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(54) **METHOD FOR THE PRODUCTION OF HUMAN THROMBIN AND USES THEREOF**

VERFAHREN ZUR HERSTELLUNG VON MENSCHLICHEM THROMBIN UND VERWENDUNGEN DAVON

PROCÉDÉ DE PRODUCTION DE THROMBINE HUMAINE ET SES UTILISATIONS

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Description

FIELD OF THE INVENTION

[0001] The invention refers to the field of biotechnology. Specifically, the invention relates to a stable recombinant mutated prethrombin-2 which is able to convert itself auto-catalytically into active α-thrombin that can be used as a haemostatic. Another aspect of the invention includes the application of mutated recombinant prethrombin-2 and the α-thrombin obtained by itself-autocatalytic activity, in diagnostics and/or prognostics of coagulation related-diseases.

BACKGROUND OF THE INVENTION

[0002] α-Thrombin plays an important role in hemostasis and hence is a highly useful protein for use as a hemostatic. Currently, α-thrombin is utilized in more than 1 000 000 patients in the USA each year [1]. The main source of α-thrombin is pooled human plasma collected from donors. The plasma is treated by complicated filtration and separation, however, no procedure is completely effective against viral particles derived from human blood [2].

[0003] Alternatively, recombinant α-thrombin can be produced that is devoid of the risks of viral particles. Thrombin is synthesized in the form of prothrombin which is one of proteolytic enzymes which are normally synthesized in their inactive form, known as proenzymes or zymogens, which can be cleaved by factor Xa or by ecarin, a protease found in snake poison, [3] at two places, Arg-Thr and Arg-Ile bonds. Cleavage at the first factor Xa site results in prethrombin-2, an inactive single-chain precursor that has the same size as α-thrombin. Activation of prethrombin-2 to α-thrombin occurs through internal rearrangement of the initial peptide chain upon cleavage of Arg-Ile bond with factor Xa or by ecarin [4]. The cleaved shorter peptide section does not leave the α-thrombin macromolecule but stays linked with the longer peptide sequence via an S-S bond.

[0004] Currently, it is impossible to directly express active recombinant α-thrombin from the gene fragment corresponding to prothrombin because the resulting protein will be always inactive prethrombin-2. The preparation of recombinant prethrombin-2 and its activation to α-thrombin by ecarin, has been described in the literature [5]. The main drawback of this procedure is the necessity to employ extremely dangerous ecarin, the primary reagent in the venom of the snake Echis carinatus [6] to activate α-thrombin. The removal of ecarin, significantly diminishes the yield and increases costs of α-thrombin preparation. Still there is no 100% assurance that recombinant α-thrombin is not contaminated with ecarin. Factor Xa can be used instead of lethal ecarin but it requires for its optimal operation a complex with factor V, platelet phospholipids and calcium [7], that also must be separated from α-thrombin.

[0005] On the other hand, enzymes, such us, α-thrombin, have been commonly used in different bioanalytical assays for detection and amplification of signal. They are employed in quantification of glucose [8], H_2O_2 [9], pesticides [10], cholesterol [11], and ethanol [12], and are the basis for ELISAs (enzyme linked immunosorbent assays) [13]. Enzyme amplification techniques [14] have been used to improve the sensitivity of several bioanalytical assays. In this sense, the state of the art discloses an assay including a double amplification cascade in which ecarin, converted prothrombin to α-thrombin to digest an artificial fluorogenic substrate [15]. As mentioned above, prothrombin is one of proteolytic enzymes which are normally synthesized in their inactive form, known as proenzymes or zymogens [16]. When the product of the proenzyme cleavage reaction catalyzes the same reaction, the process is called an autocatalytic activation [17]. Some examples of natural autocatalytic enzymes are trypsinogen, pepsinogen, or the blood coagulation factor XII [18]. The autocatalytic behavior of these enzymes could be applied for analytical purposes. Moreover, tests for α-thrombin activity are used to evaluate the rate of blood coagulation, therefore it is important to develop sensitive methods to monitor its activity.

[0006] The signal obtained by a low concentration of an enzyme can be considerably amplified by means of an autocatalytic reaction. The use of zymogens in bioanalysis is limited since all known natural autocatalytic proenzymes are unstable *in vitro* and their preparations always contain traces of corresponding active enzymes [19]. To the best of our knowledge, there are still no commercially available kits based on natural autocatalytic proenzymes. Previously, the inventors used the self-replicating DNA machines based on endonucleases to create signal amplification networks [20, 21]. Unfortunately, DNA machines are not stable in body liquids and can not be applied for the analysis of blood derived samples.

[0007] In spite of considerable research into the signal amplification in bioassays, specifically, using self-replicating enzymes and also the preparation and production of these enzymes, more studies are still needed. Moreover, it has not yet been found a proenzyme stable *in vitro* without traces of corresponding active enzymes and also with autocatalytic activity, particularly these is not disclosures in the prior art about a recombinant prethrombin-2, with autocatalytic features, which show stability *in vitro,* without traces of α-thrombin.

DESCRIPTION OF THE INVENTION

Brief description of the invention

[0008]   The present invention, overcomes these problems in the art by disclosing a new methodology for production of a mouse (SEQ ID NO: 1) and human (SEQ ID NO: 2) mutant recombinant stable prethrombin-2 which are able to convert itself auto-catalytically into active α-thrombin in the absence of ecarin or factor Xa or any other substance.

[0009]   The method for obtaining a stable mutant recombinant prethrombin-2 consists of changing the cleavage site of a recombinant proenzyme by site directed mutagenesis to obtain a stable protein that can be cleaved by the corresponding active enzyme. Specifically, the inventors have modified the cleavage site of native or wild type (WT) mouse (SEQ ID NO: 3) and human (SEQ ID NO: 4) prethrombin-2 to a α-thrombin cleavage site to obtain a stable artificial autocatalytic enzyme. The mutant recombinant proenzyme is activated by α-thrombin triggering the autocatalytic reaction. The concept of signal amplification using self-replicating enzymes can be applied to improve sensitivity of α-thrombin assays and also for the preparation of different enzymes. Therefore, the present invention displays an autocatalytic mutant recombinant enzyme, prethrombin-2, and its application for the signal amplification in bioassays for the detection of α-thrombin/prothrombin and for the synthesis of recombinant α-thrombin.

[0010]   Surprisingly, the mutated prethrombin-2 disclosed in the present invention is completely stable, without any trace of thrombin, or any other proteases or any undesired factor, component, contaminant, etc, which may initiate an undesired and/or uncontrolled cleavage of the mutant prethrombin-2. The invention described herein, allows total control of the autocatalysis of prethrombin-2 due to the unexpected stability shown by the mutated prethrombin-2 of the invention. This stability feature of mutated prethrombin-2 of present invention has as advantage its use in *in vitro* assays for detection of α-thrombin/prothrombin, with more accuracy as far as the stable mutated prethrombin-2 of the invention has not autocatalytic basal activity due to contaminant, as the ones previously mentioned, and its autocatalysis is only triggered by the amount of α-thrombin present in the assay sample.

[0011]   Thus, in a first aspect the present invention refers to a stable and autocatalytic mutant prethrombin-2, from murine or human origins (SEQ ID NO: 1 or SEQ ID NO: 2, respectively) and their corresponding DNA encoding sequences (SEQ ID NO: 5 or SEQ ID NO: 6, respectively).

[0012]   In a second aspect, the invention refers to the use of at least one of the mutated prethrombin-2 proenzymes or their nucleotide encoding sequences as mentioned above for the production of α-thrombin.

