerland). The PREP inhibitor KYP-2047 and FAP inhibitors UAMC-1110 and UAMC-1063 were synthesized at the Laboratory of Medicinal Chemistry at the University of Antwerp as published [28,29]. The 4.6 mM stock solution of *Z*-Gly-Pro-AMC was made in 100% DMSO and the 6.9 mM stock solution of Z-Gly-Pro-AMC was made in 100% methanol. Stock solutions of 100 mM KYP-2047, UAMC-1110 and UAMC-1063 were made in 100% DMSO. Stock solutions were aliquoted and stored at −20 °C. Immediately before use in the activity assays, the inhibitor stock solutions were diluted in assay buffer.

2.2. Plasma sample preparation

The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the University of Antwerp ethics committee (Belgian registration number: B300201214328). All healthy individuals gave their written informed consent. Peripheral venous blood was drawn into pre-chilled EDTA collection tubes (Greiner Bio-One, Vilvoorde, Belgium) and quickly mixed by inversion. The plasma was separated from blood cells within 90 min by centrifugation at 4 °C, 1200 ×*g* for 15 min, and the supernatant was aliquoted and stored frozen at −80 °C.

2.3. Platelet free, poor and rich plasma

EDTA blood samples were centrifuged for 15 min at 200 ×*g* to obtain platelet-rich plasma (PRP), at 2000 ×*g* to obtain platelet-poor plasma (PPP) and at 10,000 ×*g* to obtain platelet-free plasma (PFP). Centrifugation was performed at 4 °C and aliquots were stored at −80 °C.

2.4. Hemolysis

Red blood cells (RBC) were lysed by adding 4 volumes of cold distilled water followed by 30 min centrifugation at 2000 ×*g*, 4 °C. Free hemoglobin (Hb) was measured in RBC lysate by its absorbance at 576 nm (extinction coefficient = 55,540 $cm^{-1} M^{-1}$). RBC lysate was diluted in plasma to final concentrations from 0.6 to 15 g/L Hb.

2.5. Assay conditions for the kinetic measurements

Enzymatic activity was determined kinetically in a 96-well plate (half area, flat bottom, Greiner Bio-One, Vilvoorde, Belgium) measuring the initial velocities of AMC release (λ_{ex} = 380 nm, λ_{em} = 465 nm) from the substrate, using a Tecan microtiter plate reader. First, 5 µL of plasma sample was pre-incubated with 10 µL of 250 nM FAP inhibitor, 10 µL of 250 nM PREP inhibitor or 10 µL of 0.0025% (*v*/v) DMSO for 15 min at 37 °C. Next, 35 µL pre-heated *Z*-Gly-Pro-AMC (380 µM diluted in buffer) was added to obtain a final concentration of 266 µM and fluorescence was measured kinetically for 30 min at 37 °C. Fluorescence intensity was related to an AMC standard curve in the same buffer. One unit of enzymatic activity is the amount of enzyme that catalyzed the release of 1 µmol AMC from the substrate per minute under assay conditions. The following assay buffers were used: PREP assay buffer (100 mM potassium phosphate, pH 7.7, 1 mM EDTA), FAP assay buffer (100 mM Tris-HCl, pH 8, 300 mM NaF, 1 mM EDTA, 50 mM salicylic acid) and Tris buffer (100 mM Tris-HCl, pH 8, 1 mM EDTA). Except when indicated otherwise (see Results), 5 mM DTT was added to the assay buffers.

2.6. Assay conditions for the endpoint measurements

2.6.1. FAP activity

Standards and plasma samples were diluted 26-fold in FAP assay buffer. 150 µL of diluted samples was mixed with 6 µL *Z*-Gly-Pro-AMC substrate (6.9 mM in 100% methanol) and incubated for 120 min at 37 °C in a warm water bath. Reactions were stopped by adding 500 µL of 1.5 M acetic acid. Next, 500 µL of this mixture was mixed with 2 mL of deionized water and the fluorescence was measured (λ_{ex} = 380 nm, λ_{em} = 465 nm). Fluorescence intensity was related to an AMC standard curve in the same assay conditions.

