Protein-peptide based assay for the characterization of human blood coagulation factor XIII-A isopeptidase activity

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ABSTRACT

Blood coagulation factor XIII-A (FXIII-A), a member of the transglutaminase enzyme family, is best known for its fibrin clot stabilizing function during blood coagulation. It possesses amine incorporating and protein cross-linking transamidase activities, but it is also able to cleave the previously formed isopeptide bond by its isopeptidase activity. Our aim was to develop a protein-based assay for better characterization of FXIII-A isopeptidase activity. The first attempt applying the crosslinked D-dimer of fibrin as a substrate was not successful because of poor reproducibility. Then, the principle of an earlier published anisotropy based activity assay was adapted for the measurement of FXIII-A isopeptidase activity. After crosslinking the fluorescently labelled α2-antiplasmin derived peptide and S100A4(GST) lysine donor protein, this protease-resistant γ-glutamyl-ε-lysine isopeptide bond containing protein-peptide product was applied as a substrate for FXIII-A. Using this substrate and detecting decreasing anisotropy, kinetic measurement of FXIII-A isopeptidase activity was achieved at high sensitivity even in a complex biological sample and in the presence of inhibitor.

1. Introduction

Blood coagulation factor XIII-A (fibrin stabilizing factor, FXIII-A), initially named after its discoverers as Laki-Lorand factor, is one of the first described and best-known member of the transglutaminase enzyme family. Firstly, its physiological function, stabilization of blood clots making them urea insoluble, was recognized [1] and later it was revealed that transglutaminase activity is responsible for fibrin cross-linking [2,3]. FXIII-A also plays important roles in wound healing, angiogenesis and maintaining pregnancy [4]. FXIII-A, as its best known physiological function, catalyzes the Ca^{2+}-dependent formation of γ-glutamyl-ε-lysine crosslinks within fibrin clots after thrombin activation during blood coagulation. In addition, it incorporates α2-antiplasmin, one of the main inhibitors of fibrinolysis, into the blood clot, which, as a result, becomes resistant against early fibrinolysis, suggesting the involvement of FXIII-A in the regulation of the hemostatic balance between fibrin coagulation and fibrinolysis [5]. These indispensable functions make FXIII-A inhibition a potential therapeutic target in anticoagulation therapy, resulting in facilitated fibrinolysis without increased bleeding tendency [6].

FXIII-A, similar to other transglutaminases, possesses various types of calcium-dependent transglutaminase activities. The formation of

intermolecular γ-glutamyl-ε-lysine isopeptide bonds between fibrin molecules is a typical example of FXIII-A transamidase activity. The reaction mechanism has two main steps: in the first, an enzyme-substrate intermediate is formed between a reactive glutamine residue and the enzyme catalytic thiol group (Cys314; considering the removal of the first Met) followed by ammonia release. In the second step, depending on the presence of the other possible substrates, protein-protein crosslinking, amine incorporation occurs, or in the absence of eligible amine donor substrate, deamidation can occur [7,8]. Several biogenic and synthetic primary amine molecules, can be incorporated by transamidation into glutamine residues of proteins which could be either responsible for biological functions or useful as a research tool inhibiting transglutaminase mediated crosslink formation [9,10]. Recently, it was confirmed, that FXIII-A also has deamidation activity with peptide substrates when an amine-donor substrate is not present [11].

FXIII-A, similar to tissue transglutaminase (transglutaminase 2, TG2) [12], has another type of hydrolase activity: it can reverse its transamidase activity and cleave the previously formed isopeptide bond. Ichinose and Aoki demonstrated that FXIII-A is able to crosslink α2-antiplasmin to, then release it from fibrinogen [13] and some years later they also demonstrated the release of α2-antiplasmin from plasma fibrin clot [14]. The biochemical details of this isopeptidase activity

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were described first by Lorand's laboratory using several synthetic, fluorescently labelled peptides which were crosslinked with a small quencher in an isopeptide bond mimicking fashion. The isopeptidase activity of guinea pig and human TG2 and human FXIII-A were tested kinetically by the increase of fluorescent signal [15]. In the same year, Loewy et al. also published another method to detect isopeptidase activity, applying radioisotope labelling [16].

Measuring the isopeptidase activity of FXIII-A could provide a good possibility for accurate, highly sensitive determination of FXIII-A activity in plasma, particularly in the case of FXIII-A deficient patients. Zedira Gmbh. (Darmstadt, Germany) has developed a sensitive, commercial, α2-antiplasmin derived peptide-based isopeptidase substrate [17]. The unique advantage of detecting isopeptidase activity is that there is no alternative, parallel reaction and the substrate conversion always proceeds in one direction because in the product the remaining glutamate does not react again with the enzyme. However, transglutaminases frequently act on protein substrates resulting in different reaction kinetics, which could not be predicted using peptide and small amine substrates as, for example, used in the Zedira isopeptidase assay. In addition, previously, we developed TG2 mutants which demonstrate enhanced isopeptidase activity on peptide substrates using the Zedira assay, but decreased activity in the case of protein-peptide based substrates, suggesting the need for protein-peptide based kinetic isopeptidase activity assays in the field [12].

In the current study, first, we made an attempt to apply a newly developed commercial monoclonal antibody (Zedira) against the crosslinked fibrin degradation product D-dimer for monitoring at the protein level the change of isopeptide bond content as a result of FXIII-A activity. Then, we successfully adapted our previously developed protein-peptide substrate based method which follows anisotropy change during the cleavage of isopeptide bond between a protein (S100A4(GST)) and a fluorescently labelled peptide substrate (FLpepPI2) to characterize FXIII-A isopeptidase activity.