[0013]   In another aspect, the invention refers to mutant pre-thrombin-2 for use in the *in vitro* detection of α-thrombin/prothrombin. In another aspect, the invention refers to mutant pre-thrombin-2 for use in the *in vitro* diagnosis and/or prognosis of coagulation-related diseases. The coagulation-related diseases are selected from the group comprising: haemophilia; thrombosis; inherited prothrombin deficiency, either Type I also known as hypoprothrombinemia, or Type II, also known as dysprothrombinemia; acquired prothrombin deficiency; hypoprothrombinaemia syndrome; and von Willebrand disease.

[0014]   In a third aspect, the invention refers to a kit for detecting α-thrombin/prothrombin in a sample comprising the mutant prethrombin-2 as mentioned above, which comprises at least one of the mutated prethrombin-2 proenzymes disclosed in the present invention. The kit disclosed herein also could include other reagents, i.e.: assay reagents, buffers and sterile saline or another pharmaceutically acceptable emulsion and suspension base. In addition, the kits may include instructional materials containing directions (e.g., protocols) for the practice of the assay methods of this invention.

[0015]   In another aspect, the present invention refers to the use of the kit disclosed herein for the *in vitro* diagnosis and/or prognosis of coagulation-related diseases. The coagulation-related disease is selected from the group comprising: haemophilia, thrombosis, inherited prothrombin deficiency Type I or Type II, acquired prothrombin deficiency and von Willebrand disease.

[0016]   In another aspect, the present invention refers to a method for preparing active α-thrombin using the mutant recombinant prethrombin-2 disclosed in the present invention.

[0017]   In still another aspect, the invention refers to an *in vitro* method of diagnosis and/or prognosis of coagulation-related diseases which comprises determining in a sample from a subject, the expression or activity level of α-thrombin/prothrombin using the kit disclosed herein and the comparison of said expression level with respect to the expression values obtained from healthy controls. The coagulation-related disease is selected from the group comprising: haemophilia, thrombosis, inherited prothrombin deficiency Type I or Type II, acquired prothrombin deficiency and von Willebrand disease. The α-thrombin/prothrombin expression or activity levels are analyzed in a sample of blood. For this purpose is extremely important that the prethrombin-2 to be used in the amplification reaction wherein the *in vitro* assay is based was stable, because traces of thrombin or any other proteases contaminating the prethrombin-2 to be used in thrombin detection in biological samples would render artefacts.

[0018]   Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. However, for ease of reference, some of these terms will now be defined.

[0019] The expression or activity level of a disease-associated α-thrombin/prothrombin is information in a number of ways. For example, a differential expression of a disease-associated α-thrombin/prothrombin compared to a control may be used as a diagnostic that a patient suffers from the disease. Expression or activity levels of a disease-associated α-thrombin/prothrombin may also be used to monitor the treatment and disease state of a patient. Furthermore, expression or activity levels of the disease-associated α-thrombin/prothrombin may allow the screening of drug candidates for altering a particular expression profile or suppressing an expression profile associated with the disease. In the present invention, the terms "expression or activity" in relation with α-thrombin/prothrombin levels are used interchangeably.

[0020] The term "sample" refers preferably to a sample of a fluid such as a solution and more preferably refers to a sample of a body fluid. Samples of body fluids can be obtained by well known techniques and include, preferably, samples of blood, plasma, serum, more preferably, samples of plasma.

[0021] The term "subject" as used herein refers to an animal, preferably a mammal, most preferably a human being, including both young and old human beings of both sexes which may suffer from or are predisposed to a pathology. The subject according to this aspect of the present invention may suffer from a pathology associated with abnormal expression or activity of coagulation pathway. The terms "subject" and "patient" could be used interchangeably throughout the present invention.

[0022] The term "stable prethrombin-2" means, within present invention, a prethrombin-2 in a sample free of thrombin or any other proteases or any undesired factor, component, contaminant, etc, which may trigger autocatalytic activity of prethrombin-2 itself.

Figure Legends

[0023]

Figure 1. Sequence alignment of the cleavage site of wild type (WT) and mutant prethrombin-2. FXa cleavage site present in WT prethrombin-2, corresponding to residues IDGRIV, was changed to IVPRGV which corresponds to a α-thrombin (Th) cleavage site. Changed amino acids are underlined.

Figure 2. Analysis of the purified mouse mutant prethrombin-2. **A)** 12% SDS-PAGE stained with Coomassie Brilliant Blue. **B)** Nitrocellulose membrane probed with anti-His-tag mouse monoclonal antibodies. Lane 1, molecular mass references; lane 2, 0.3 μg mutant prethrombin-2.

Figure 3. Stability assay of human mutant prethrombin-2. Evolution of fluorescence intensity in samples containing human wild type recombinant prethrombin-2 (1.6×10^{-6} M) **(a)** or human mutant recombinant prethrombin-2 (1.6×10^{-6} M) **(b),** with the fluorogenic substrate rhodamine 110, bis-(p-tosyl-L-glycyl-L-prolyl-L-arginine amide) (4×10^{-6} M) (Invitrogen) in 50 mM Tris-HCl, pH 9.0, 150 mM NaCl.

Figure 4. Evolution of fluorescence intensity in samples containing **a)** 2.2×10^{-12} M human wild type α-thrombin and 2.1×10^{-7} M human mutant prethrombin-2; **b)** 2.2×10^{-12} M human wild type α-thrombin and 2.1×10^{-7} M mouse mutant prethrombin-2; **c)** 2.2×10^{-12} M human wild type α-thrombin; **d)** 2.1×10^{-7} M human mutant prethrombin-2; **e)** 2.1×10^{-7} M mouse mutant prethrombin-2.

Figure 5. Time courses of mutant prethrombin-2 activation and self-replication, **a)** 5.38×10^{-9} M cleaved mutant prethrombin-2 and 2.3×10^{-7} M untreated mutant prethrombin-2; **b)** 5.38×10^{-9} M cleaved mutant prethrombin-2; **c)** 2.3×10^{-7} M untreated mutant prethrombin-2.

Figure 6. A) Evolution of fluorescence intensity in the presence (curves **a-h**) of 5.3×10^{-7} M mutant prethrombin-2 or its absence (curves **i-p**). The samples contained different concentrations of human α-thrombin: a) and i) 5×10^{-12} M; b) and j) 3.75×10^{-12} M; c) and k) 2.5×10^{-12} M; d) and 1) 1.5×10^{-12} M; e) and m) 7.5×10^{-13} M; f) and n) 5×10^{-13} M; g) and o) 2.5×10^{-13} M; h) and p) 0 M. **B)** Calibration curve of α-thrombin in the presence (curve a) and absence (curve **b)** of mutant prethrombin-2.

Figure 7. A) Evolution of fluorescence intensity in human plasma samples in the presence of 1.5×10^{-6} M mutant prethrombin-2 (curves **a-f**) or its absence (curves **g-1**). The samples contained 6×10^{-8} M ecarin and different volumes of human plasma: a) and g) 2.6 nL; b) and h) 2 nL; c) and i) 1.3 nL; d) andj) 0.67 nL; e) and k) 0.33 nL; f) and 1) 0 nL. **B)** Calibration curve of human plasma in the presence (curve **a)** or absence (curve **b)** of mutant prethrombin-2.

Detailed description of the invention

[0024] Present invention relates to a mutant prethrombin-2 characterized by having SEQ ID NO: 1 or SEQ ID NO: 2 or their nucleotide encoding sequences characterized by SEQ ID NO: 5 or SEQ ID NO: 6, respectively.