2.6.2. PREP activity

20 µL plasma sample was pre-incubated for 15 min with 100 µL PREP assay buffer at 37 °C in a warm water bath. The reaction was started by the addition of 6 µL *Z*-Gly-Pro AMC substrate (4.6 mM in 100% DMSO). After 120 min the reaction was stopped by the addition of 500 µL 1.5 M acetic acid. Next, 500 µL of this mixture was mixed with 2 mL of deionized water and the fluorescence was measured (λ_{ex} = 380 nm, λ_{em} = 465 nm). Fluorescence intensity was related to an AMC standard curve in the same assay conditions.

2.7. FAP and PREP antigen quantification

FAP antigen concentration was determined in plasma samples using the Meso Scale discovery R-plex immuno-assay technology (Meso Scale Diagnostics, Rockville, Maryland, USA), according to the manufacturer's instructions. Competitive PREP ELISA kits were purchased from MyBioSource (San Diego, California, USA) and Gentaur Belgium (Kampenhout Belgium) and used according to the manufacturers instructions.

2.8. Validation of the assay

Validation of the combined FAP-PREP activity assay was conducted according to the guidelines of the International Committee on Harmonization [30]. The method was validated for linearity, accuracy, specificity, intra-and inter-day precision, lower limit of detection and lower limit of quantitation. Statistical tests were performed using SPSS version 23.

3. Results

3.1. Assay development

3.1.1. Definition of measured activities

In the combined FAP-PREP fluorescent activity assay the following activities will be measured: (1) FAP activity by the addition of a specific PREP inhibitor (50 nM KYP-2047), (2) PREP activity by the addition of a specific FAP inhibitor (50 nM UAMC-1110 or UAMC-1063) and, (3) the 'total activity' in absence of any inhibitor, which represents both FAP and PREP activity.

3.1.2. Buffer composition

To combine these measurements in one single activity assay we selected an assay buffer in which both FAP and PREP activity could be measured optimally. Three different buffers were screened: (1) a buffer previously used for PREP activity assays (PREP buffer) [14], (2) a buffer previously used for FAP activity assays (FAP buffer) [9] and (3) Tris-buffer. The final choice used for the validation of the assay was the FAP buffer supplemented with DTT: 0.1 M Tris-HCl buffer, pH 8, 300 mM NaF, 1 mM EDTA, 50 mM salicylic acid and 5 mM DTT.

3.1.3. Effect of DTT

Activities were tested in presence or absence of 5 mM DTT for FAP and PREP buffer. As already described before, the addition of DTT is necessary to measure PREP activity [26,27]. Therefore, we analyzed the effect of DTT on FAP activity. We demonstrated that a concentration of 5 mM DTT does not affect the FAP activity in plasma (Fig. 1A and B). When DTT concentrations were higher than 10 mM, FAP activity decreased. In further measurements, a concentration of 5 mM DTT was used in the assay buffer.

3.1.4. Effect of DMSO

The specific inhibitors for FAP and PREP are stored in a DMSO stock solution. In the final assay conditions there is still residual DMSO from these stock solutions. Therefore, the effect of DMSO on FAP and PREP activity was analyzed. DMSO causes a decrease in both FAP and PREP activity, however the effect on PREP activity is only seen at concentrations higher than 1%, FAP activity decreases already at concentration of 0.001% DMSO. The concentration of 0.0005% DMSO that we use in the combined assay does not affect PREP or FAP activity (Fig. 1C and D). The substrate stock solution must not contain DMSO, but can be made in methanol.