2. Materials and methods

2.1. Materials

All materials were purchased from Sigma (St Louis, MO, USA) unless otherwise indicated. The FLpepT26 (5FAM-HQSYVDPWMLDH-NH$_2$), FLpepPI2 (5FAM-NQEQVSPLTLLK-NH$_2$) peptides were obtained from GenScript (Piscataway, NJ, USA). The FLpepT26 and FLpepPI2 peptides have a molecular mass of 1885 and 1727 Da, respectively, but generally appear in the 15% SDS-polyacrilamide gel as 14 or 12 kDa diffuse bands, respectively (Fig. 2D). GPRP peptide, an inhibitor of fibrin aggregation was purchased from Cayman Europe (Tallinn, Estonia). Recombinant commercial FXIII-A produced in insect cells (T027), fibrin degradation products (FDP; F012), cross-linked fibrin degradation

products (xFDP; F011), DD-XLink-mab (A076), FXIII-Assay substance (A101) and ZED1301 site specific irreversible inhibitor of FXIII-A (A108) were produced by Zedira (Darmstadt, Germany).

2.2. Expression and purification of proteins

For isopeptidase substrate production, N-terminal glutathione S-transferase (GST)-tagged S100A4, a member of the S100 small, Ca^{2+} binding EF-hand containing protein family (pETARA-S100A4; Uniprot code: P26447; Mw: 39,559 Da), was expressed in E. coli Rosetta 2(DE3), an E. coli BL21 derivative strain containing a vector, expressing 7 barely present eukaryotic tRNAs in bacterial cells (Novagen, Darmstadt, Germany), exactly as described in the earlier published method [12]. Human recombinant TG2 was expressed and purified also based on the earlier published method [12].

2.3. Large scale production of the crosslinked FLpepT26-S100A4(GST), FLpepPI2-S100A4(GST)

5 μM FLpepT26 or FLpepPI2 and 12.8 μM S100A4(GST) were incubated at 37 °C for 90 min in the presence of 5 nM TG2, 5 mM Ca^{2+} in 20 mM HEPES reaction buffer, pH 8.0 containing 150 mM NaCl, 5 mM dithiothreitol (DTT), 0.01% Tween 20. The reaction was stopped by the addition of 10 mM EDTA (final concentration) and FLpepT26-S100A4(GST) or FLpepPI2-S100A4(GST) with unmodified S100A4(GST) were separated from the free, unbound FLpepT26 or FLpepPI2 peptide by a centrifugal concentrator filter (Amicon ultra, 10 kDa, Millipore, Billerica, MA, USA). Then, the buffer was replaced by 20 mM MOPS buffer, pH 6.8, containing 0.5 mM EDTA, 150 mM NaCl, 5 mM DTT, 0.01% Tween-20 due to the slightly acidic pH preference of the isopeptidase activity [12]. Due to the co-purification of the crosslinked isopeptide bond containing products and S100A4(GST) their ratios were calculated based on the total protein concentration (determined by Bio-Rad Protein Assay) and its fluorescein content (absorption at 493 nm) using 79600 M^{-1} cm^{-1} as the molar extinction coefficient for fluorescein. Under optimized conditions the FLpepPI2-S100A4(GST) content was approximately 7% on average in the reaction product, meaning that in the purified mixture 5 μg of FLpepPI2-S100A4(GST) and S100A4(GST) corresponds to 259.5 nM FLpepPI2-S100A4(GST) in 35 μl of the isopeptidase assay mixture.

To exclude the potential effect of remaining TG2 in the substrate used for the formation of isopeptide bond, and uncrosslinked S100A4(GST), after separation of free peptide substrates (FLpepT26/PI2), control experiments were performed. On the one hand, in the absence of any freshly added enzyme into the reaction mixture, containing 5 mM CaCl$_2$, we were unable to detect any fluorescence polarization change compared to the EDTA and iodoacetamide control, suggesting that there is no remaining TG2 activity in the substrate. On

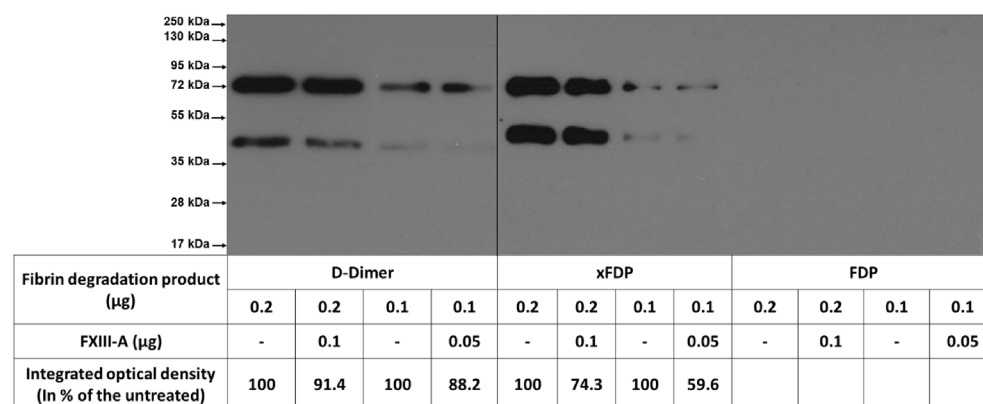| Fibrin degradation product (μg)	D-Dimer				xFDP				FDP			
	0.2	0.2	0.1	0.1	0.2	0.2	0.1	0.1	0.2	0.2	0.1	0.1
FXIII-A (μg)	-	0.1	-	0.05	-	0.1	-	0.05	-	0.1	-	0.05
Integrated optical density (In % of the untreated)	100	91.4	100	88.2	100	74.3	100	59.6				