[0025] In a particular embodiment of the invention, the mutant prethrombin-2 is SEQ ID NO: 2 or their nucleotide encoding sequence characterized by SEQ ID NO: 6.

[0026] In another particular embodiment of the invention, the mutant prethrombin-2 characterized by having SEQ ID NO: 2 or by their nucleotide encoding sequence: SEQ ID NO: 6 which is from human origin.

[0027] In another particular embodiment of the invention, the mutant prethrombin-2 disclosed in the present invention is characterized by having autocatalytic activity and by being stable *in vitro*.

[0028] Present invention also relates to the use of the mutated prethrombin-2 mentioned above for the production of α-thrombin. Furthermore, present invention also relates to mutated prothrombin-2 for use in the *in vitro* detection of α-thrombin/prothrombin in biological samples.

[0029] Present invention also discloses the use of the mutated prethrombin-2 mentioned above for the manufacture of a kit for the *in vitro* diagnosis and/or prognosis of coagulation-related diseases.

[0030] In a particular embodiment of the kit of the present invention, the coagulation-related disease is selected from the group comprising: haemophilia, thrombosis, inherited prothrombin deficiency Type I or Type II, acquired prothrombin deficiency and von Willebrand disease.

[0031] Present invention also discloses a mutant prethrombin-2 mentioned above for use in the *in vitro* diagnosis and/or prognosis of coagulation-related diseases. In a particular embodiment, the coagulation-related disease is selected from the group comprising: haemophilia, thrombosis, inherited prothrombin deficiency Type I or Type II, acquired prothrombin deficiency and von Willebrand disease.

[0032] Present invention also relates to a method for preparing α-thrombin which comprises the following steps:

1. Modify, preferably by site-direct mutagenesis, the DNA sequence encoding for factor Xa cleavage site in wild type prethrombin-2 gene to a DNA sequence encoding for α-thrombin cleavage site.
2. Insert the mutated DNA prethrombin-2 into an expression vector.
3. Transform a host cell with the vector disclosed in previous step.
4. Culturing the transformed host cell of previous step in an appropriate culture medium.
5. Harvest transformed cells and solubilize proteins in inclusion bodies.
6. Isolating the supernatant to obtain purified mutant prethrombin-2.
7. Contacting the purified mutant prethrombin-2 obtained in previous step with α-thrombin.
8. Separating the α-thrombin obtained in previous step 7.

[0033] In a preferred embodiment of the invention, the method disclosed herein it is characterized in that the amino acid sequence of factor Xa cleave site in wild type prethrombin-2 is SEQ ID NO: 17.

[0034] In a preferred embodiment of the invention, the method disclosed herein it is characterized in that the amino acid sequence of the thrombin cleave site in mutant prethrombin-2 is SEQ ID NO: 18.

[0035] In a preferred embodiment of the invention, the method disclosed herein it is characterized in that the DNA sequence of the wild type prethrombin-2 is selected from the group comprising SEQ ID NO: 7 or SEQ ID NO: 8.

[0036] In a preferred embodiment of the invention, the method disclosed herein it is characterized in that the vector is preferably a plasmid. Any vector or plasmid known in the prior art for the same purpose could be used. In this sense, any vector suitable for expression in bacteria containing T7 promoter and coding a N-terminal His tag may be employed. On the other hand, any vector suitable for expression in yeast, containing galactose or methanol inducible promoters and coding a His tag may also be used in present invention. More particularly the plasmid is pET-TEV-prethr-2 plasmid or pQE9-hprethr-2 plasmid.

[0037] In a preferred embodiment of the invention, the method disclosed herein it is characterized in that the host cell is selected from the group comprising: bacterial cells, animal cells and/or yeast.

[0038] In a more preferred embodiment of the invention the bacterial host cell is *E. coli,* preferably, *E. coli* XL1-blue, or *E. coli* BL21 or any strain derived thereof.

[0039] In a more preferred embodiment of the invention the host cell is a yeast selected from the group comprising: *Saccharomyces sp.* or *Pichia pastoris* or any strain derived thereof.

[0040] In a preferred embodiment of the invention, the method disclosed herein it is characterized in that the ratio prethrombin-2: α-thrombin of step 7 ranged 1:10^{-6}.

[0041] In a preferred embodiment of the invention, the method disclosed herein it is characterized in that the purification of α-thrombin comprises at least one step of techniques of affinity chromatography.

[0042] Present invention also discloses a kit for detecting α-thrombin/prothrombin in a sample comprising the mutant prethrombin-2, murine or human as disclosed herein.

[0043] Present invention also discloses the use of the kit mentioned above for the *in vitro* diagnosis and/or prognosis of coagulation-related diseases.

[0044] In a preferred embodiment, the coagulation-related disease is selected from the group comprising: haemophilia, thrombosis, inherited prothrombin deficiency Type I or Type II, acquired prothrombin deficiency and von Willebrand disease.

[0045] Present invention also disclosed an *in vitro* method of diagnosis and/or prognosis of coagulation-related diseases which comprises the determination in a sample from a subject of the expression or activity level of α-thrombin/prothrombin using the kit mentioned above and the comparison of said expression or activity level with respect to the values for the

same expression or activity obtained from healthy controls.

[0046] In a preferred embodiment of the *in vitro* method, the coagulation-related disease is selected from the group comprising: haemophilia, thrombosis, inherited prothrombin deficiency: Type I or Type II, acquired prothrombin deficiency and von Willebrand disease.

[0047] In a preferred embodiment of the *in vitro* method, the sample of the subject is selected from blood, plasma and/or serum, more preferably, samples of plasma.

[0048] Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described.

[0049] The following examples illustrate the invention and should not be considered in a limiting sense, but rather in an illustrative sense of the invention.

Example 1. Cloning and site specific mutations of prethrombin-2 gene.

[0050] The present invention discloses the change the cleavage site of a recombinant proenzyme, prethrombin-2, by site directed mutagenesis to obtain a stable artificial autocatalytic enzyme. To achieve this purpose, the inventors have cloned the WT gene of prethrombin-2 and modified the factor Xa cleavage site in it to a thrombin cleavage site to obtain a stable artificial autocatalytic enzyme. The mutation strategy relies on converting the factor Xa cleavage site (Arg-Ile bond) into the α-Thrombin cleavage site by directed mutagenesis to create a self-replicative protease.

[0051] Briefly, for this approach, the sequences corresponding to human and mouse prethrombin-2 genes were cloned and five single mutations were introduced by, as mentioned above, site-directed mutagenesis in order to create a mutant prethrombin-2.

[0052] First, the vector pCMV-SPORT6 containing the full length mouse prothrombin cDNA was obtained from Geneservice (UK). The prethrombin-2 fragment was amplified from the same vector by PCR using PfuTurbo (Stratagene) and PCR nucleotide mix (Promega). Amplification primers were SEQ ID NO: 9 and SEQ ID NO: 10 containing BamHI and XhoI restriction sites respectively. The PCR product was run on an agarose gel, purified with QIAquick gel extraction kit (Qiagen) and digested with BamHI and XhoI (Takara). Afterwards, the fragment was purified using the QIAquick PCR purification kit (Qiagen) and ligated into the pET-TEV expression vector, a pET-19b vector modified by introducing a TEV protease cleavage site (generously provided by Dr. Lars Backman) giving pET-TEV-prethr-2 plasmid. The correctness of the inserted sequence was verified (MWG Germany). pET-TEV-prethr-2 plasmid was used as a template to mutate the prethrombin-2 gene. Site-direct specific mutations were introduced using the following primers: SEQ ID NO: 11 and SEQ ID NO: 12 with QuikChange II Site -Directed Mutagenesis Kit (Stratagene). The correctness of the mutant sequence was verified (MWG Germany).