3.2. Assay validation

3.2.1. Specificity

In this assay, the commercially available substrate *Z*-Gly-Pro-AMC that can only be cleaved by enzymes with endopeptidase activity, is used. Other members of the dipeptidyl peptidase (DPP) family, such as DPP IV, DPP II, DPP9, DPP8 or proline specific carboxypeptidase (PRCP) only have exopeptidase activity and are not able to cleave the Z-Gly-Pro-AMC substrate. To attribute the Z-Gly-Pro releasing activity exclusively to FAP or PREP, a specific PREP and FAP inhibitor was used respectively. The specificity of these inhibitors is described in detail in literature (Supplementary information) [28,29,31]. To be certain that both inhibitors are specific, the assay was performed in presence of both FAP and PREP inhibitors. The residual activity was 0.036 ± 0.006 U/L, which is negligible. In conclusion, the assay is specific for FAP and PREP.

3.2.2. Linearity

The FAP, PREP and total activity was determined for 8 plasma dilutions in the range of 0.1 to 2 U/L (Fig. 2). Linear regression analysis showed good linearity for the three measured conditions (FAP/PREP/total) with initial velocities of 0 to 600 RFU/min using the routine setting of the fluorescence reader. R^2 values were 0.9858 for FAP, 0.9930 for PREP and 0.9981 for PREP and FAP combined.

3.2.3. Sensitivity

The limit of detection (LOD) is the lowest amount of analyte that can be detected, but not measured accurately. The LOD is determined as 3.3 times the standard deviation of the fluorescence signal (RFU/min) of 10 blank solutions divided by the slope of the AMC calibration curve. The LOD for the combined FAP-PREP assay is 0.009 U/L.

The limit of quantitation (LOQ) is the lowest amount of analyte that can be measured precisely and accurately. The LOQ is determined as 10 times the standard deviation of RFU/min of 10 measured blank solutions, divided by the slope of the AMC calibration curve. The LOQ for the combined FAP-PREP assay is 0.027 U/L.

3.2.4. Precision

The within-run coefficient of variation, determined by measuring 10 plasma samples with various FAP and PREP activities several times on the same day ($n = 3$), was 3.1% for PREP and 4.4% for FAP.

The between-run coefficient of variation, determined by measuring 10 samples with various FAP and PREP activities on different days (n = 3) was 7.3% for PREP and 11.8% for FAP.

3.2.5. Accuracy

The accuracy of the combined assay was determined by comparing this newly developed assay with previously used individual endpoint assays for FAP and PREP [9,14]. Plasma samples from 25 healthy volunteers were measured for FAP and PREP activity with both the new combined kinetic assay and the old endpoint assay. The kinetic assay showed a good correlation with the endpoint assays, both for FAP and PREP. The correlation coefficient for PREP and FAP endpoint versus kinetic assays was 0.83 and 0.91 respectively (Fig. 3A and B). PREP activities measured with the endpoint method were lower than when measured with the kinetic assay; this can be explained by the different pH and composition of the assay buffer. As can be seen in Fig. 1A, PREP activity is higher in FAP assay buffer (kinetic method) than in PREP assay buffer (endpoint).

In addition, the FAP antigen was measured using the R-plex human FAPα/seprase antibody set from Meso Scale Discovery in the 25 plasma samples. FAP antigen level and FAP activity in plasma displayed a good correlation (correlation coefficient of 0.9), adding evidence that the kinetic combined FAP-PREP assay allows accurate measurement of FAP presence in plasma (Fig. 3C). The commercial competitive PREP ELISA kits did not meet reasonable requirements for between-run variation and selectivity. Therefore PREP antigen could not be correlated with PREP activity (Supplementary information).

Using the data of Fig. 3, FAP activity ranged between 51 and 92% of the total FAP-PREP activity with an average of 67%. The average FAP/PREP activity ratio was 1.95. FAP and PREP activity did not appear to be correlated.