Fig. 1. An attempt to detect reversibility of fibrin crosslinking by FXIII-A at the protein level using a crosslinked fibrin degradation product specific monoclonal antibody.
DD-Xlink-mab (Zedira) recognizes the isopeptide bond containing fibrin degradation products. Based on the manufacturer webpage the band(s) at 72 kDa represents γ-γ or γ'-γ fibrin dimers while there is no information concerning the other band around 40 kDa. After a 2 h incubation, the effect of activated FXIII-A on the level of isopeptide bond in the crosslinked fibrin degradation products was visualized on representative Western blot images. The upper band intensities were calculated by ImageJ and the values are given as a percentage of the untreated controls. Representative Western blots are presented of more than 3 independent experiments.
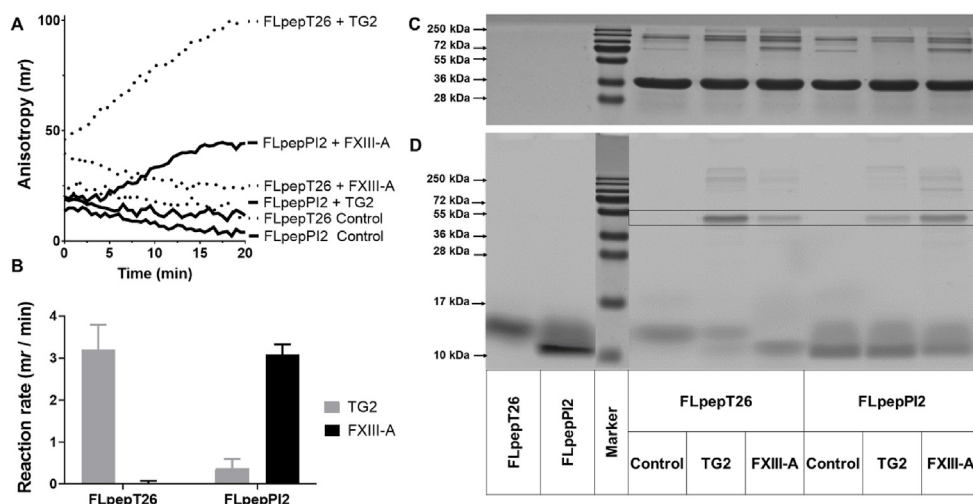
Fig. 2. Incorporation of FLpepPI2 peptide into S100A4 protein catalyzed by FXIII-A applied for kinetic detection of FXIII-A transamidase activity at the protein-peptide level.
The fluorescence polarization change was followed using BioTek Synergy H1 Green FP filter cube (A; representative picture, Ex/Em: 485/525; mirror: 510 nm) during TG2 or FXIII-A (5 nM) catalyzed transamidase reaction in the presence of 100 nM FLpepT26 or FLpepPI2 and 5 μM S100A4(GST) substrates. The reaction rates (B) were calculated based on the first approximately linear range of the kinetic curves or after a short lag period in the case of FXIII-A. Data are presented as the mean ± SD from two independent experiments performed in triplicate. To visualize the crosslinked product, after denaturation, the reaction mixtures were subjected to 15% SDS-PAGE and proteins were immediately detected by a PharosFX Plus Molecular Imager (D). The asterisk marks the crosslinked protein-peptide product. Finally, the gel was Coomassie stained to compare the amount of S100A4(GST) proteins in the loaded samples (C, representative gel images).

the other hand, 5 μg of the uncrosslinked S100A4(GST) demonstrated only a slight (5.5%) inhibitory effect on FXIII-A isopeptidase activity using Zedira A101 substrate based on the manufacturer instructions (data not shown).

2.4. Preparation of samples after kinetic reactions for SDS-PAGE analysis

The activity assays were stopped by adding 6x denaturation buffer (300 mM Tris-HCl, pH 6.8, 0.43 M β-mercaptoethanol, 12% (m/v) SDS, 20% (v/v) glycerol, 0.06% (m/v) bromophenol blue) and the samples were boiled for 10 min. SDS-PAGE was performed using 15% Tris-Glycine gels. The fluorescence was detected immediately by means of a PharosFX Plus Molecular Imager (Ex/Em: 488/530 nm; Bio-Rad).

2.5. Kinetic transamidase activity measurement

A fluorescence polarization assay, originally published by Kenniston and co-workers [18], was used to detect the crosslinking activity of transglutaminases based on our earlier publication [12] with some modifications. Briefly, 5 nM TG2 or FXIII-A enzyme and 100 nM FLpepT26 or FLpepPI2 peptide were incubated with 5 μM S100A4(GST) in 20 mM HEPES buffer, pH 7.5, containing 150 mM NaCl, 5 mM DTT, 0.01% Tween-20 in the presence of 10 mM EDTA and 2 mM iodoacetamide (IA) or 5 mM Ca^{2+} as a negative control or test reaction, respectively. The final volume of the reaction mixture was 35 μl in Untreated Polystyrene Black Microplates (Nunc, Thermo Scientific, Denmark; catalog# 262260) and contained 500 mU thrombin when FXIII-A activity was tested. The reaction was started by the addition of enzyme solution and performed at 37 °C, measuring the change in fluorescence polarization (FP) value by a Synergy H1 microplate reader (GreenFP filter cube, Ex: 485 nm, Em: 528 nm; Gain: 75; BioTek, Winooski, VT, USA). The reaction rates were calculated in terms of anisotropy per minute from the initial slopes of the kinetic curves in the case of TG2, while in the case of FXIII-A after an approximately 4 min lag period.

2.6. Kinetic isopeptidase activity measurement

In 35 μl reaction volume on 384-well Untreated Polystyrene Black Microplates, 380 nM of the FLpepT26-S100A4(GST) or 259.5 nM of the FLpepPI2-S100A4(GST) crosslinked substrates (5 μg of FLpepT26-S100A4(GST) or FLpepPI2-S100A4(GST) and S100A4(GST)) were used in 20 mM MOPS, pH 6.8, reaction buffer containing 100 mM NaCl,

5 mM $CaCl_2$, 6 mM glycine methyl ester, 3 mM DTT, 0.1% Polyethylene-glycol 8000 and various concentration of recombinant human TG2 or FXIII-A. In the case of FXIII-A the reaction mixture contained 500 mU human thrombin. Normal human plasma samples were analyzed in the presence of 2 mM GPRP peptide and 5 mg/ml polybrene. The reaction was started by the addition of enzyme solution and performed at 37 °C, measuring the change in fluorescence polarization (FP) value by means of a Synergy H1 microplate reader (GreenFP filter cube; Gain: 63). The negative control contained 10 mM EDTA and 2 mM iodoacetamide to inhibit transglutaminase activity. The reaction rates were calculated in terms of anisotropy per minutes from the initial slopes of the kinetic curves in the case of TG2, while in the case of FXIII-A after an approximately 4 min lag period.