[0053] The vector pOTB7 containing the full length human prothrombin cDNA was obtained from Geneservice (UK). The prethrombin-2 fragment was amplified from the same vector by PCR using PfuTurbo (Stratagene) and PCR nucleotide mix (Promega). Amplification primers were SEQ ID NO: 13 and SEQ ID NO: 14 containing SalI and HindIII restriction sites respectively. The PCR product was run on an agarose gel, purified with QIAquick gel extraction kit (Qiagen) and digested with SalI and HindIII (Takara). Afterwards, the fragment was purified using the QIAquick PCR purification kit (Qiagen) and ligated into the pQE9 expression vector (Qiagen), giving pQE9-hprethr-2 plasmid. The correctness of the inserted sequence was verified (MWG Germany). pQE9-hprethr-2 plasmid was used as a template to mutate the human prethrombin-2 gene. Site-direct specific mutations were introduced using the following primers SEQ ID NO: 15 and SEQ ID NO: 16 with QuikChange II Site -Directed Mutagenesis Kit (Stratagene). The correctness of the mutant sequence was verified (MWG Germany).

[0054] As a result, the, the FXa cleavage site present in wild type (WT) prethrombin-2, corresponding to residues with SEQ ID NO: 17 was changed to residues wth SEQ ID NO: 18 which corresponds to the thrombin cleavage site. Figure 1 shows the alignment of the FXa and thrombin cleavage sites of mutant prethrombin-2 and WT respectively. Changed amino acids are underlined. Thrombin selectively cleaves Arg-Gly bonds in fibrinogen and other polypeptides. Studies on the thrombin cleavage site from 30 different polypeptides revealed that the optimum cleavage site has the structure of P4-P3-Pro-Arg-P1'-P2' where P4 and P3 are hydrophobic amino acids and P1'and P2' are non acidic amino acids. It was also observed that polypeptides which contained Gly at P1' were especially susceptible to thrombin cleavage [24].

Example 2. Expression and purification of mutant prethrombin-2

[0055] The plasmid contained the mutated sequences of human and mouse prethrombin-2 were used to transform *Escherichia coli* cells and the human and mouse mutant prothrombin-2 proteins were expressed and purified.

[0056] Particularly, *Escherichia coli* BL21 (DE3) cells were transformed by heat shock with the plasmid containing the mutant mouse prethrombin-2 gene. Cells were grown at 37°C in Luria-Bertani media supplemented with 100 μg/ml ampicillin to reach an OD600 ≈ 0.7. Protein expression was induced by adding 0.1 mM isopropyl thio-β-D-galactoside. Cells were grown for 4 h at 37°C and harvested by centrifugation. Protein purification from inclusion bodies was performed

as described in Soejima et al. [25] with some modifications. After protein refolding, the sample was dialyzed against 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, during two days at 4 °C without stirring. The dialyzed sample was centrifuged and the resulting supernatant was filtered. The pass-through fraction was loaded on a nickel HisTrapTM HP affinity column (GE Healthcare) controlled by an ÄKTA purifier equipment (GE Healthcare). Protein was eluted with a linear gradient from 0 to 500 mM of imidazole in 50 mM Tris-HCl, pH 7.6, 150 mM NaCl. Protein purity was investigated by 12 % SDS-PAGE.

[0057] Electrophoretic analysis of the purified protein showed a single band corresponding to the expected size (Figure 2A). Protein identity was confirmed by Western blot using antibodies against His-tagged polypeptides (Figure 2B).

[0058] For the purification of the human mutated prethrombin-2, *E. coli* XL1-blue cells were transformed by heat shock with the plasmid containing the mutant human prethrombin-2 gene. Cells were grown at 37°C in Luria-Bertani media supplemented with 100 μg/ml ampicillin to reach an OD600 ≈ 0.6. Protein expression was induced by adding 1 mM isopropyl thio-β-D-galactoside. Cells were grown for 3 h at 37°C and harvested by centrifugation. Protein purification from inclusion bodies was performed as described before for the mouse mutant prethrombin-2. After protein refolding, the sample was dialyzed against 50 mM Tris-HCl, pH 7.0, at 4 °C without stirring. The dialyzed sample was centrifuged and the resulting supernatant was filtered. The pass-through fraction was loaded on a HiTrapTM HeparinHP affinity column (GE Healthcare) controlled by an ÄKTA purifier equipment (GE Healthcare). Protein was eluted with a linear gradient from 0 to 2 M NaCl in 50 mM Tris-HCl, pH 7.0. Protein purity was also investigated by 12 % SDS-PAGE (Figure 2A).

Western blot

[0059] Proteins, mouse mutant prethrombin-2 and human mutant prethrombin-2, were separated by 12% SDS-PAGE and electroblotted onto a nitrocellulose membrane. The membrane was then incubated for 1 h in phosphate-buffered saline (PBS), containing 0.05% Tween 20, and 10% defatted milk. Afterwards, the membrane was washed with PBS containing 0.05% Tween 20 and incubated with anti-His mouse monoclonal antibodies (GE Healthcare) for 1 h. Then, the membrane was washed with PBS containing 0.05% Tween 20 and further incubated with secondary rabbit antibodies antimouse IgG conjugated to alkaline phosphatase (Sigma-Aldrich) for 1 h. After washing with PBS containing 0.05% Tween 20, protein bands were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution (Fluka) (Figure 2B).

Example 3. Stability assay of mutant prethrombin-2

[0060] Stability assays were performed by mixing human WT or human mutant prethrombin-2 (1.6 x 10^{-6} M) both produced by recombination in *E. coli,* with the fluorogenic substrate rhodamine 110, bis-(p-tosyl-L-glycyl-L-prolyl-L-arginine amide) (4×10^{-6} M) (Invitrogen) in 50 mM Tris-HCl, pH 9.0, 150 mM NaCl. The fluorescence of the resulting solutions was monitored using λexcitation= 498 nm and λemission= 521 nm.

[0061] Stability assay measures in real time the enzymatic activity of active α-thrombin in the presence of the fluorogenic substrate (p-tosyl-Gly-Pro-Arg)2- rhodamine 110 (Invitrogen). This substrate contains the amino acid sequence recognized by α-thrombin, which is a peptidase. Thrombin cleavages the amino acid releasing the fluorescent rhodamine. During the test, it was measured the increase in fluorescence over time. The more enzyme activity of α-thrombin higher the cleavage speed, and therefore the release of rhodamine, which causes the increase in fluorescence. Figure 3 shows that the WT human recombinant prethrombin-2 (a) is less stable than the mutant human recombinant prethrombin-2 discloses in the present invention (b). The test with mutated recombinant prethrombin-2 of the invention shows no increase in fluorescence for 15 h (55 000 sec). This implies that in preparation of mutated recombinant prethrombin-2 of the invention there are not residues of active α-thrombin, neither are being produced during the time that test takes place. On the other hand, the test with WT human recombinant prethrombin-2 displays an increase in the fluorescence from the beginning of the test. This increase in fluorescence indicates the presence of active α-thrombin in the preparation; even though WT human recombinant prethrombin-2 was also obtained by genetic engineering in E. *coli.* Therefore the stability *in vitro* shown by mutant human prethrombin of present invention was due to the mutagenesis operated herein.