3.3. Robustness

3.3.1. Effect of presence of platelets in plasma

FAP and PREP activity were measured in platelet rich, platelet poor and platelet free plasma. FAP activity is not influenced by the presence of platelets in the plasma, PREP activity however, is significantly

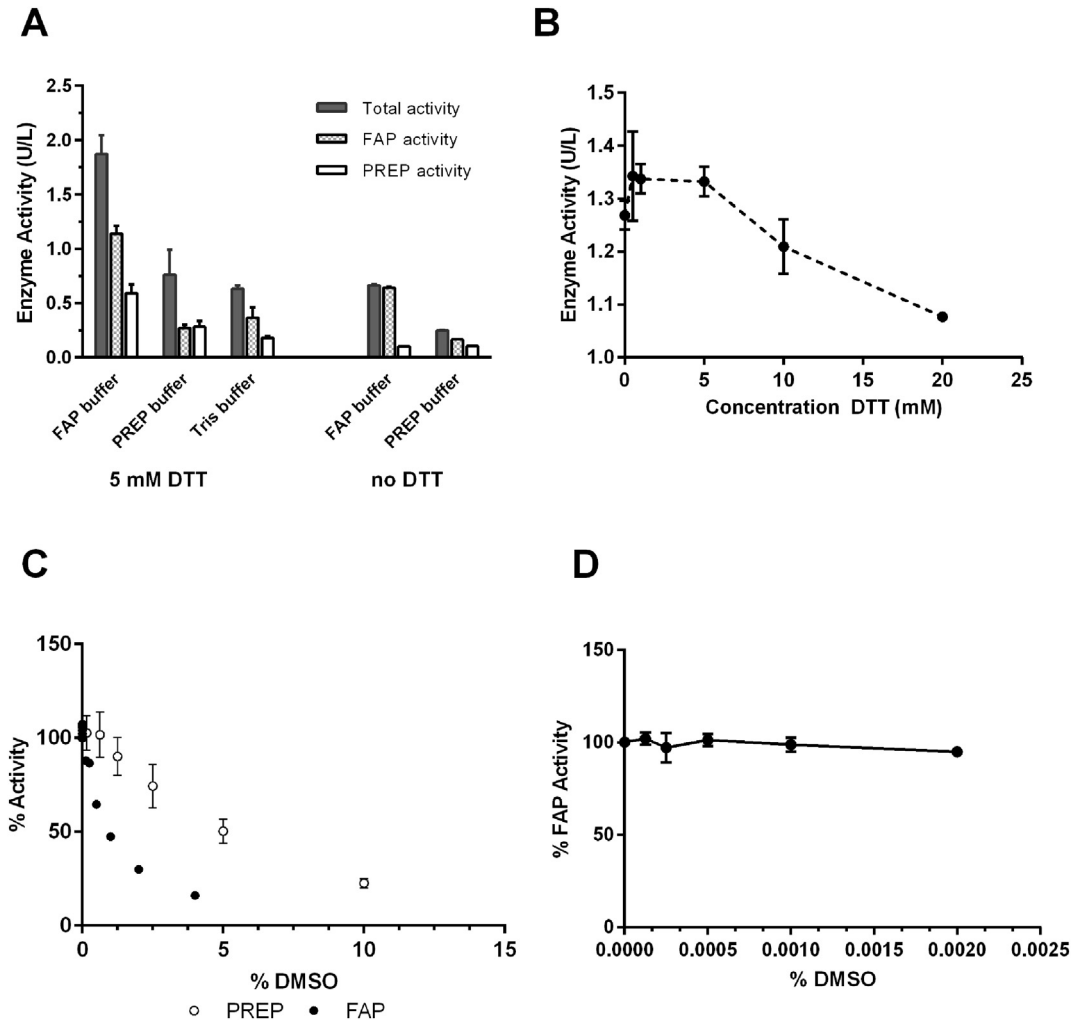

Fig. 1. (A) Plasma proline specific endopeptidase activities in different buffers, in presence and absence of 5 mM DTT. Columns represent the mean values and whiskers the SD ($n \geq 3$). (B) Effect of increasing DTT concentrations on total activity in FAP buffer. (C) Effect of DMSO on the PREP (○) and FAP (●) activity measured in FAP buffer. (D) Effect of small percentages of DMSO (0–0.0025%) on FAP activity. Symbols represent mean value and whiskers SD ($n = 2$).