2.7. Reaction conditions and western blot to test the specificity and applicability of DD-Xlink-mab for detection of isopeptide bond

Crosslinked fibrin degradation product (xFDP) and fibrin degradation product (FDP) were incubated with or without recombinant human FXIII-A in the presence of 500 mU thrombin, 10 mM $CaCl_2$ in 20 mM MOPS reaction buffer, pH 6.8, containing 100 mM NaCl, 5 mM $CaCl_2$, 6 mM glycine methyl ester, 3 mM DTT, 0.1% Polyethylene-glycol 8000. The reaction mixtures were incubated at 37 °C for 2 h. Then, the reactions were stopped by the addition of 6x denaturation buffer and the samples were boiled for 10 min. SDS-PAGE was performed using 10% Tris-Glycine gels, which was followed by a blotting step performed with a Bio-Rad Trans-blot SD Semi-dry Transfer Cell. For blocking, the membrane was incubated in 5% (m/v) low-fat milk powder in TTBS (0.1% (v/v) Tween-20 in TBS) for 1 h at room temperature or overnight at 4 °C. DD-XLink-mab primary (Zedira; 1/7500) and Goat anti-mouse IgG(H + L) (1/10000; Advansta, San Jose, CA, USA) secondary antibodies were diluted in 0.5% low-fat milk powder containing TTBS. Finally, WesternBright ECL HRP substrate (Advansta) was used for signal development.

2.8. Data analysis

Data analysis, curve fitting, kinetic calculations and statistics were performed using GraphPad Prism 6 and 8 software (Graphpad Software Inc. La Jolla, CA, USA) using the appropriate incorporated equations and tools mentioned where it is appropriate.

3. Results and discussion

3.1. An attempt to detect isopeptidase activity of FXIII-A on fibrin D-dimer using a monoclonal antibody and western blotting

The role of FXIII-A in blood coagulation and fibrinolysis is a good example of how transglutaminases are involved in essential biological processes by protein crosslinking and later by the cleavage of the previously formed covalent bond. The incorporation of α2-antiplasmin and later its release from the clot is already known. However, there is no evidence as to whether FXIII-A can cleave the previously formed isopeptide bond between fibrin subunits. The FXIII-A catalyzed covalent crosslink between the γ-γ subunits of fibrin/fibrinogen (D-dimer) can be recognized using a recently developed monoclonal antibody by Zedira Gmbh. for differentiation between fibrin degradation products which contain or do not contain an isopeptide bond (Fig. 1). Circulating D-dimer, containing covalent crosslinks between fibrin subunits, is a good marker of blood coagulation and an indicator of sufficient activation and level of FXIII-A.

We thought that the application of this monoclonal antibody could also help in the protein-based detection of FXIII-A isopeptidase activity. We incubated the cross-linked fibrin degradation products (xFDP and D-dimer) in the presence of activated FXIII-A. To facilitate isopeptidase activity, glycine methyl ester and pH 6.8 was applied. After 2 h incubation, by evaluating the integrated pixel densities on the film using ImageJ, we have observed regularly, but not consistently, an apparent 10–20% decrease in the level of the antibody recognized xFDP band (upper band on Fig. 1 was analyzed). Based on several repetitions of the experiment, sometimes with extended incubation time, we can conclude that this application of the antibody combined with Western blot is not applicable to accurately monitor the level of isopeptide bond between the fibrin subunits. The reason could be technical difficulties associated with the sample loading, electrophoresis, blotting method, or during the incubation period, additional crosslinking can cause a still undetectable shift in the size of the D-dimer influencing the accurate determination of the isopeptide bond. But, the antibody-based detection of crosslinked fibrin degradation product could still be a good qualitative marker of FXIII-A transamidase activity during blood coagulation.

Probably due to a still unknown steric regulation, FXIII-A does not cleave the previously formed isopeptide bond between fibrin molecules. After fibrinolytic and proteolytic degradation of the blood clot at peptide bonds, ε-(γ-glutamyl)lysine dipeptide remains in the circulation due to the resistance of the isopeptide bond to such cleavage. Eventually, however, the isopeptide bond is cleaved by cyclo-transferases which are significantly expressed in the intestine and kidney [19,20]. Therefore, a higher level of ε-(γ-glutamyl)lysine dipeptide in the serum is a good marker of blood coagulation events and over activation of transglutaminases in other diseases [21,22].

In the literature, an enzyme named destabilase isolated from medicinal leech saliva was reported to accelerate fibrinolysis through hydrolyzing FXIII-A formed crosslinks [23]. Baskova and co-workers demonstrated that this enzyme is able to enhance the lysis of blood clots by hydrolyzing the isopeptide bond. We also cloned, expressed and purified by one-step affinity chromatography the destabilase 3 isoform [24]. After incubation of xFDP and D-dimer with the recombinant destabilase 3 the level of DD-Xlink-mab recognized xFDP was lower than that in the reaction mixture lacking enzyme (approximately 20% decrease based on integrated optical density analysis; data not shown).
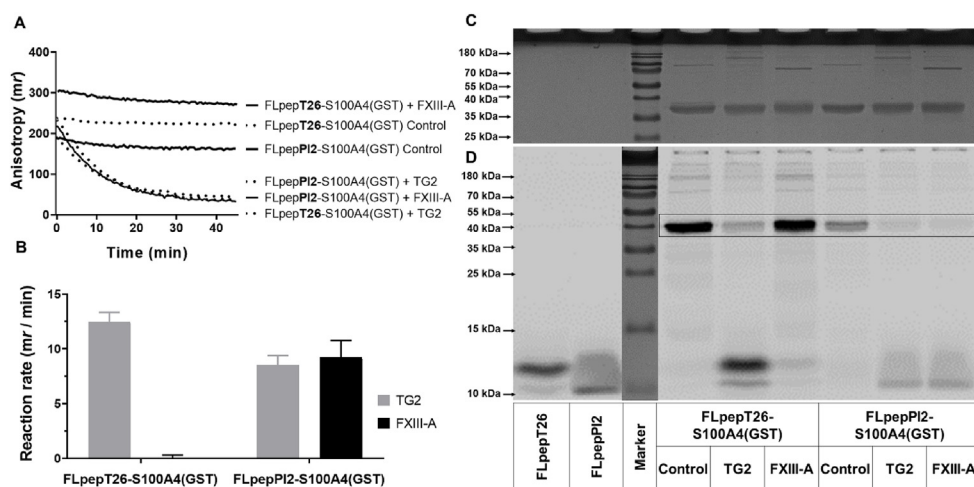
3.2. Protein-peptide based method for FXIII-A transamidase activity measurement