Example 4. α-Thrombin activity assays

[0062] In order to verify whether human α-thrombin could cleave mutated recombinant human and mouse pre-thrombins-2, were incubated both mutated recombinant proteins with WT human α-thrombin (Sigma Aldrich) in the presence of the fluorogenic substrate (p-tosyl-Gly-Pro-Arg)2-rhodamine 110 (Invitrogen). Thrombin activity assays were performed in a Varioskan Flash microplate reader (Thermo Scientific) using black microwell plates at room temperature. Samples (100 μl final volume) were incubated in 50 mM Tris-HCl, pH 9.0, 150 mM NaCl with 4×10^{-6} M rhodamine 110, bis-(p-tosyl-L-glycyl-L-prolyl-L-arginine amide) (Invitrogen) and the fluorescence of the resulting solution was monitored

using λexcitation= 498 nm and λemission= 521 nm.

[0063] The results obtained from the experiment display that in the presence of WT human α-thrombin, the protease activity increased considerably with time (Figure 4, curves a, b), compared to the samples where only WT α-thrombin was added (Figure 4, curve c). On the other hand, in the absence of WT α-thrombin, both mutants did not present enzymatic activity (Figure 4, curves d, e), demonstrating their stability under the experimental conditions. These results clearly indicate that WT α-thrombin is able to cleavage both mutant recombinant prethrombins-2 to generate active enzymes with α-thrombin activity. Exogenous α-thrombin activates the mutant recombinant prethrombin-2 converting it into endogenous α-thrombin which in turn cleaves other macromolecules of prethrombin-2 and the fluorogenic substrate in the course of this autocatalytic reaction.

Example 5. Mutant prethrombin-2 self-replication assays

[0064] Another experiment proved that generated endogenous α-thrombin is able to cleavage mutated recombinant prethrombin-2 to maintain the autocatalytic process. A solution of mutated prethrombin-2 was treated with α-thrombin immobilized on agarose. Mouse mutant prethrombin-2 was cleaved using Thrombin CleanCleave™ Kit (Sigma-Aldrich) as described in manufacturer's directions. The protein solution was then filtered through a 0.22 μm filter to eliminate any remaining. To investigate the autocatalytic reaction, 5.38×10^{-9} M cleaved mouse mutant prethrombin-2 was mixed with and without 2.3×10^{-7} M untreated mouse mutant prethrombin-2 and with the fluorogenic substrate (p-tosyl-Gly-Pro-Arg)2- rhodamine 110 (Invitrogen). Then, the evolution of the fluorescence intensity was monitored using λexcitation= 498 nm and λemission= 521 nm.

[0065] Figure 5 shows that in the presence of the cleaved mutant prethrombin-2 and excess of the untreated mutant prethrombin-2 (Figure 5 curve a), the fluorescence signal increased considerably in comparison with the sample where only the cleaved mutant prethrombin-2 was included (Figure 5 curve b). The untreated mutant prethrombin-2 treated with the buffer solution used to wash α-thrombin on agarose demonstrated no activity (Figure 5 curve c). Consequently, growth in the protease activity was not caused by exogenous α-thrombin that could have detached from agarose. This experiment confirms the ability of endogenous α-thrombin derived from mutated prethrombin-2 to participate in the autocatalytic reaction.

Example 6. Detection of α-thrombin.

[0066] Different concentrations of human α-thrombin (Sigma-Aldrich) were mixed with and without untreated 5.3×10^{-7} M mouse mutant prethrombin-2 disclosed herein. The reactions were carried out in 50 mM Tris-HCl, pH 9.0, 150 mM NaCl with 4×10^{-6} M rhodamine 110, bis-(p-tosyl-L-glycyl-L-prolyl-L-arginine amide) (Invitrogen) and the fluorescence of the resulting solution was monitored using λexcitation= 498 nm and λemission= 521 nm.

[0067] Figure 6A shows evolution of the fluorescence intensities for varying amounts of human α-thrombin mixed with the fluorogenic substrate in the presence (Figure 6 curves a-h) or absence (Figure 6 curves i-p) of mutated prethrombin-2. The fluorescence intensities demonstrated exponential growth in the presence of the mutant, pointing to the autocatalytic character of the reaction. In such case calibration plots of the first derivative of fluorescence intensity with respect to time dF/dt, representing the rate of reaction after 2 hours, versus analyse concentration (Figure 6B) are more informative that the conventional plots [26]. On the basis of the obtained calibration curves we calculated the detection limit of the systems operating without mutated prethrombin-2 (0.488 pM, S/N = 3, n = 3) and with the mutant (10 fM, S/N = 3, n = 3).

Example 7. Detection of prothrombin in human plasma

[0068] The present invention displays the advantage of the amplified assay for prothrombin over a conventional non amplified quantification method (Figure 7). Varying concentrations of pooled human plasma (Sigma-Aldrich) were mixed with 6×10^{-8} M ecarin (Sigma-Aldrich) with or without 1.5×10^{-6} M mouse mutant prethrombin-2. The reactions were carried out in 50 mM Tris-HCl, pH 9.0, 150 mM NaCl with 4×10^{-6} M rhodamine 110, bis-(p-tosyl-L-glycyl-L-prolyl-L-arginine amide) (Invitrogen) and the fluorescence of the resulting solution was monitored using λexcitation= 498 nm and λemission= 521 nm.

[0069] The detection limits of amplified (7.7 pL, S/N = 3, RSD=12%, n=3) and non amplified (710 pL, S/N=3, RSD=6%, n=3) assays were calculated according to the calibration plots (Figure 7B) depicting dF/dt at fixed time of 60 min versus volume of human plasma per microplate well. Taking into consideration that human plasma usually contains 90 μg/ml of prothrombin [27] the assay with autocatalytic amplification allowed to detect as low as 0.693 pg of prothrombin per microplate well. The conventional non amplified assay allowed to quantify as low as 63.9 pg of the analyte per well. Thus, employment of autocatalytic amplification cascade allows diminishing the volume of human plasma needed for the prothrombin assay by two orders of magnitude.

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SEQUENCE LISTING

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Claims

1. Mutant prethrombin-2 **characterized by** having SEQ ID NO: 1 or SEQ ID NO: 2 or their nucleotide encoding sequences **characterized by** SEQ ID NO: 5 or SEQ ID NO: 6, respectively.

2. Mutant prethrombin-2 according to claim 1 **characterized by** having autocatalytic activity and by being stable *in vitro.*

3. Use of the mutant prethrombin-2 of claims 1 to 2 for the production of α-thrombin.

4. Mutant prethrombin-2 of claims 1 to 2 for use in the *in vitro* detection of α-thrombin/prothrombin.

5. Mutant prethrombin-2 of claims 1 to 2 for use in the *in vitro* diagnosis and/or prognosis of coagulation-related diseases.

6. Mutant prethrombin-2 for use according to claim 5 wherein the coagulation-related disease is selected from the group comprising: haemophilia, thrombosis, inherited prothrombin deficiency Type I or Type II, acquired prothrombin deficiency, hypoprothrombinaemia syndrome and von Willebrand disease.

7. Method for preparing α-thrombin which comprises the following steps:

   1. Modify, preferably by site directed mutagenesis, the DNA sequence encoding for factor Xa cleavage site in wild type prethrombin-2 gene to a DNA sequence encoding for α-thrombin cleavage site.
   2. Insert the mutated DNA prethrombin-2 into an expression vector.
   3. Transform a host cell with the vector disclosed in previous step.
   4. Culturing the transformed host cells of previous step in an appropriate culture medium.
   5. Harvest transformed cells and solubilize proteins in inclusion bodies.
   6. Isolating the supernatant to obtain purified mutant prethrombin-2.
   7. Contacting the purified mutant prethrombin-2 obtained in previous step with α-thrombin.
   8. Separating the α-thrombin obtained in previous step 7.