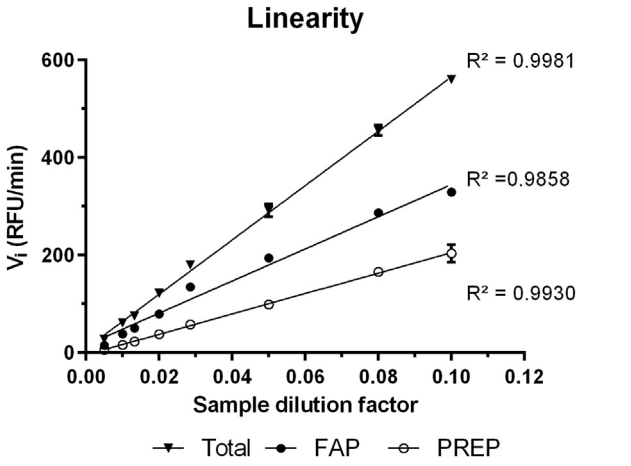

Fig. 2. Linearity of the measured initial velocity for total (▼), FAP (+ KYP-2047, ●), and PREP (+ UAMC-1063, ○) in function of sample dilution. Mean and SD are shown (n = 2). Vi is the initial rate and RFU is relative fluorescence units.

increased by the presence of platelets (Fig. 4).

3.3.2. Effect of hemolysis on FAP and PREP activity read-out

The effect of hemolysis was analyzed by the addition of lysed red blood cells to the plasma sample. The amount of lysis was expressed as the concentration of free hemoglobin, which is released when the RBC lyse.

Hb concentrations above 1.61 ± 0.09 g/L caused a decrease in the FAP signal (Fig. 5A). This can be explained by the reduction of the fluorescent signal of AMC due to the absorption of excitation/emission light by hemoglobin. The deviation of FAP activity at these Hb concentrations could be corrected via an AMC calibration in the hemolytic sample (Fig. 5B).

In contrast to FAP activity, hemolysis had a more pronounced effect on the PREP activity read-out. Increasing Hb concentrations caused a significant increase in PREP activity (Fig. 5C). This can be explained by the fact that PREP is present in RBC and that, besides Hb, hemolysis also releases PREP, which consequently leads to a rise in PREP activity, even after correction by calibration in the hemolytic sample (Fig. 5D). The threshold for accurately measuring PREP activity is 1.5 ± 0.3 g/L free Hb.