In order to develop real-time monitoring methods for characterization of FXIII-A activities at the protein-peptide level, we examined whether FXIII-A accepts as substrates the S100A4(GST) recombinant protein and FLpepT26 or FLpepPI2 fluorescently labelled dodecapeptides. Earlier, we had improved a fluorescence anisotropy based assay which applied bovine serum albumin (BSA) and FLpepT26 (fluorescently labelled T26 peptide) for monitoring TG2 transamidase activity [18]. BSA was replaced by S100A4(GST) which is an excellent substrate for TG2. It has only one transglutaminase reactive lysine residue (Lys100 in S100A4) with good accessibility on its C-terminal [25]. Peptide T26 (HQSYVDPWMLDH) is a phage display selected TG2 specific artificial dodecapeptide containing only one reactive glutamine residue [26]. Peptide PI2 (NQEQVSPLTLLK) is also a dodecapeptide but derived from the N-terminal sequence of α2-antiplasmin and it is an excellent substrate for both FXIII-A and TG2 [27]. In the current assay, crosslinking transamidase activity was followed by the measurement of exponentially increasing fluorescent anisotropy (Fig. 2A). Then the activity values were calculated based on the linear regression of the first phase of the increasing fluorescent polarization (R-squared values were close to 1; Fig. 2B). In the presence of FLpepT26 and S100A4(GST) FXIII-A did not generate an easily detectable anisotropy change. But, after the tests were completed, SDS-PAGE analysis of the reaction mixture demonstrated the appearance of a faint fluorescent band at approximately 42 kDa in the case of both enzymes. This confirms that FLpepT26 peptide is incorporated into S100A4(GST) by both enzymes, but in the case of FXIII-A to a very low extent (Fig. 2D, loading control on Fig. 2C). T26 peptide was developed as a TG2 preferred peptide substrate and it has only low reactivity with FXIII-A, explaining the undetectable kinetic signal [26]. Then FLpepT26 peptide was replaced by FLpepPI2 peptide and we have detected an increase of the fluorescent polarization in the presence of active FXIII-A and lower reactivity with TG2 (Fig. 2A). The SDS-PAGE analysis (Fig. 2D) confirmed that TG2 can accept FLpepPI2 peptide as a substrate [27,28] and incorporate it into S100A4(GST), but to a lower extent than FXIII-A.

The application of a natural lysine donor FXIII-A substrate, fibrinogen, was also considered in the assay development. But fibrinogen has several transglutaminase reactive residues, both lysine and glutamine, and has a strong tendency to aggregate. Crosslinking of S100A4(GST) and fluorescently labelled dodecapeptides generates an easily detectable anisotropy change (more than 25 m*r*; Fig. 2A) demonstrating that selection of substrate molecules which have only one reactive residue in an exposed position and having similar size to S100A4(GST) could lead to the development of a widely applicable kinetic activity test for each transglutaminase family member. Earlier, Yamada and Meguro published for FXIII-A activity measurement a fluorescence anisotropy based activity assay using casein and mono-dansyl cadaverine as substrates [29]. Later, Hauser and co-workers analyzed in detail the possibilities, advantages and disadvantages of monitoring fluorescence anisotropy changes caused by the increase of size and paralleling the change of rotation parameters of fluorescently labelled substrates as a consequence of the transamidase activity [30]. They suggested the application of rhodamine B fluorophore instead of fluorescein due to its favorable spectral property. They also concluded that a fluorescence anisotropy based assay is a convenient and simple way to follow transglutaminase activity, which correlates with the amount of the enzyme. Our experiments using different peptide substrates raise the possibility of developing isoenzyme specific protein-peptide based assays to measure the activity of various co-expressed transglutaminases from the same biological samples.

Real-time monitoring of FLpepPI2 and S100A4(GST) crosslinking could serve as a protein-peptide based kinetic FXIII-A assay. However, under this assay condition transamidation, deamidation and isopeptide cleavage can occur simultaneously, which could decrease the assay precision. The incorporation of α2-antiplasmin by FXIII-A into Aα-chain of fibrinogen molecules reaches an equilibrium level in which steric hindrance and the parallel isopeptide cleavage could be also involved. These features could influence the determination of FXIII-A level in a biological sample, but monitoring isopeptidase activity probably would avoid these concerns.

Fig. 3. Cleavage of FLpepPI2-S100A4(GST) protein-peptide based substrate provides the possibility of kinetically following FXIII-A isopeptidase activity.

Fluorescence polarization change (A) during isopeptidase activity of 150 nM TG2 or FXIII-A enzyme in the case of 380 nM FLpepT26-S100A4(GST) or 259.9 nM FlpepPI2-S100A4(GST) substrates (5 μg mixture of S100A4(GST)-FLpepT26/PI2 and S100A4(GST)) was followed by BioTek Synergy H1 Green FP filter cube (Ex/Em: 485/525; mirror: 510 nm). The reaction rates (B) were calculated based on the first approximately linear range of the kinetic curves. Data are presented after control subtraction as the mean ± SD from two independent experiments performed in triplicate. To visualize the cleavage, after denaturation, the reaction mixtures were separated by 15% SDS-PAGE and proteins were immediately detected by a PharosFX Plus Molecular Imager (D). The asterisk marks the crosslinked protein-peptide substrate. Finally, the gel was Coomassie stained to visualize the loaded S100A4(GST) (C, representative images).