8. Method according to claim 7 wherein the amino acid sequence of the factor Xa cleave site in wild type prethrombin-2 is SEQ ID NO: 17.

9. Method according to claim 7 wherein the amino acid sequence of thrombin cleave site in mutant prethrombin-2 is SEQ ID NO: 18.

10. Method according to claim 7 wherein the DNA sequence of the wild type prethrombin-2 is selected from the group comprising SEQ ID NO: 7 or SEQ ID NO: 8.

11. A kit for detecting α-thrombin/prothrombin in a sample comprising the mutant prethrombin-2 of claims 1 to 2.

12. Use of the kit of claim 11 for the *in vitro* diagnosis and/or prognosis of coagulation-related diseases.

13. Use according to claim 12 wherein the coagulation-diseases are selected from the group comprising: haemophilia, thrombosis, inherited prothrombin deficiency Type I or Type II, acquired prothrombin deficiency and von Willebrand disease.

14. An *in vitro* method of diagnosis and/or prognosis of coagulation-related diseases which comprises the determination in a sample from a subject of the activity level of α-thrombin/prothrombin using the kit of claim 11 and the comparison of said activity level with respect to the values for the same activity obtained from healthy controls.

15. An *in vitro* method according to claim 14 wherein the coagulation-related disease is selected from the group comprising: haemophilia, thrombosis, inherited prothrombin deficiency Type I or Type II, acquired prothrombin deficiency and von Willebrand disease.

Patentansprüche

1. Mutiertes Präthrombin-2, **dadurch gekennzeichnet, dass** es die SEQ ID NO: 1 oder SEQ ID NO: 2 oder die dafür kodierenden Nukleotidsequenzen, **gekennzeichnet durch** die SEQ ID NO: 5 bzw. SEQ ID NO: 6, aufweist.

2. Mutiertes Präthrombin-2 nach Anspruch 1, **dadurch gekennzeichnet, dass** es eine autokatalytische Aktivität aufweist und *in vitro* stabil ist.

3. Verwendung des mutierten Präthrombins-2 nach den Ansprüchen 1 bis 2 zur Herstellung von α-Thrombin.

4. Mutiertes Präthrombin-2 nach den Ansprüchen 1 bis 2 zur Verwendung beim *in-vitro*-Nachweis von α-Thrombin/Prothrombin.

5. Mutiertes Präthrombin-2 nach den Ansprüchen 1 bis 2 zur Verwendung bei der *in-vitro*-Diagnose und/oder Prognose von Gerinnungsstörungen.

6. Mutiertes Präthrombin-2 zur Verwendung nach Anspruch 5, wobei die Gerinnungsstörung ausgewählt wird aus der Gruppe umfassend: Hämophilie, Thrombose, angeborenen Prothrombinmangel Typ I oder Typ II, erworbenen Prothrombinmangel, Hypoprothrombinämie-Syndrom und von-Willebrand-Krankheit.

7. Verfahren zur Herstellung von α-Thrombin, das folgende Schritte umfasst:

   1. Umwandlung, vorzugsweise durch ortsgerichtete Mutagenese, der DNA-Sequenz, die für die Faktor-Xa-Schnittstelle im Wildtyp-Präthrombingen kodiert, in eine DNA-Sequenz, die für die α-Thrombin-Schnittstelle kodiert;
   2. Einfügen der mutierten Präthrombin-2-DNA in einen Expressionsvektor;
   3. Transformieren einer Wirtszelle mit dem im vorhergehenden Schritt beschriebenen Vektor;
   4. Züchten der transformierten Wirtszellen des vorhergehenden Schritts in einem geeigneten Kulturmedium;
   5. Ernten der transformierten Zellen und Solubilisierung der Proteine in Einschlusskörpern;
   6. Isolierung des Überstandes, um gereinigtes mutiertes Präthrombin-2 zu erhalten;
   7. Inkontaktbringen des gereinigten, im vorhergehenden Schritt erhaltenen mutierten Präthrombins-2 mit α-Thrombin;
   8. Abtrennen des im vorhergehenden Schritt 7 erhaltenen α-Thrombins.

8. Verfahren nach Anspruch 7, wobei die Aminosäuresequenz der Faktor-Xa-Schnittstelle im Wildtyp-Präthrombin-2

die SEQ ID NO: 17 ist.

9. Verfahren nach Anspruch 7, wobei die Aminosäuresequenz der Thrombin-Schnittstelle im mutierten Präthrombin-2 die SEQ ID NO: 18 ist.

10. Verfahren nach Anspruch 7, wobei die DNA-Sequenz des Wildtyp-Präthrombins-2 ausgewählt wird aus der Gruppe umfassend die SEQ ID NO: 7 oder die SEQ ID NO: 8.

11. Kit zum Nachweis von α-Thrombin/Prothrombin in einer Probe, der das mutierte Präthrombin-2 nach den Ansprüchen 1 bis 2 umfasst.

12. Verwendung des Kits nach Anspruch 11 zur in-vitro-Diagnose und/oder Prognose von Gerinnungsstörungen.

13. Verwendung nach Anspruch 12, wobei die Gerinnungsstörungen ausgewählt werden aus der Gruppe umfassend: Hämophilie, Thrombose, angeborenen Prothrombinmangel Typ I oder Typ II, erworbenen Prothrombinmangel und von-Willebrand-Krankheit.

14. *In-vitro*-Verfahren zur Diagnose und/oder Prognose von Gerinnungsstörungen, das die Bestimmung des Aktivitäts-grades von α-Thrombin/Prothrombin in der Probe eines Subjektes unter Verwendung des Kits nach Anspruch 11 und den Vergleich des besagten Aktivitätsgrades mit den Werten für die gleiche, aus gesunden Kontrollen erhaltenen Aktivität umfasst.

15. *In-vitro*-Verfahren nach Anspruch 14, wobei die Gerinnungsstörung ausgewählt wird aus der Gruppe umfassend: Hämophilie, Thrombose, angeborenen Prothrombinmangel Typ I oder Typ II, erworbenen Prothrombinmangel und von-Willebrand-Krankheit.

Revendications

1. Préthrombine 2 mutante **caractérisée en ce qu'**elle a une SEQ ID NO: 1 ou SEQ ID NO: 2 ou leurs séquences codantes de nucléotides **caractérisées par** une SEQ ID NO: 5 ou SEQ ID NO: 6, respectivement.

2. Préthrombine 2 mutante selon la revendication 1, **caractérisée en ce qu'**elle présente une activité autocatalytique et **en ce qu'**elle demeure stable in vitro.

3. Utilisation de la préthrombine 2 mutante selon les revendications 1 à 2 pour la production de thrombine α.

4. Préthrombine 2 mutante selon les revendications 1 à 2 destinée à être utilisée pour la détection in vitro de la thrombine α / prothrombine.

5. Préthrombine 2 mutante selon les revendications 1 à 2 destinée à être utilisée pour le diagnostic et/ou le pronostic in vitro des maladies liées à la coagulation.

6. Préthrombine 2 mutante destinée à être utilisée selon la revendication 5, dans laquelle la maladie liée à la coagulation est sélectionnée dans le groupe comprenant : l'hémophilie, une thrombose, un déficit héréditaire en prothrombine de Type I ou Type II, un déficit acquis en prothrombine, le syndrome d'hypoprothrombinémie et la maladie de von Willebrand.