4. Discussion

Because PREP has a broad substrate specificity [13,32] and FAP is

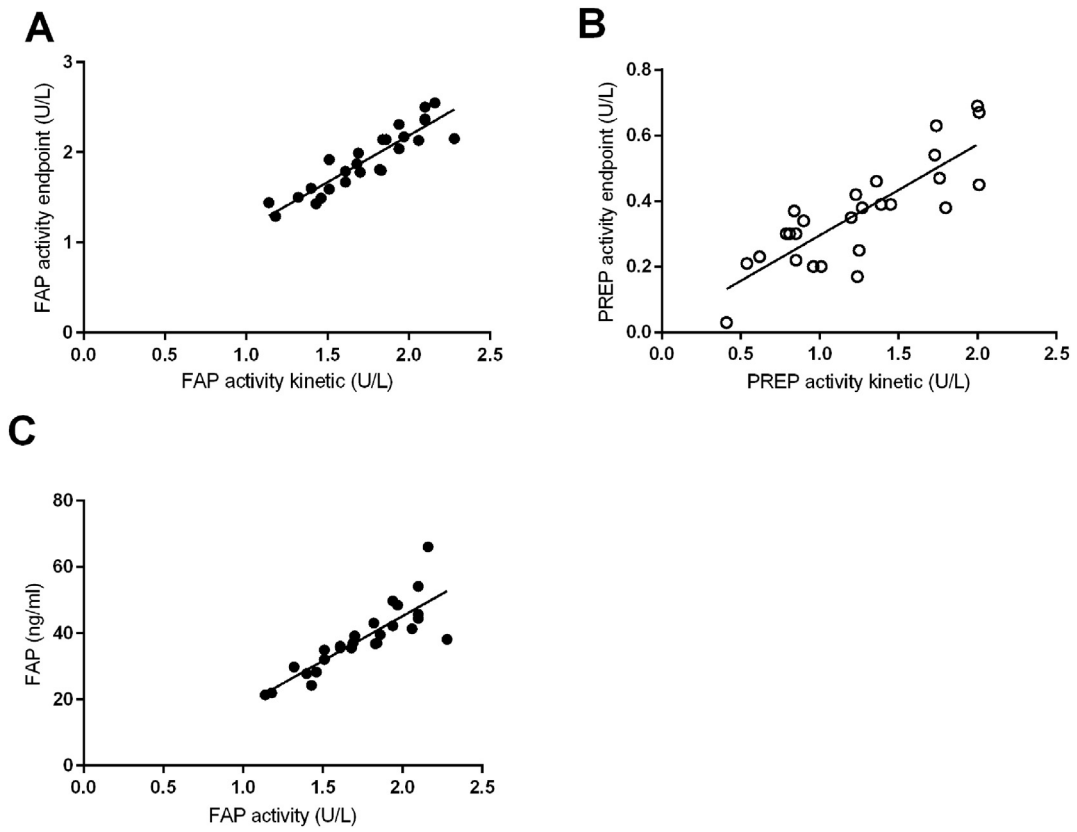

Fig. 3. Accuracy of the combined FAP/PREP assay was demonstrated by comparing FAP (A) and PREP (B) activities measured in 25 healthy volunteers by both the old endpoint method and new kinetic method. Correlation coefficient of FAP activity was 0.91, that of PREP activity 0.83. (C) Correlation between FAP antigen and FAP activity in plasma of 25 healthy volunteers. Correlation coefficient is 0.9.

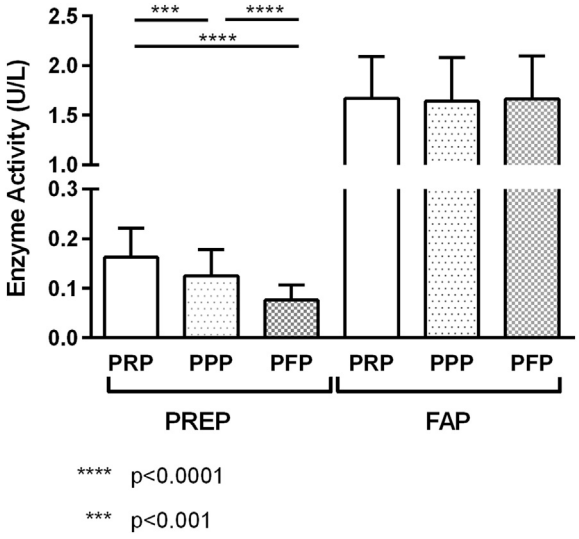

Fig. 4. PREP and FAP activity in platelet-rich (PRP), platelet-poor plasma (PPP), and platelet-free plasma (PFP) from healthy individuals ($n = 45$). In contrast to the FAP specific activity, the measured activity for PREP is significantly altered by the concentration of platelets. Averaged group values are shown with the respective SD. *** $p < .001$ and **** $p < .0001$ (repeated measures 2-way ANOVA).

more restricted to the Gly-Pro motif [24,25,28,29], it is hard to distinguish these two peptidases using readily available substrates. For PREP there are no selective substrates available that can be used in activity assays. In most published studies *Z*-Gly-Pro-AMC, [14,16,18–20] or Z-Gly-Pro-β-napthylamide [21] are used as substrate, whether or not in combination with a specific PREP inhibitor. Two research groups published the use of selective FAP substrates: one that is called 3144-AMC [24] and the other based on the natural FAP-substrate FGF21 [25]. However none of the selective FAP substrates are commercially available.