3.3. Adaptation of a protein-peptide based kinetic isopeptidase assay for FXIII-A (by replacement of the TG2 specific peptide component)

Previously, we described a protein-peptide based isopeptidase activity assay to characterize TG2 isopeptidase activity [12]. We hypothesized that if the crosslinking of a protein and labelled peptide is detectable following the change of fluorescent anisotropy, the cleavage of the isopeptide bonds could be also detectable in the same way, but with reverse signal direction. In addition, the Lys100 in S100A4 may be in a unique steric position, making it a good substrate for other transglutaminases. Since there is no detailed study concerning the specificity of FXIII-A isopeptidase activity, first, we tested FLpepT26-S100A4(GST) as a potential isopeptidase substrate (Fig. 3A). In agreement with our expectation, FXIII-A did not cleave the crosslink in FLpepT26-S100A4(GST) which was also confirmed by SDS-PAGE analysis demonstrating similar fluorescence band intensity of isopeptide bond containing substrate around 42 kDa in the control and in the presence of FXIII-A in the reaction mixture (Fig. 3D; loading control on Fig. 3C).

As a next step, the peptide part of the crosslinked protein-peptide substrate, FLpepT26 was replaced by FLpepPI2 peptide. However, FXIII-A accepts FLpepPI2 as a substrate with a higher velocity and incorporation ratio than TG2. Therefore, to prevent any unwanted effect of remaining FXIII-A later, S100A4(GST) and FLpepPI2 were crosslinked by TG2 to prepare the optimal substrate for the final assay conditions. After a short 4 min lag period (activation of FXIII-A), the anisotropy change followed an exponential decay. Using linear regression based on this approximately linear phase (between 4 and 14 min) of the decrease, reaction rates were calculated and are presented in Fig. 3B after subtracting the blank values. Indeed, the FLpepPI2-S100A4(GST) served as a substrate providing sufficient signal to monitor kinetically FXIII-A isopeptidase activity at the protein-peptide level (Fig. 3A and B). The cleavage of the isopeptide bond was also demonstrated by SDS-PAGE, showing a decreased amount of fluorescence labelled crosslinked product (around 42 kDa) and a stronger peptide band compared to the control (around 12 kDa; Fig. 3D).

This confirms our hypothesis that the replacement of the peptide part in the substrate molecule, can produce a protein-peptide based substrate for other transglutaminase family members. Sugimura et al. published an artificial, phage display selected dodecapeptide, F11 peptide [26], which also serves as a better substrate of FXIII-A than of TG2. The application of F11 peptide in the crosslinked substrate could theoretically further improve the specificity of this assay.

3.4. Sensitivity, linearity, detection limit of the protein-peptide based kinetic isopeptidase assay for FXIII-A

In order to test the potential applications of the protein-peptide based FXIII-A kinetic isopeptidase assay, first, the effect of increasing FLpepPI2-S100A4(GST) concentration on the reaction rate was measured in the presence of 150 nM FXIII-A. The activity values followed saturation kinetics, and a curve was fitted using the Michaelis-Menten equation (Fig. 4A, coefficient of determination: $R^2 = 0.928$). The activity attained a plateau phase and K_m and V_{max} parameters were calculated as 4.10 ± 0.76 nM and 54.92 ± 1.7 mr/min/nM FXIII-A, respectively, using GraphPad Prism.

This K_m is extremely low compared to other published values for human FXIII-A substrates. For example, Oertel and co-workers [17] published a fluorometric method which was also developed based on the isopeptidase activity of FXIII-A; the K_m value for their modified peptide substrate was 19.8 ± 2.8 μM. In the case of the widely applied transglutaminase assay when the released ammonia was followed from the α2-antiplasmin derived dodecapeptide (PI2) the K_m(app) was 530 μM [27]. When TG2 transamidase activity was measured by following the fluorescence anisotropy change using DMC and fluorescently labelled cadaverine substrates the apparent K_m value for DMC was in the subnanomolar range and for cadaverine analogues even lower [30]. Due to the same detection method, it is likely that in our assay complex interactions between the concentration of fluorophore labelled substrate molecules and fluorescence anisotropy (for short review of this effect see Ref. [30]) are responsible for the observed maximal reaction rate. Under the current conditions, the low K_m value cannot be explained solely on the basis of kinetics. One of the simplest explanation could be that protein-peptide based substrates have a higher affinity for the enzyme than small peptides or amines, due to a higher number of potential interacting residues between them. Considering the explicit meaning of K_m, either the substrate affinity is high towards the enzyme or the conversion rate of the substrate is low, or both together can result in a low K_m value.