7. Méthode de préparation de la thrombine α qui comprend les étapes suivantes:

1. Modification, de préférence par mutagénèse dirigée, de la séquence d'ADN codant le site de clivage de facteur Xa dans un gène de préthrombine 2 de type sauvage en une séquence d'ADN codant le site de clivage de thrombine α.
2. Insertion de la préthrombine 2 d'ADN muté dans un vecteur d'expression.
3. Transformation d'une cellule hôte avec le vecteur mentionné dans l'étape précédente.
4. Culture des cellules hôtes transformées de l'étape précédente dans un milieu de culture approprié.
5. Recueil des cellules transformées et solubilisation des protéines dans des corps d'inclusion.
6. Isolement du surnageant afin d'obtenir la préthrombine 2 mutante purifiée.

7. Mise en contact de la préthrombine 2 mutante purifiée obtenue dans l'étape précédente avec la thrombine α.

8. Séparation de la thrombine α obtenue dans l'étape 7 précédente.

8. Méthode selon la revendication 7 où la séquence d'acides aminés du site de clivage du facteur Xa dans la préthrombine 2 de type sauvage est la SEQ ID NO: 17.

9. Méthode selon la revendication 7 où la séquence d'acides aminés du site de clivage de la thrombine dans la préthrombine 2 mutante est la SEQ ID NO: 18.

10. Méthode selon la revendication 7 où la séquence d'ADN de préthrombine 2 de type sauvage est sélectionnée dans le groupe comprenant la SEQ ID NO: 7 ou la SEQ ID NO: 8.

11. Kit de détection de la thrombine α / prothrombine dans un échantillon comprenant la préthrombine 2 mutante des revendications 1 à 2.

12. Utilisation du kit de la revendication 11 pour le diagnostic et/ou pronostic in vitro de maladies liées à la coagulation.

13. Utilisation selon la revendication 12 où les maladies liées à la coagulation sont sélectionnées dans le groupe comprenant : l'hémophilie, une thrombose, un déficit héréditaire en prothrombine de Type I ou Type II, un déficit acquis en prothrombine et la maladie de von Willebrand.

14. Méthode in vitro de diagnostic et/ou de pronostic des maladies liées à la coagulation comprenant, la détermination dans un échantillon provenant d'un sujet du niveau d'activité de la thrombine α / prothrombine en utilisant le kit de la revendication 11, et la comparaison dudit niveau d'activité par rapport aux valeurs obtenues pour la même activité chez des sujets témoins en bonne santé.

15. Méthode in vitro selon la revendication 14 où la maladie liée à la coagulation est sélectionnée dans le groupe comprenant : l'hémophilie, une thrombose, un déficit héréditaire en prothrombine de Type I ou Type II, un déficit acquis en prothrombine et la maladie de von Willebrand.
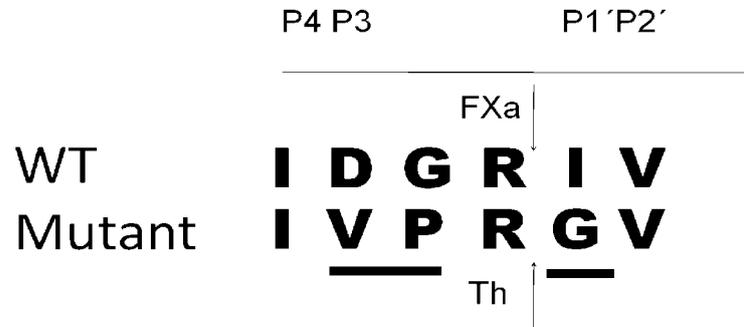
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Figure 2

Figure 3
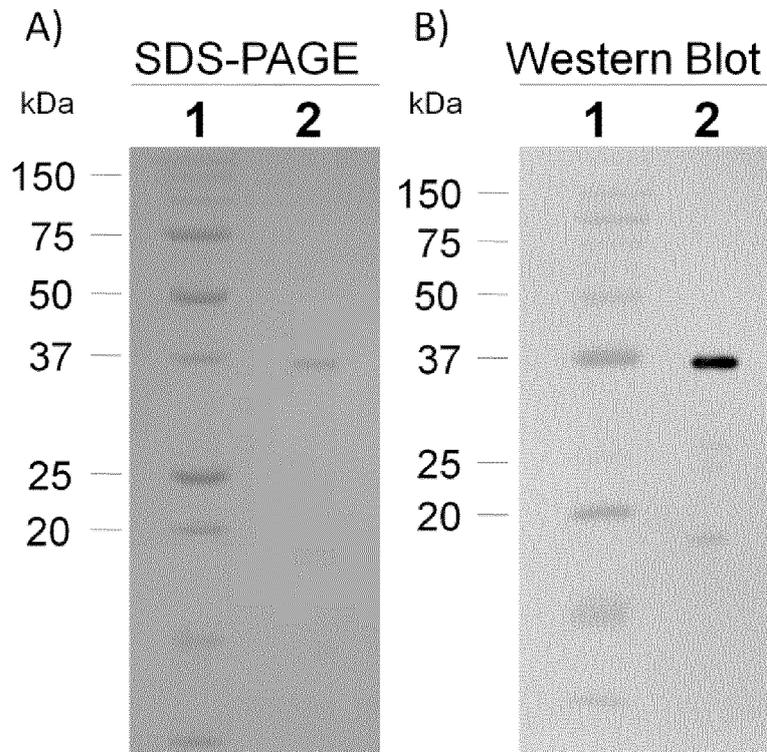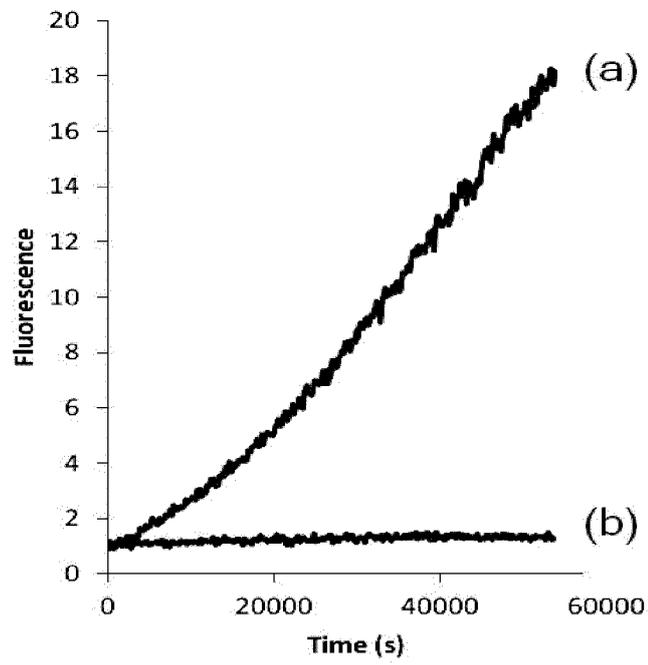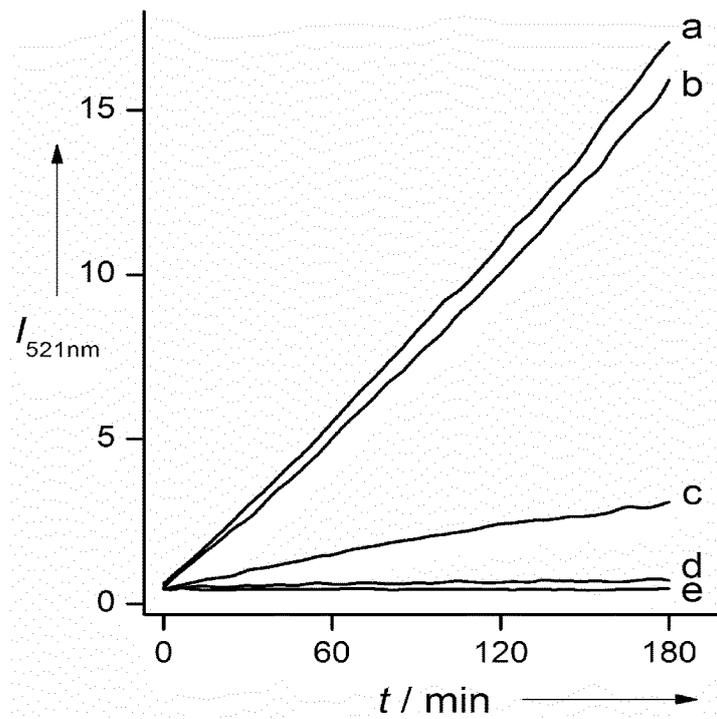