We developed and validated an activity assay where post-prolyl endopeptidase activity is measured in plasma samples by the use of the Z-Gly-Pro-AMC substrate and where selectivity for FAP or PREP is created by the use of a selective PREP or FAP inhibitor. The PREP inhibitor KYP-2047 and FAP inhibitor UAMC-1110 are commercially available. The use of inhibitors to create a selective enzyme activity assay is a trusted strategy as it is also used to discriminate the related dipeptidyl peptidases DPP IV, DPP8 and DPP9 [33]. Some research groups already used KYP-2047 to ensure the specificity of their PREP assays [19,21]. However, because experimental errors add up when 2 relatively large activities are subtracted from each other, it is more accurate to measure PREP in presence of a FAP inhibitor.

In addition, effort was taken to select the most suitable assay buffer to measure both PREP and FAP in similar conditions. The importance of the presence of DTT cannot be underestimated as PREP contains 16 cysteines with free thiol groups that need to be protected from oxidation. As can be seen in Fig. 1A, using FAP buffer without DTT, the PREP activity is minimal and the FAP activity approaches the total activity. Whereas the intracellular environment - where most of the PREP protein resides – is sufficiently reducing, oxidative processes in blood and in processed plasma samples are likely to affect the activity of PREP via oxidation of cysteine residues located on the surface and in the substrate binding site [26,27]. Many disorders (including inflammation, immune disease and cancer) wherefor FAP and PREP were investigated as biomarkers are also characterized by oxidative stress markers in the circulation. There is at least one study on relapsing-remitting multiple