As a next step, to obtain data on the linearity and sensitivity of the assay we measured the correlation between FXIII-A concentration and activities in the presence of 259.5 nM FLpepPI2-S100A4(GST) substrate (Fig. 4B). When FXIII-A activity was measured up to 150 nM concentration, the Pearson correlation coefficient was 0.9387 with 0.8811 R-square value, suggesting a strong, but not exactly linear correlation between FXIII-A concentration and activity in the whole examined concentration range. The lowest measurable FXIII-A concentration was 1.0 nM. When the data were reanalyzed in a smaller, up
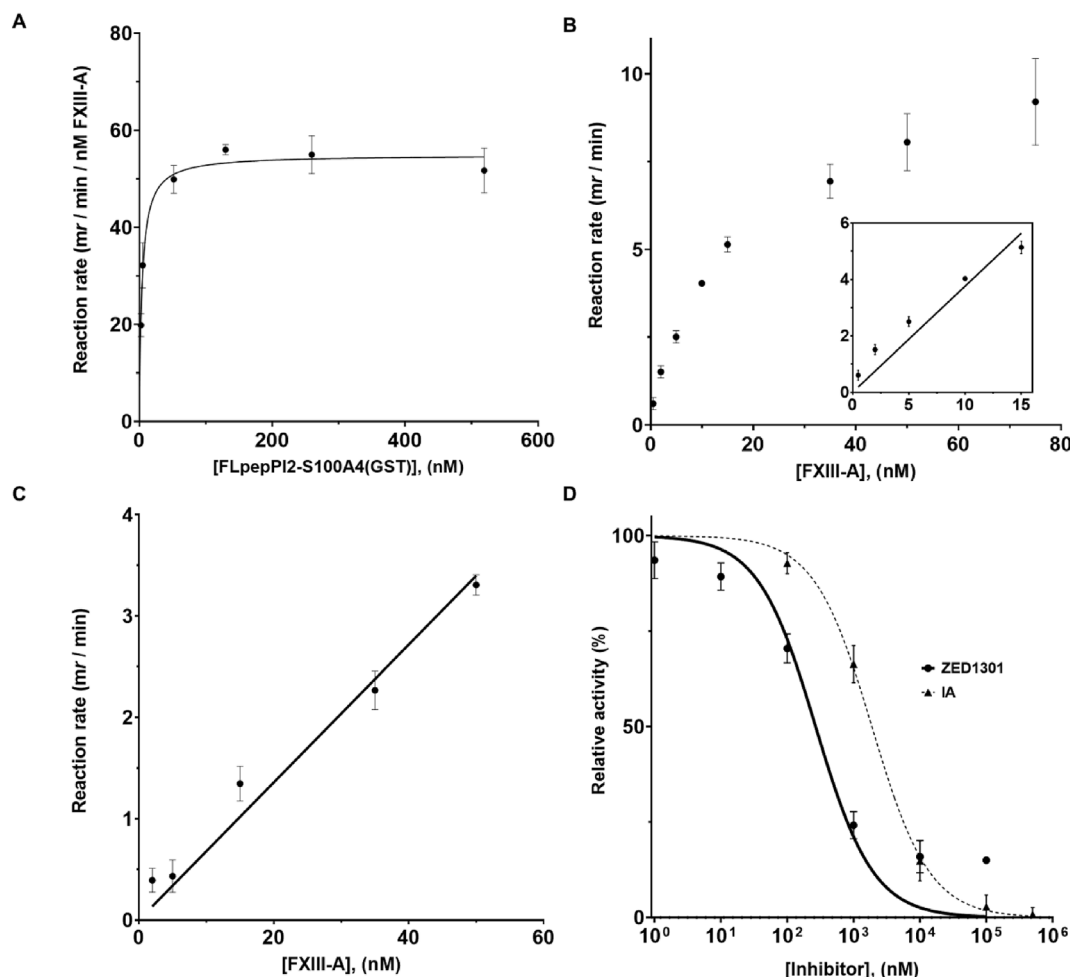
Fig. 4. Kinetic parameters of the FLpepPI2-S100A4(GST) protein-peptide based substrate and sensitivity, linearity and potential applications of the newly developed FXIII-A isopeptidase activity assay.
Determination of the Michaelis constant in the presence of 150 nM FXIII-A (A) and the correlation between FXIII-A concentration and activity in the absence (B) and presence of normal human plasma (C). The inset highlights the correlation around the physiological FXIII-A concentration. Dose-dependent inhibitory effect of iodoacetamide (IA, solid line) and ZED1301 FXIII-A specific inhibitor (dashed line) on the isopeptidase activity of 50 nM FXIII-A (D). The reaction rates were calculated based on the approximately linear range of the kinetic curves. Data are presented as mean ± SD from two independent experiments performed in triplicate.

to 15 nM FXIII-A concentration range (Fig. 4B inset), the correlation coefficient was 0.9907 with 0.9816 R-square value. For plasma FXIII-A activity measurement, the sample is usually mixed with the reaction mixture in a 1:10 ratio. The mean of physiological FXIII-A concentration is approximately 50 nM [17] and the reference interval is between 69 and 143% [27], which by taking into account the 10 times dilution, overlaps with the linear range of our protein-peptide based assay, suggesting that it could determine the physiological FXIII-A level in the human plasma. This FXIII-A concentration range is smaller than that in the case of the Hauser and co-workers [30] published fluorescence anisotropy assay measuring TG2 activity, where the optimal enzyme concentration range was 0.5–5 µg/ml, which corresponds to approximately 5–63 nM enzyme (FXIII-A) in our assay.

In order to further evaluate the applicability of the assay, normal human plasma was added at a 1:10 dilution to the reaction buffer which contained a various concentration of recombinant human FXIII-A. To obtain an appropriate blank value in the presence of plasma, the transglutaminase activity of endogenous FXIII-A was inhibited by chelating Ca^{2+} using 10 mM EDTA and adding iodoacetamide as an –SH group reactive alkylating agent (Fig. 4C). The reaction rate of the normal plasma was 1.22 ± 0.18 mr/min without recombinant FXIII-A addition, which was subtracted from the activity values before plotting.

Interestingly, in the presence of normal human plasma, the corrected activities resulted in better correlation parameters but lower activity values. Up to 50 nM recombinant FXIII-A concentration, the Pearson correlation coefficient was 0.9950, with 0.9901 R-square value and the coefficient of determination for the fitted line was 0.9422. The smallest clearly measurable FXIII-A concentration (sensitivity) was at 5 nM recombinant FXIII-A. Below this concentration, the differentiation from the blank anisotropy change value was not possible. The diagnostic test reactions usually contain the plasma at a 1 to 10 dilution. This corresponds to 5 nM FXIII-A in the assay conditions when FXIII-A level is physiological in the plasma [17], suggesting that in the examined experimental setting, the diagnostic application of the assay is limited.