Figure 4

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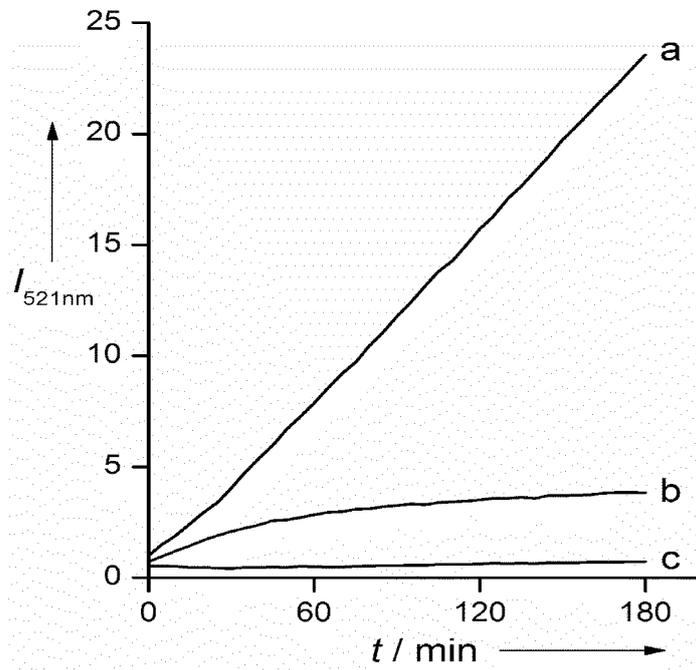
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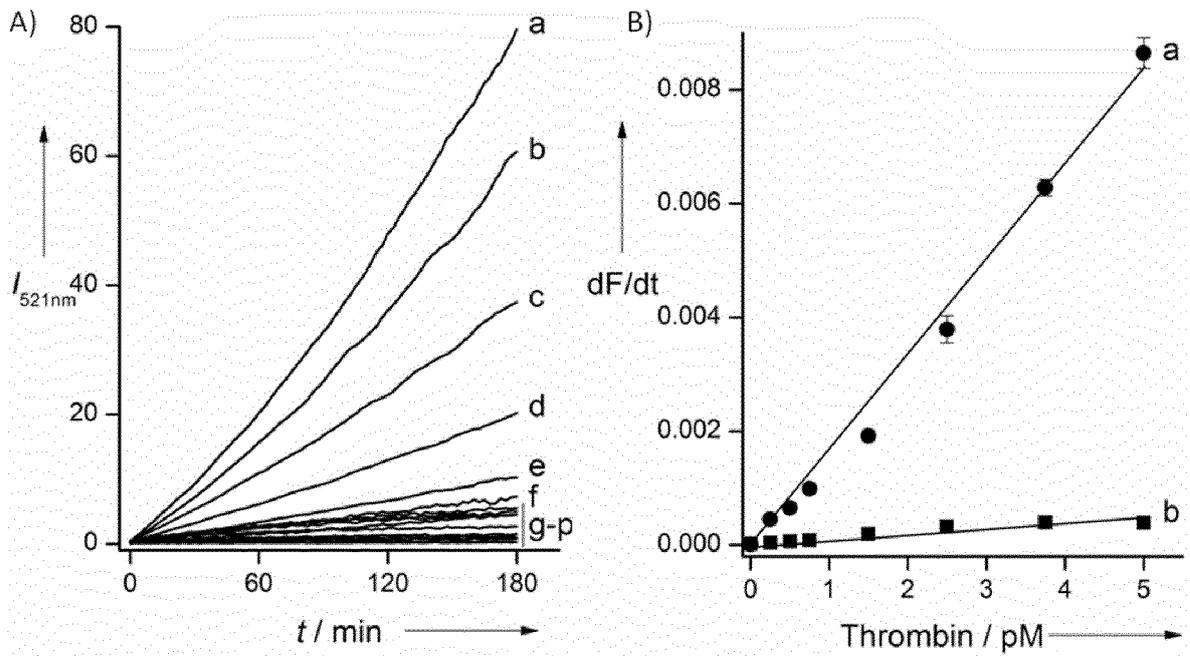
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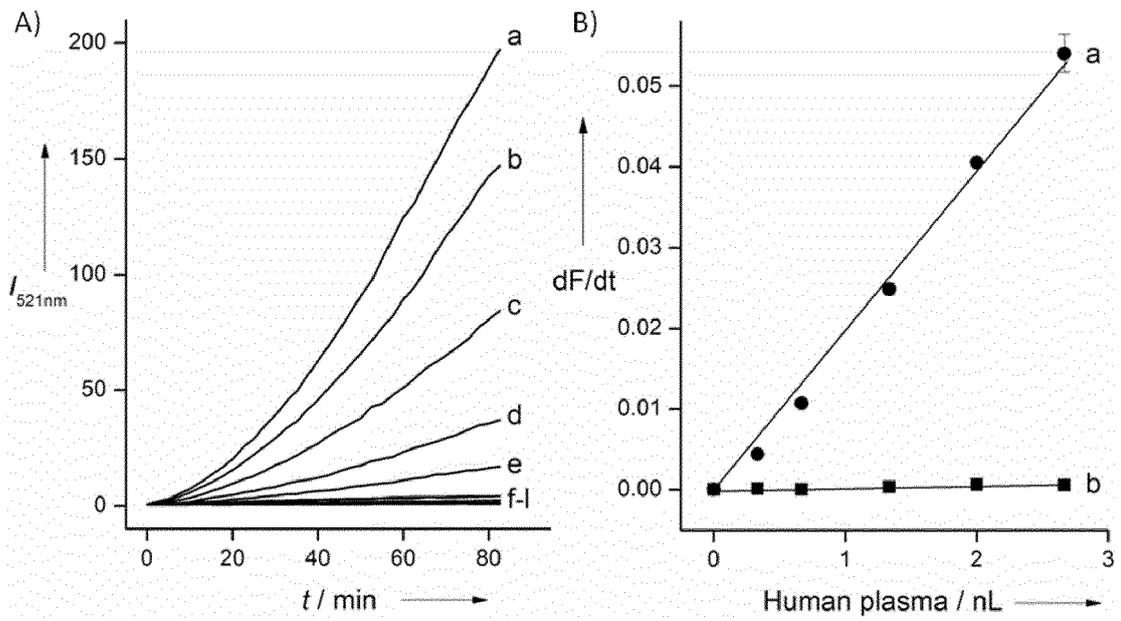
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