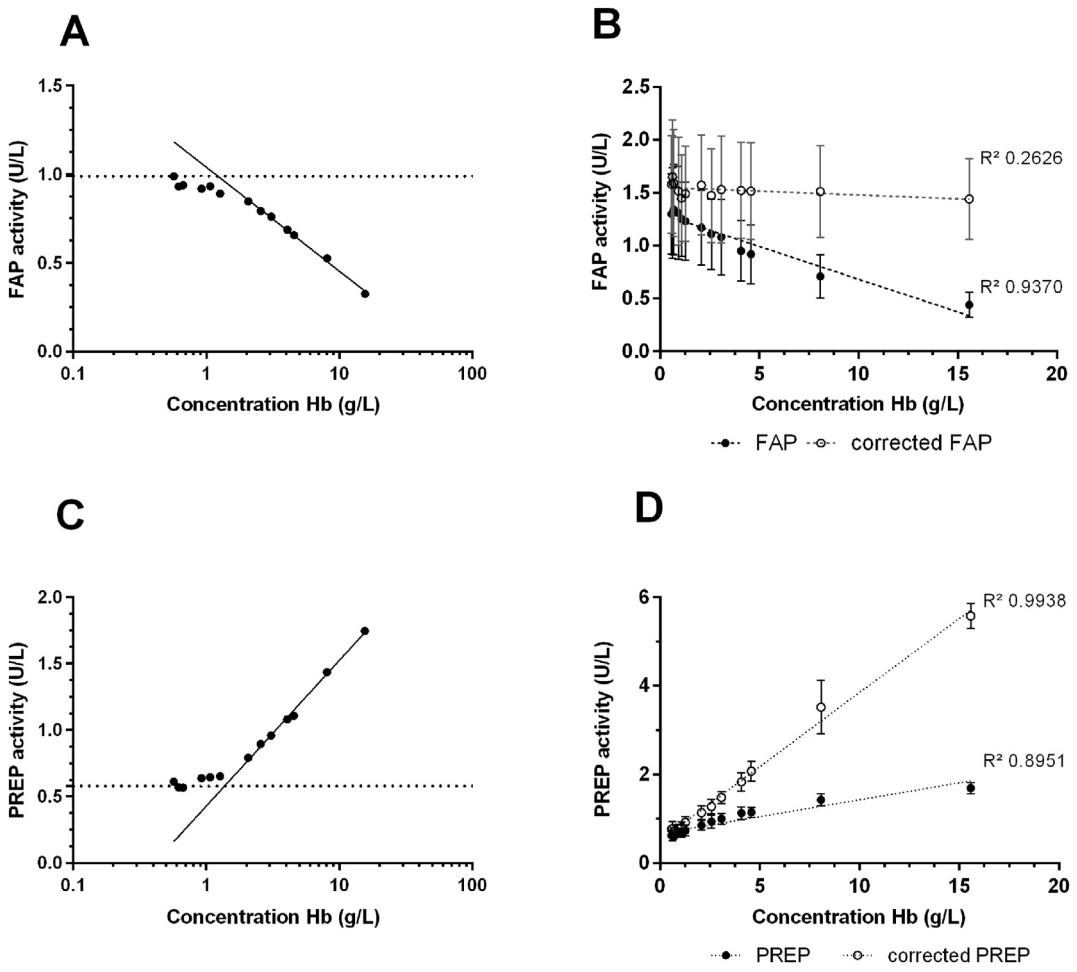

Fig. 5. (A) Effect of hemolysis on FAP activity. Lysis of RBCs is represented as concentrations of free Hb. The threshold of deviation for FAP activity is 1.61 ± 0.09 g/L Hb. (B) The deviation caused by hemolysis (●) can be corrected via an AMC calibration in the hemolytic sample (○). (C) Effect of hemolysis on PREP activity. Lysis of RBCs is represented as concentrations of free Hb. The threshold of deviation for PREP activity is 1.48 ± 0.28 g/L Hb. (D) The deviation caused by hemolysis (●) cannot be corrected via an AMC calibration in the hemolytic sample (○).

sclerosis where a significant difference in PREP activity between study groups disappeared after addition of a reducing agent in the assay [18].

A concentration of 5 mM DTT is ideal as this concentrations does not affect FAP activity. Furthermore, 50 mM salicylic acid was added to the buffer to ensure the linearity of FAP activity measurements in plasma. Salicylic acid binds to human serum albumin and may prevent an interaction between components of the reaction mixture with albumin. NaF, believed to preserve glucose levels by inhibiting glycolysis, also had a stabilizing effect on FAP activity for reasons yet to be discovered.

Both FAP and PREP are clinically interesting proteins as they are involved in several pathological processes. FAP is highly induced during inflammation, activation of hepatic stellate cells in liver cirrhosis and strongly expressed by mesenchymal cells of remodeling tissue. FAP is also a key regulator during tumor growth and metastasis [33]. PREP on the other hand is associated with neurological and neurodegenerative disorders [34,35], including neuro-inflammation [36]. Therefore, this validated combined FAP and PREP activity assay will facilitate further characterization of FAP and/or PREP as putative diagnostic, prognostic or predictive biomarkers in certain pathological disorders. However, it is important to take into account that PREP as biomarker is not without challenges, as we have seen that the presence of platelets or hemolysis will cause an increase in PREP activity. Therefore it is important to document the pre-analytical phase and to discard hemolytic samples from the analysis. This problem is less of an issue for FAP and hence FAP seems to be the better candidate biomarker compared to PREP. In general, PREP research would benefit from the development of a good immuno-assay to complement activity measurements.

In conclusion we believe that the combined assay described here is a breakthrough in the measurement of FAP as well as PREP activity in plasma samples.

Author contributions

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Disclosure

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.04.063.

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