In bleeding disorders, the accurate determination of clotting factor levels is essential for the diagnosis of the disease and for checking the efficiency of factor complementation treatment. Measuring FXIII-A activity provides an excellent tool for the identification of FXIII-A deficiency [31]. Sensitive methods have been developed to determine FXIII-A transamidase activity using fluorescently, radioisotope or biotin labelled amines, but their application is difficult and time-consuming, due to technical difficulties and safety issues [31]. Microtiter plate based methods provide good reproducibility when using various substrates for coating the plate and various biotin labelled substrates for

the detection of their incorporation, but these methods are still time-consuming and relatively laboratory extensive [32,33]. In 1971 Lorand et al. published a continuous fluorescent method based on the incorporation of dansyl-cadaverine into dimethylated casein, which is capable of automated determination of FXIII-A activity in plasma [34]. Today, there are two widely used commercial assays for screening FXIII-A level (Berichrom and Technochrom), and both depend on the first catalytic step of the transamidase reaction, which is followed by ammonia release [35,36]. This feature makes possible the real kinetic monitoring of the reaction rate during a short period. The advantage of these assays is that they detect a change which is relatively independent of the second substrate and measures the potential parallel deamidation activity of FXIII-A [11,31]. Unfortunately, using our method, the detection and differentiation of the most severe cases of FXIII-A deficiency where the enzyme level is less than 5% of the normal value leading to severe bleeding disorders, is not possible. However, the coupled kinetic test that detects ammonia release is able to differentiate between this severe form of FXIII-A deficiency and the mild FXIII-A deficiency, which causes mild or not observable bleeding when FXIII-A activity in the plasma is between 5 and 30% of the normal value [37].

The optimization of the assay for a simple microfluidic platform could be the subject of the next study. The further purification of the substrate from the unmodified S100A4(GST) and application of F11 peptide instead of PI2 could also improve this method.

As inhibition of FXIII-A is a therapeutic goal to prevent unwanted blood clot stabilization, the application of a protein-peptide based assay for drug testing would be useful because in vivo FXIII-A acts on protein substrates. The effect of iodoacetamide (IA), a general transglutaminase inhibitor and ZED1301, a FXIII-A specific peptidomimetic inhibitor were tested in the anisotropy based FXIII-A assay (Fig. 4D). In the presence of IA and ZED1301 dose-dependent inhibitions were detected with 1.89 ± 0.29 μM and 268 ± 56 nM IC50 values, respectively. However, the IC50 values are not comparable between different test conditions, but this iodoacetamide concentration is relatively high in the case of 50 nM FXIII-A concentration. We speculate that this higher iodoacetamide concentration correlates with the potentially higher affinity of the protein-peptide substrates than a small substrate molecule, suggesting that the efficiency of other FXIII-A inhibitors in vivo could be significantly different from the values generated in such assays using small substrate molecules. Application of a kinetic fluorescence anisotropy based method for inhibitor testing is highly desirable because efficient inhibitors frequently have fluorescence properties, but this does not influence the detected anisotropy change significantly.

During the last few years, we have made some effort to disconnect the isopeptidase and transamidase activities of transglutaminases. In the case of TG2, mutation of W278F and W332F residues, which participate in the formation of the substrate-binding cleft and active site, resulted in mutants which prefer transamidase or isopeptidase activity, respectively. The problem is that these mutants express their special property only on small substrates. When their activities were measured using the developed protein-peptide based assay, this property disappeared. The TG2 W332F mutant possessed lower isopeptidase activity than the wild type, and the TG2 W278F mutant showed higher isopeptidase activity than the TG2 W332F mutant with the FLpepT26-S100A4(GST) substrate [12].

The disconnection of isopeptidase and cross-linking activities in a FXIII-A mutant, which can cleave isopeptide bond with high reaction speed and does not crosslink proteins, could have therapeutic importance, because it could enhance the release of α2-antiplasmin from the blood clot and cleave isopeptide bonds between fibrin molecules contributing to destabilization, fibrinolysis and consequent elimination of unwanted blood clots. Based on the structural homology between TG2 and FXIII-A, in an attempt to increase the substrate-binding in the active site cleft of the enzyme, we made W316F and W371F mutant FXIII-A proteins (corresponding to the W278F and W332F in TG2 proteins, respectively). But, FXIII-A W371F was completely inactive,

and FXIII-A W316F possessed low transamidase and isopeptidase activities (both in small peptide- and protein-based assays; data not shown).

4. Conclusions

In vivo most of the transglutaminase catalyzed reactions act on proteins, but the in vitro assays generally use peptides or small molecules as substrates, often due to economic reasons. In addition, activity assays measuring absorbance in the UV region or fluorescence are frequently not applicable to test transglutaminase activity in biological samples. This makes it difficult to obtain relevant conclusions when regulatory ligands and inhibitors are tested as possible therapeutic transglutaminase agents. FXIII-A has an emerging therapeutic potential to cure thrombotic diseases, preventing the stabilization of unwanted blood clots. The methods developed in the present study provide tools to generate reliable and relevant results concerning the effect of developed regulatory molecules. Following the anisotropy changes during crosslinking or isopeptide cleavage offers a relatively sensitive and easily measurable method to monitor FXIII-A transglutaminase and isopeptidase activities on protein substrates. Moreover, our system, combining the application of the same substrates to test opposite directions of transglutaminase catalyzed reactions decreases the drawbacks generated by the different binding properties of various applied substrates in the thus far developed assays in the field.

Another important issue is the presence of various transglutaminases and their reactions in living systems. The present assay system provides the basis for monitoring both directions of transglutaminase reactions in complex, homogeneous biological samples and for high-throughput screening of effectors and mutant transglutaminases. Our long term vision is to produce appropriate assays for the development of non-immunogenic transglutaminase mutants or abzymes which are able to cleave isopeptide bonds and can reverse progressive, deadly, non-curable diseases such as various types of fibrosis and thrombotic diseases.

Declaration of competing interest

None.

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